Oxidative Stress and Diseases

Nick Gilmour

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Edited by **Nick Gilmour**

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Contents

	Preface	**VII**
Section 1	**Introduction**	**1**
Chapter 1	**Introductory Chapter** Volodymyr I. Lushchak and Dmytro V. Gospodaryov	**3**
Section 2	**General Aspects**	**11**
Chapter 2	**Oxidative Stress:** **Cause and Consequence of Diseases** Dmytro Gospodaryov and Volodymyr Lushchak	**13**
Section 3	**Cardiovascular Diseases**	**39**
Chapter 3	**Reactive Oxygen Species and Cardiovascular Diseases** Vitor Engrácia Valenti, Luiz Carlos de Abreu, Celso Ferreira and Paulo H. N. Saldiva	**41**
Chapter 4	**Adipocytokines, Oxidative Stress** **and Impaired Cardiovascular Functions** Ana Bertha Zavalza Gómez, María Cristina Islas Carbajal and Ana Rosa Rincón Sánchez	**71**
Chapter 5	**Oxidative Stress in the Carotid Body:** **Implications for the Cardioventilatory** **Alterations Induced by Obstructive Sleep Apnea** Rodrigo Iturriaga and Rodrigo Del Rio	**103**
Chapter 6	**Oxidative Damage in Cardiac Tissue from Normotensive** **and Spontaneously Hypertensive Rats: Effect of Ageing** Juliana C. Fantinelli, Claudia Caldiz, María Cecilia Álvarez, Carolina D. Garciarena, Gladys E. Chiappe de Cingolani and Susana M. Mosca	**119**

Chapter 7 **Oxidative Stress and Mitochondrial Dysfunction in Cardiovascular Diseases** 135
Sauri Hernández-Reséndiz, Mabel Buelna-Chontal, Francisco Correa and Cecilia Zazueta

Chapter 8 **Role of Oxidized Lipids in Atherosclerosis** 167
Mahdi Garelnabi, Srikanth Kakumanu and Dmitry Litvinov

Chapter 9 **Oxidatively Modified Biomolecules: An Early Biomarker for Acute Coronary Artery Disease** 189
Sarawut Kumphune

Section 4 Diabetes Mellitus 215

Chapter 10 **Oxidative Stress in Diabetes Mellitus: Is There a Role for Hypoglycemic Drugs and/or Antioxidants?** 217
Omotayo O. Erejuwa

Chapter 11 **Diabetes, Oxidative Stress, Antioxidants and Saliva: A Review** 247
Natheer H. Al-Rawi

Chapter 12 **Oxidative Stress and Novel Antioxidant Approaches to Reduce Diabetic Complications** 255
Sih Min Tan, Arpeeta Sharma and Judy B. de Haan

Chapter 13 **Evaluation of Oxidative Stress and the Efficacy of Antioxidant Treatment in Diabetes Mellitus** 289
Nemes-Nagy Enikő, V. Balogh-Sămărghiţan, Elena Cristina Crăciun, R. Morar, Dana Liana Pusta, Fazakas Zita, Szőcs-Molnár Terézia, Dunca Iulia, Sánta Dóra and Minodora Dobreanu

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Preface

Information related to oxidative stress and diseases has been highlighted in this profound book. The formulation of hypothesis of oxidative stress in the 1980s aroused the interest of biomedical and biological sciences which exists in the present day scenario as well. The contributions in this book educate the readers on the role of reactive oxygen species in distinct pathologies in animals as well as humans. The book is designed in such a way that it covers particular groups of pathologies like diabetes, cardiovascular diseases, and related general aspects. The aim of this book is to serve as a useful source of information for readers including researchers, students, etc.

This book is the end result of constructive efforts and intensive research done by experts in this field. The aim of this book is to enlighten the readers with recent information in this area of research. The information provided in this profound book would serve as a valuable reference to students and researchers in this field.

At the end, I would like to thank all the authors for devoting their precious time and providing their valuable contribution to this book. I would also like to express my gratitude to my fellow colleagues who encouraged me throughout the process.

Editor

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Section 1

Introduction

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1

Introductory Chapter

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1. Introduction

The term "oxidative stress" was first defined by Helmut Sies (1985) as "Oxidative stress" came to denote a disturbance in the prooxidant-antioxidant balance in favor of the former". In order to reflect the findings of last 25 years in the field, such as plural ROS roles and dynamics of their levels we recently proposed one more definition such as "Oxidative stress is a situation when steady-state ROS concentration is transiently or chronically enhanced, disturbing cellular metabolism and its regulation and damaging cellular constituents" (Lushchak, 2011b). Understanding of mechanisms of reactive oxygen species (ROS) formation and operation of the systems responsible for ROS elimination were necessary prerequisites for such formulation. Fenton reaction, enzymatic systems like cytochromes P450, xanthine oxidase or respiratory chains were identified as ROS producers. Studies on catalase and peroxidase since the beginning of 20th century (Loew, 1900; Popov & Zvyagilskaya, 2007), and discovery of superoxide dismutase by McCord and Fridovich (1969), lead to suggestion, that cells have specialized systems for conversion of ROS to less reactive compounds. After introduction, the term "oxidative stress" has been accreting with medical issues. Today, one could find in literature connection of oxidative stress to almost all of well-known diseases. The most important of them are cardiovascular and neurodegenerative ones, diabetes, cancer, viral and bacterial infections, taking millions of lives every year.

In the simplest case, pathology originates from the perturbations in either reactive species formation, their elimination or in both simultaneously. Many of real situations are much more complicated, that is difficult to determine the crucial event for disease origin. In some cases, gene mutations can be responsible for the imbalance in ROS metabolism. In other ones, a range of environmental influences would produce metabolic changes. Antioxidant therapy seems to be useful in both cases. It is often important to know, if oxidative stress was a primary event leading to the disease or it was developed during the disease.

Diseases caused by gene polymorphism are curable hard, and here only really emerging gene therapy could be the best solution. In addition, environment can be changed easier. We need to understand how environmental changes may induce oxidative stress and perturb redox processes. This field is rather broad. Food toxins or even some of usual meals supposed to be safe, cigarette smoke or polluted air, car exhaust fumes or pesticides can be prerequisites for enhanced oxidant formation or impairment in antioxidant defence system.

Evidences for connection of oxidative stress with the stresses induced by other factors are promptly gained. The potency of transition metals, some herbicides and carbohydrates to promote oxidative stress was recently showed (Lushchak et al., 2009a; Lushchak et al., 2009b; Lushchak, 2011; Semchyshyn et al., 2011). The same thing is concerned to many physical factors like heat, sound or ionizing irradiations. After all, inflammation induced by traumatic event or pathogenic agent like viral, bacterial or protist infections can result in oxidative stress. Disturbances in ROS metabolism, caused by multiple external factors or by DNA mutations, lead, eventually, to progressive tissue damage and subsequent degeneration.

Identification of specific targets for ROS is one more thing important for the development of appropriate therapy. Moreover, place of ROS formation and their targets determine often particular connection with certain pathology. Proteins, nucleic acids and lipids are the most critical targets for ROS and their derivatives. Important enzymes, standing on crossroads of metabolic pathways, are frequently inactivated at excessive ROS formation not counterbalanced by antioxidants. Glyceraldehyde-3-phosphate dehydrogenase, aconitase, glucose-6-phosphate dehydrogenase and superoxide dismutase are the most studied examples (Bagnyukova et al., 2005; Lushchak, 2007; Grant, 2008; Di Domenico et al., 2010; Avery, 2011). The list is indeed much longer including representatives for almost all metabolic pathways in different tissues, as well as ion transporters (Unlap et al., 2002), receptors (Anzai et al., 2000), and other proteins. Polyunsaturated fatty acid residues of diverse lipids are mainly subjected to oxidation by ROS in this class of compounds. Protein oxidation results in formation of carbonylated or glutathionylated derivates, whilst non-enzymatic lipid oxidation yields 4-hydroxy-2-nonenal, isoprostanes, malondialdehyde and diene conjugates (Hermes-Lima, 2004b). Reactive species, particularly hydroxyl radical, are also involved in carbohydrate oxidation, what is especially harmful for nucleic acid pentose backbones (Gutteridge & Halliwell, 1988; Hermes-Lima, 2004b). Nucleotides are not any exception. Mutagenesis resulted from guanosine oxidation is widely described (reviewed in Hermes-Lima, 2004b). Cells may possess even special receptors for some products of oxidation, e.g. receptors for F_2-isoprostanes and advanced glycation end products (Fukunaga et al., 1997) or scavenger receptors for oxidized low-density lipoproteins (Ashraf & Gupta, 2011). Increase in ROS production was found to be also regulated via specific receptors (Thannickal & Fanburg, 2000). Production of ROS driven by transforming growth factor-β1, by receptors for endothelial or platelet-derived growth factors, as well as for angiotensin II or advanced glycation end-products (Thannickal & Fanburg, 2000) are among the most discussed examples. These facts suggest robust cellular control for ROS metabolism.

Oxidized derivatives of proteins and lipids may also damage other molecules exacerbating consequences of oxidative stress. For instance, 4-hydroxy-2-nonenal was shown to modify proteins through the interaction with amino group of lysine, cysteine or histidine residues. That results in the formation of Michael adducts. The formed adducts can impair considerable number of metabolically important proteins like transporters of glucose and glutamate, GTP-binding proteins, ion-motive ATPases and so forth (reviewed in (Mattson, 2009)). Ability to initiate protein carbonylation was also demonstrated for MDA (Burcham & Kuhan, 1996).

Nowadays, the knowledge about important signalling role for some ROS has gained in addition to their known deleterious roles (Thannickal & Fanburg, 2000; Dröge, 2002). It is known that ROS, namely hydrogen peroxide, can regulate c-Jun N-terminal kinase pathway, apoptosis initiation, tumour suppression by means of p53, ion channels and G-protein-coupled receptors (Thannickal & Fanburg, 2000; Dröge, 2002; Ushio-Fukai, 2009).

The term "reactive oxygen species" has itself seems become insufficient. It would be difficult to speak today about oxidant metabolism considering only ROS. In many contemporary studies, ROS are examined along with reactive nitrogen species (RNS), reactive carbon species (RCS), reactive chlorine species (RChS), and reactive sulphur species (RSS) (Hermes-Lima, 2004b; Ferreri et al., 2005). Formation for most of them is driven by specialized systems and is finely controlled (Dröge, 2002). It suggests a bunch of important roles for these highly reactive molecules. Some of these roles may even not be discovered. More and more interactions between ROS, RNS, RCS and RSS are found from study to study. The formation of peroxinitrite, a powerful oxidant and RNS, in reaction between nitric oxide and superoxide anion radical is a commonly known example in this case. Similarly, thiyl radicals, which are considered to be RSS, can be formed under the interaction of peroxyl or hydroxyl radical with thiol-containing compounds (Ferreri et al., 2005). Thus, once the formation of ROS has overwhelmed cellular detoxifying capacity, there is a big potential for generation of other highly reactive molecules with different properties and targets.

Ischemia, atherosclerosis, stroke and different types of inflammation were, probably, the first recognized pathological states closely connected with oxidative stress. The strong association between ROS and pathological states were disclosed here. At all these states, probability of ROS formation is much higher than in normal physiological state. For instance, mitochondria of ischemic cells increase the steady-state level of electrons which may escape electron carriers under reperfusion leading to one-electron reduction of oxygen (Hermes-Lima, 2004a). During inflammatory processes, ROS are produced purposely by NADPH oxidases (Lassègue & Griendling, 2010). In both these cases ROS seem to accompany disease flow, but are not the cause. A relation between oxidative stress and commonly known neurodegenerative disorders and diabetes was also found. These diseases are believed to be caused by ROS. It is known that alloxan, a compound broadly used for experimental diabetes induction, is a redox-cycling compound damaging insulin-producing pancreatic β-cells (Lenzen, 2008). Alzheimer's and Parkinson's diseases are connected with impairment of mitochondrial function resulting in enhanced ROS generation (Henchcliffe & Beal, 2008). The key proteins composing protein aggregates in Parkinson's and Alzheimer's diseases, α-synuclein and β-amyloid, respectively, were found to be capable to produce ROS themselves (Atwood et al., 2003; Wang et al., 2010). Diabetic complications are found to be induced by the formation of advanced glycation end products which interact with specialized receptors and promote ROS production (Forbes et al., 2008).

The term "human disease" has been defined as a condition worsening usual human being and working capacity, and in some cases leading to death. Illness state is also a disorder of homeostasis connected with impairment of important parts of either whole organism, or specific proteins, whole cells, and even whole tissues and organs. In this context, ROS role as damaging agents would seem to be evident in disease origin. Despite that ROS in many

works are described in their halo of harmfulness, especially in concern with diseases, there is also a complementary view on beneficial role of ROS in adaptation to stress (Ristow & Schmeisser, 2011; Lushchak, 2011a). Protein oxidation may also not always be harmful. Particularly, reversible oxidation of some key enzymes may respond to metabolic reorganization promoting to some extent cell adaptation to enhanced ROS production (Grant, 2008). Even protein carbonylation may have signalling role in vascular system (Wong et al., 2010) and in some examples activates proteins (Lee & Helmann, 2006). These findings should also be taken into account at analysis of association between oxidative stress and particular diseases. Participation of ROS in signaling, their roles in regulation of apoptosis and cell adaptation significantly complicate our view on them as a cause of diseases. Consequently, the view on oxidative stress should also be altered. Now, it is emerging impression that oxidative stress is not only the state when oxidation prevails. It is more resemble to the state of disturbance redox control mechanisms when "harmful" and undesirable for cell survival oxidation is prevailing, and physiological functions of ROS are altered or reprogrammed to promot cell death (Jones, 2006). Using this approach, one can suggest that cell death may result not only from several dozens of oxidized proteins and lipids. If we would not have any oxidation events, disturbance of physiological ROS metabolism might turn several dozens of processes in wrong direction. It may have more systemic effects, spread on whole organism, rather than causing cell death in particular tissue.

In the current book, the most topical issues of connection between oxidative stress and broadly known pathologies are examined. They include presumably cardiovascular diseases, hypertension and diabetes. Some attention is paid to well-known neurological diseases and cancer. Issues like reproduction, immunity, hormonal disorders are also affected. Some chapters are devoted to discussion on antioxidant therapy, though antioxidant clue goes through all other chapters as well. It is worth noting, knowledge highlighted in this book is collected all over the world. It implies the topic is long ago out of particular laboratories and elaborated by medical scientists in many countries. In some points concerns of the authors coincide, in other ones they are unique. Thus, the book mirrors many different aspects of pathological roles of ROS. We did not aim to make it comprehensive as much as possible. It is rather impossible taking into account that oxidative stress today has many faces. If someone would like to get specific knowledge on this topic from the beginning, the best advice would be to choose firstly the branch among incomprehensive canopy of oxidative stress studies. The book aimed to show how the field is studied in different countries and what is common for all investigations.

The connection between oxidative stress and diseases is mentioned in introduction of almost every article in the field. However, there is a difference between *in vitro* studies, studies on cell cultures, laboratory animals and clinical studies with humans. The last ones are most complicated for perception, but they provide a picture of reality. In this context, it is a pleasure to realize that some of the authors of this book are physicians whose studies are conducted on patients. The results from these studies are always more difficult for interpretation than those from model experiments carried out at cultivated cells. Nevertheless, clinical studies are highly complicated for understanding of ROS contribution in illness state. Once the implication of ROS in particular disease found, it suggests

possibility of antioxidant therapy. However, how it is mentioned in one of the chapters, under some conditions antioxidants may act also as pro-oxidants. Following redox pioneers, John Gutteridge and Barry Halliwell, here one could say "pro-oxidants can be better for you in some circumstances" (Gutteridge & Halliwell, 2010). Moreover, modulation of signalling pathways linked with ROS may be more effective than simple antioxidant therapy. Most of known antioxidants can act also as signalling molecules, but there are also many compounds important for signaling that are not antioxidants. Other crucial thing is prophylactics. Cardiovascular diseases, diabetes, obesity, metabolic syndrome, neurological and hormonal disorders, impairment in kidney and liver functioning, mentioned in the book and described in terms of free radical biology, are not always strictly genetically conditioned. They are lifestyle and life condition pathologies often with onset in late age. So, they can be prevented. It is, probably, the most important conclusion that can be drawn from the generalized data. Even genetically caused pathologies could be attenuated by wisely arranged prophylactics if the defect is not too serious. That is also the reason for the accumulation, generalization and systematization knowledge obtained at different levels, with different models and clinical studies. We hope that this book will disclose, at least partially, the state of the problem worldwide and the current directions of laboratories focused on studies for implication of ROS in different pathologies. We also believe that it will help researchers to find weak places in current understanding and advise them quite novel and non-standard approaches to find and decipher mechanisms of diseases.

Finally, we would like to thank all authors for their contributions and hard work to match and unify the "philosophy" of this book. We also thank to our colleagues from Precarpathian National University and University of Tampere who supported us and helped us in preparation and edition of the chapters, especially to those who raised complex questions and promoted us to answer them. We are also grateful to the "In-Tech" Publisher personnel, especially Ms. Sasa Leporic, who assisted us in the arrangement of the book and scheduling our activities.

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Section 2

General Aspects

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2

Oxidative Stress: Cause and Consequence of Diseases

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1. Introduction

Oxidative stress, termed as an imbalance between production and elimination of reactive oxygen species (ROS) leading to plural oxidative modifications of basic and regulatory processes, can be caused in different ways. Increased steady-state ROS levels can be promoted by drug metabolism, overexpression of ROS-producing enzymes, or ionizing radiation, as well as due to deficiency of antioxidant enzymes. The plethora of ways leading eventually to oxidative stress is depicted in Figure 1. This chapter has several aims. The main aim is to show examples which mirror our knowledge on the role of oxidative stress in origin of diseases as well as its significance in disease complications. It was it that oxidative stress is described over developed pathology and nothing is told, if it was initial, triggering event or it was a consequence of the metabolic shift caused by other factors. Virtually, it would be important to develop a cure for specific diseases. The other aim of this chapter is to spotlight the role of enzyme deficiencies in promotion of oxidative stress during pathological conditions. These deficiencies are often caused by mutations in genes coding antioxidant or related enzymes, i.e. by genetic polymorphism. Mutations are preconditions for many diseases, which cannot be prevented in most cases. Nevertheless, complete understanding of the ways from the gene to disease symptoms is necessary condition for successful therapy. Attention should be paid also to full or partial loss of the enzyme activity via exogenous factors or via other indirect reasons. Many connections can be drawn between certain pathologies and oxidative modification of proteins. Most antioxidant and related enzymes are targets for oxidative modification. Hence, if oxidative stress was primary event, possible oxidative modifications of antioxidant enzymes may exacerbate diseases and define cell destiny. Finally, it is worth mentioning advantages and disadvantages of diverse models which serve for disclosing of mechanisms underlying ROS contribution to diseases. Predominant number of studies is conducted on mice and cell cultures. Significant insights were received with use of lower organisms like budding yeast, nematodes and fruit flies. All model organisms and cell cultures have certain limitations and disadvantages. So far, the largest benefit can be brought out from complex studies, involving many model systems and investigating phenomena from different points of view.

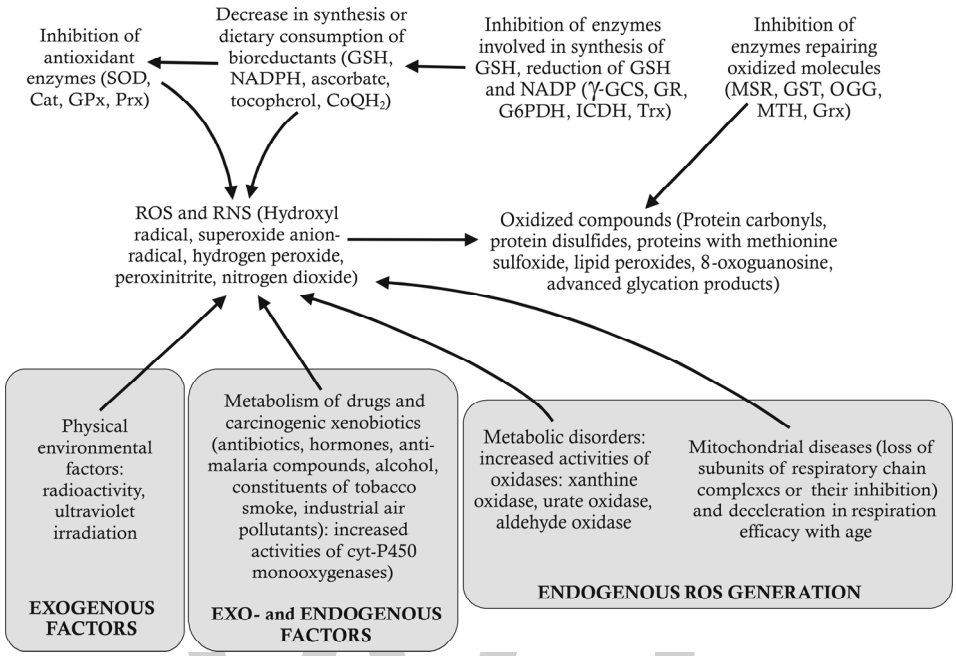

Fig. 1. Ways leading to oxidative stress. Reactive oxygen species are constantly produced in cells by electron transport chains and some enzymes, like xanthine oxidase, aldehyde oxidase, cytochrome-P450 monooxygenases, etc. Increased ROS production may be promoted by exogenous factors (temperature variation, radioactivity, ultraviolet irradiation, xenobiotics), metabolic disorders or inherited diseases affecting electron transport chain. Deficiencies in antioxidant enzymes or impaired metabolism of low-molecular-mass antioxidants will also lead to elevation of ROS concentration over steady-state level. Negative consequences of elevated ROS level can be alleviated by repair enzymes. Abbreviations: SOD – superoxide dismutase, Cat – catalase, GPx – glutathione-dependent peroxidase, Prx – peroxyredoxin, GSH – reduced glutathione, $CoQH_2$ – ubiquinol, γ-GCS – γ-glutamylcysteine synthetase, GR – glutathione reductase, G6PDH – glucose-6-phosphate dehydrogenase, ICDH – isocitrate dehydrogenase, Trx – thioredoxin, MSR – methionine sulfoxide reductase, GST – glutathione S-transferase, OGG – 8-hydroxy-2′-deoxyguanosine glycosylase, MTH – oxidized purine nucleotide triphosphatase, Grx – glutaredoxin.

2. Genetic polymorphism of antioxidant and related enzymes

Genetic polymorphism is frequently related to large number of pathologies. Enzymes involved in defence against ROS are not an exception. All enzymes contributing to antioxidant defence can be classified to really antioxidant ones, dealing directly with ROS as substrates, and auxiliary ones (we will call them also related to or associated antioxidant enzymes). The latter enzymes respond for reparation or degradation of oxidatively modified molecules, maturation and posttranslational modification of antioxidant enzymes, metabolism of low molecular mass antioxidants, etc. As a rule, genetic polymorphisms of

enzymes of these two big groups may lead to oxidative stress and consequent diseases, among which cancer, neurodegeneration, cardiovascular disorders, and diabetes are most frequently mentioned. Some of them, like diabetes, cardiovascular and neurodegenerative diseases, are connected with cell death. On the other hand, cancer presents opposite side, and is marked by abnormal cell proliferation. Indeed, ROS guide to cell death when their targets are proteins or lipids. Cell proliferation can be promoted, at least partially, by oxidative modification of nucleic acids and subsequent mutations. Nevertheless, several exceptions from this "rationale" have already been described. For example, it is known that nitration of transcription factor p53 by peroxynitrite, one of the reactive nitrogen species, is associated with human glioblastoma (Halliwell, 2007). In many other cases, oxidative modification of proteins leads to cell damage. Examples which confirm this are reviewed elsewhere (Nyström, 2005) and some of them will be described below. The functions and cellular roles of antioxidant or associated enzymes will define the consequences which happen in case of polymorphism of the genes coding these enzymes.

2.1 Glucose-6-phosphate dehydrogenase deficiency

The most striking example among polymorphisms of genes coding enzymes related to antioxidant defence is well-known deficiency in glucose-6-phosphate dehydrogenase (G6PDH) which leads to favism. In this case, there is no very strong phenotype; only additional exogenous factors, like drugs or certain types of food, tolerated by unaffected individuals, can reveal the pathology. Lifestyle, diet or adventitious diseases may exacerbate consequences of decreased enzyme activity. Opposite situation is also possible: deficiencies in antioxidant and related enzymes may exacerbate other pathologies, namely infectious and neurodegenerative diseases, as well as cancer. Glucose-6-phosphate deficiency was one of the first known to mankind deficiencies of auxiliary antioxidant enzymes. Favism caused by this enzymopathy is known from ancient times and is exhibited as haemolytic anaemia induced by consumption of broad beans (*Vicia faba*) (Beutler, 2008). First report, discovering contribution of G6PDH deficiency to sensitivity to an anti-malaria drug, primaquine, was published more than half century ago (Alving et al., 1956). Association of G6PDH deficiency with favism was drawn soon after that, when several independent studies found that this disorder is attributed only to individuals with low G6PDH activity (Sansone & Segni, 1958; Zinkham et al., 1958). The mechanism for the haemolytic anaemia, which develops in response to ingestion of *V. faba* beans at G6PDH deficiency, is consistent with several observations. *V. faba* contains polyhydroxypyrimidine compounds, prone to redox-cycling, like glycoside vicine and its aglycone divicine, as well as isouramil (Fig. 2). It was shown that they were involved in production of superoxide anion radical and hydrogen peroxide (Baker et al., 1984). Primary product is likely superoxide anion radical, because it was shown that all three compounds could promote release of iron from ferritin (Monteiro & Winterbourn, 1989). Oxidation of iron ion in iron-sulphur clusters was found to be related to the effect of superoxide anion radical (Avery, 2011). Indeed, iron ion release was inhibited by superoxide dismutase for divicine and isouramil, while combination each of the three compounds with ferritin promoted lipid peroxidation in liposomes (Monteiro & Winterbourn, 1989). Interestingly, alloxan, compound chemically related to divicine and isouramil, is widely known as a superoxide generator, and is used for experimental induction of diabetes (Lenzen, 2008).

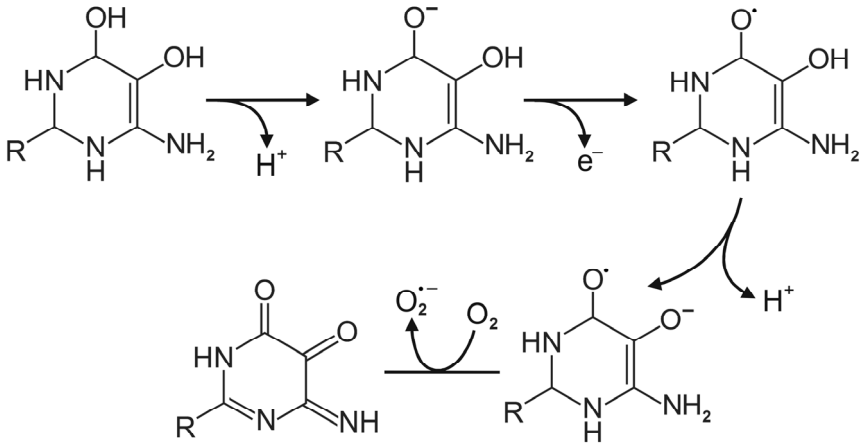

Fig. 2. Redox modifications of compounds from broad beans (*Vicia faba*). R is amino group for divicine and hydroxyl-group for isouramyl. Modified from (Chevion et al., 1982).

Formation of NADPH, catalyzed by G6PDH and 6-phosphogluconate dehydrogenase in pentose phosphate pathway, is thought to serve for a general antioxidant defence, and is frequently described by scheme shown in Fig. 3.

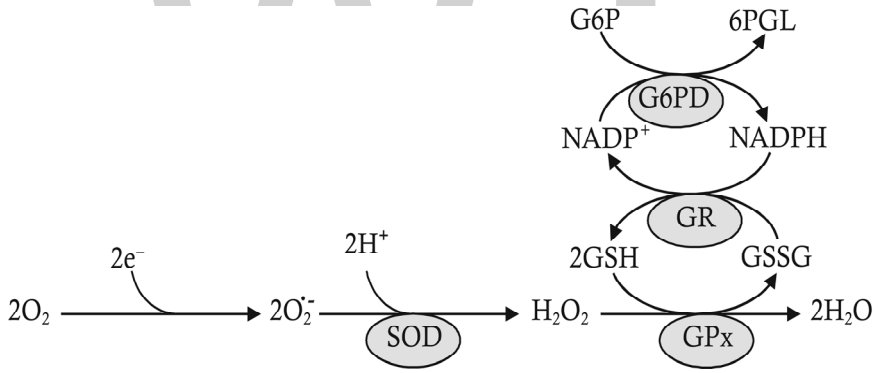

Fig. 3. Functional cooperation between G6PDH and GPx, explaining importance of G6PDH for erythrocyte survival. Abbreviations: SOD, superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; G6PDH, glucose-6-phosphate dehydrogenase; GSH, reduced glutathione; GSSG, oxidized glutathione; $NADP^+$, oxidized nicotineamide adenine dinucleotide phosphate; NADPH, reduced nicotineamide adenine dinucleotide phosphate; G6P, glucose-6-phosphate; 6PGL, 6-phosphogluconolactone. Indeed, SOD produces one molecule of oxygen and one molecule of hydrogen peroxide. This reaction is more complex than it is represented on the figure and goes in two subsequent stages. One NADPH molecule is produced in reaction catalyzed by G6PDH per G6P molecule, and one more at the next step of the pentose phosphate pathway catalysed by 6-phosphogluconate dehydrogenase.

According to Fig. 3, superoxide dismutase produces hydrogen peroxide, which, in turn, is reduced in glutathione peroxidase reaction to water with concomitant oxidation of glutathione. This reaction needs reduced glutathione which acts as a reductant. Glutathione reductase maintains concentration of reduced glutathione using NADPH as an electron and proton donor. Hence, oxidative stress, caused by redox-cycling compounds, and advanced oxidation of haemoglobin and other proteins in erythrocytes is seemed to underlie the anaemia. It is believed that NADPH may be also needed for catalase operation (Kirkman & Gaetani, 2007). There are also indirect hints that G6PDH may play a role in assembly of iron-sulphur clusters, the main cellular target for superoxide anion radical attack. In particular, in bacteria *Escherichia coli*, G6PDH and SOD belong to the same regulon, namely SoxRS (Demple, 1996; Lushchak, 2001). It is known that NADPH may be absolutely necessary for iron-sulfur cluster formation (Fig. 4) as well as for haem synthesis (Wingert et al., 2005).

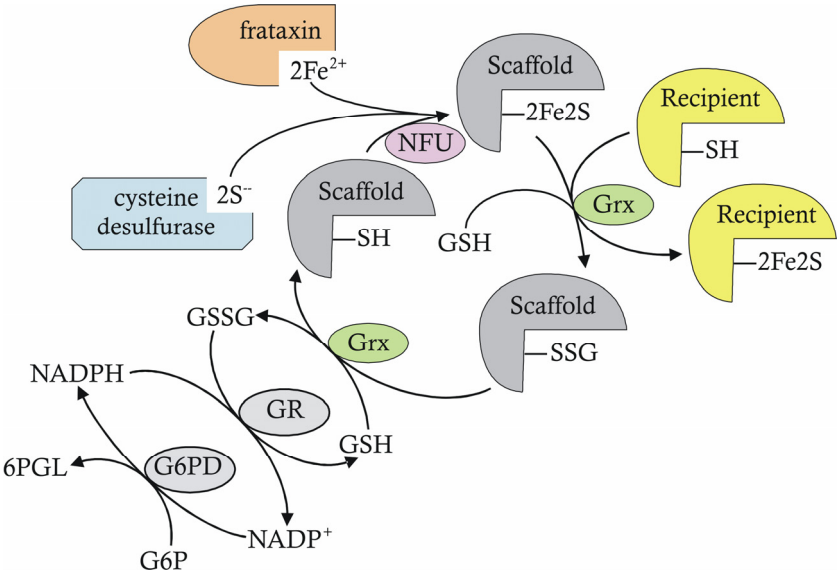

Fig. 4. Role of NADPH in iron-sulfur cluster assembly. Frataxin and cysteine desulfurase provide iron and sulfur for the clusters. NFU – alternative scaffold protein involved in assembly of iron-sulfur clusters. The clusters are assembled on other scaffold proteins and transferred to acceptor proteins like aconitase, succinate dehydrogenase, and ferredoxin. Glutaredoxins (e.g., Grx5) are supposed to be responsible for delivery of the clusters to recipient proteins using GSH. NADPH is spent for reduction of glutathione. Modified from (Bandyopadhyay et al., 2008) and (Tamarit et al., 2003).

In this case, produced NADPH is used also to maintain pool of reduced glutathione, while the latter goes for assembly of iron-sulfur clusters. It was demonstrated that glutaredoxin 5 is particularly responsible for the assembly of iron-sulfur clusters and haem synthesis (Wingert et al., 2005), where it takes part in iron-sulfur cluster delivery to proteins (Lill & Muhlenhoff, 2006). Other enzymes involved in the assembly of the clusters, ferredoxins, may also depend on NADPH (Pain et al., 2010).

Deficiency in G6PDH is widespread among people in tropical countries where a high risk of malaria is concomitantly observed (Cappellini & Fiorelli, 2008). It is believed that the G6PDH-deficient phenotype gives an adaptive advantage to survive under malaria threat (Cappellini & Fiorelli, 2008; Nkhoma et al., 2009). Indeed, progression of protist *Plasmodium falciparum*, causing malaria, is impossible in G6PDH-deficient erythrocytes because of cell "suicide" (Föller et al., 2009). In this context, it was shown that *P. falciparum* propagation in normal erythrocytes needs 5'-phosphoribosyl-1-pyrophosphate (PRPP) synthetase activity. On the other hand, PRPP synthetase was shown to be strongly dependent on the level of reduced glutathione (GSH). Since G6PDH-deficient erythrocytes possess low GSH level, the activity of PRPP synthetase is low in these cells and restricts parasite growth rate (Roth et al., 1986).

Some other pathologies are also associated with G6PDH deficiency. They are diabetes (Niazi, 1991; Gaskin et al., 2001; Carette et al. 2011), vascular diseases (Gaskin et al., 2001), and cancer (Ho et al., 2005). It is possible that induced oxidative stress in particular cells can be a ground for these phenotypes. It was shown that GSH may react with superoxide anion radical (Winterbourn & Metodiewa, 1994) providing partial defence against this ROS. In this case, decreased GSH pool in G6PDH-deficient individuals enhances their sensitivity to redox-active compounds, producing superoxide. Superoxide is able to react also with nitric oxide, leading to the formation of rather harmful oxidant peroxynitrite (Lubos et al., 2008). However, relation of this reaction to diabetes and vascular diseases is not because of peroxynitrite production and subsequent oxidative damage, but rather because of decrease in nitric oxide level. The latter is an important second messenger in certain signalling pathways particularly related to vasodilation (Förstermann, 2010). There is some probability also that individuals with G6PDH-deficiency may fail to regulate properly blood pressure (Matsui et al., 2005). Despite possible impairment in nitric oxide production, there is also other way to connect G6PDH deficiency with vascular diseases. It is known, that development of vascular diseases depends on the levels of homocysteine and folate, intermediates in metabolism of sulfur-containing amino acids (Stipanuk, 2004; Joseph et al., 2009; Rimm & Stampfer, 2011). Production of two these metabolites depends on GSH and NADPH levels in cells (Leopold & Loscalzo, 2005).

Data regarding association of G6PDH deficiency with cancer are controversial, because some studies demonstrated that G6PDH-deficient patients may additionally suffer from cancer (Pavel et al., 2003), while others state opposite (Cocco et al., 1989, 2007). Nevertheless, both situations are possible. In particular, there is a large data body indicating that different cancer types are developed at increased DNA damage. It often happens under polymorphism in enzymes contributing to DNA repair, what will be discussed below. Under conditions which would promote oxidative DNA damage, antioxidant function of could prevent cancer. On the other hand, NADPH supply at certain conditions may be even harmful leading to enhanced oxidative damage and cancer development. Indeed, it was shown that G6PDH was particularly responsible for cell growth and frequently correlated with cell growth (Ho et al., 2005). Tian and colleagues (1998) found that cancer cells possessed several times higher G6PDH activity. The positive correlation between tumour progression and G6PDH activity was found also for humans (Batetta et al., 1999).

Increased NADPH supply resulting from G6PDH overexpression can lead to so-called "reductive stress" (Rajasekaran et al., 2007; Lushchak, 2011). Enhanced activity of G6PDH, a lipogenic enzyme, was found at diabetes and obesity (Gupte, 2010). In humans, G6PDH is

regulated by many transcription factors, in particular, SREBP-1a (sterol regulatory element binding protein) (Amemiya-Kudo et al., 2002), AP-1 (Kletzien et al., 1994) and Sp1 (Franzè et al., 1998). It was shown that elevation of G6PDH activity might lead to enhanced lipid synthesis (Salati et al., 2001; Park et al., 2005; Lee et al., 2011) and to possible reductive stress (Dimmeler & Zeiher, 2007; Rajasekaran et al., 2007; Ralser & Benjamin, 2008).

Despite intensive investigations, information on lifespan and adventitious diseases of G6PDH-deficient patients remains scarce. It is complicated to understand compensatory mechanisms for a loss of the enzyme important for the production of NADPH and pentose phosphates, and to anticipate all possible sides, both negative and positive ones and, therefore, further studies are needed.

2.2 Catalase deficiency

At 1947, Japanese otolaryngologist Shigeo Takahara firstly described catalase deficiency, called acatalasemia, for a child with oral ulcer (Kirkman & Gaetani, 2007). Since that time, many studies on acatalasemia were performed. It was noted, that some patients with acatalasemia, namely those with Japanese and Peruvian types, suffer from progressive oral ulcers known as Takahara's disease. The cause is considered to consist in ability of oral Streptococci to produce hydrogen peroxide which may promote death of mouth mucosa cells in acatalasemic patients (Ogata et al., 2008). In fact, several pathogenic bacteria, like *Streptococcus pneumoniae* or *Mycoplasma pneumoniae* may produce hydrogen peroxide by means of their oxidase systems and therefore might be rather dangerous to acatalasemic patients. Nevertheless, Brennan and Feinstein (1969) on the model of acatalatic mice demonstrated that *Mycoplasma pulmonis*, a pathogenic H_2O_2-producing mollicute, may cause fast development of the disease in the animals (Brennan & Feinstein, 1969). Interestingly, acatalatic mice faster recovered after disease, probably because of bacterial autointoxication by high hydrogen peroxide concentrations.

Catalase deficiency is also associated with diabetes mellitus (Góth, 2008). This association is attributed for Hungarian hypocatalasemic patients. They were shown to possess higher levels of homocysteine and lower levels of folate (Leopold & Loscalzo, 2005). It hints, on one hand, to abnormalities of sulfur metabolism, but on the other hand, it is commonly known that higher homocysteine levels are related to cardiovascular diseases (Lubos et al., 2007), the fact we mentioned above in the context of G6PDH deficiency.

Despite high importance of antioxidant enzymes for cell survival, their loss is usually not characterized by severe phenotype. That is probably because cells have many counterparts of antioxidant enzymes. For example, in addition to G6PDH, NADPH can also be produced by NADPH-dependent isocitrate dehydrogenase, malic enzymes or $NAD(P)^+$-transhydrogenase. Catalase can be substituted by other hydrogen peroxide utilizing enzymes, namely glutathione peroxidase, cytochrome c peroxidase, thioredoxin peroxidase, etc. Superoxide dismutase deficiency can be compensated to some extent by ions of transition metals (Batinic-Haberle et al., 2010) or other proteins which may exhibit weak superoxide dismutase activity, e.g. ceruloplasmin (Goldstein et al., 1982). Nevertheless, it should also be taken into account that often whole deletion of some enzymes leads to the lethal phenotypes in mice models. Particularly, knockout on manganese-containing

superoxide dismutase (Mn-SOD) causes early lethality of experimental mice (Halliwell, 2007). It is also supposed that complete lack of G6PDH activity leads to prenatal death. Almost all mutations on G6PDH present mutations of single nucleotide or several nucleotide deletions without frameshift in the coding region (Ho et al., 2005). Notably, knockouts on whole gene, causing full loss of the activity, can also be investigated with experimental models, like mice (see also below), but are rarely found in reality.

2.3 Polymorphism of Cu,Zn-SOD and protein aggregation

Special attention should be paid to polymorphism of genes coding SOD. More than 100 nucleotide substitutions for the gene SOD1 coding human cytosolic copper- and zinc-containing SOD (Cu,Zn-SOD) were described (Valentine et al., 2005). Point mutations in SOD1 gene lead often to the pathologies by the mechanism different of that for other antioxidant or related enzymes, like described above catalase and G6PDH. It is known that several mutations in SOD1 gene are associated with cases of familial amyotrophic lateral sclerosis (ALS), a neurodegenerative disease which is characterized by paralysis and subsequent death (Vucic & Kiernan, 2009). Mutations in SOD1 can also be found in individuals with sporadic ALS. Moreover, it was proposed that Cu,Zn-SOD may account for all cases of ALS (Kabashi et al., 2007). Mechanisms of the disease development are still unknown, but there are many evidences that oxidative stress, developed in neurons, is rather caused by unexpected pro-oxidative activity of SOD than by the loss of the activity at all (Liochev & Fridovich, 2003). To the present knowledge, molecules of mutated SOD are also assembled into insoluble, amyloid-like proteinaceous aggregates (Valentine & Hart, 2003). It was found that the aggregates cause harm to the cells not only via oxidative stress, but also via inhibition of glutamate receptors (Sala et al., 2005; Tortarolo et al., 2006) and induction of apoptosis (Beckman et al., 2001). A pioneer in SOD studies, Irwin Fridovich, presented some examples of unusual activities of SOD, such as oxidase-like or reductase-like ones (Liochev & Fridovich, 2000). His works and data of other authors suggest that SOD, being mutated or placed in specific conditions, may produce more harmful ROS than hydrogen peroxide, i.e. hydroxyl radical (Yim et al., 1990; Kim et al., 2002). Some studies suggested that SOD aggregation can be triggered by higher susceptibility to oxidation of mutated protein (Rakhit et al., 2002; Poon et al., 2005). Indeed, Cu,Zn-SOD is considered to be rather stable, resistant to many, deleterious to other proteins, compounds (Valentine et al., 2005). Though it was also found that Cu,Zn-SOD is susceptible to oxidative modification (Avery, 2011). Moreover, some mutations may convert the enzyme into the form more susceptible to oxidation. Many substitutions of amino acids in polymorphic Cu,Zn-SOD variants do not present change from less susceptible to oxidation amino acid residues to more susceptible ones. For example, one of the most common substitutions, A4V, is a change from one nonpolar side chain to the other one at N-terminus (Schmidlin et al., 2009). However, even such substitutions may result in conformational changes which, in turn, can lead to unmasking of easily oxidizible amino acid gropus and exposure them to protein surface. Regarding A4V mutation, it is known that alanine at N-terminus of Cu,Zn-SOD is acetylated (Hallewell et al., 1987). In some cases, this posttranslational modification may prevent protein from oxidation (Seo et al., 2009) or ubiquitination (Arnesen, 2011). Mutations can also make the enzyme more vulnerable to oxidation in view of that Cu,Zn-

SOD is bearing ions of transition metal. Latter case suggests possibility of metal catalyzed oxidation, and it was shown in some studies that SOD is prone to oxidation (Kabashi et al., 2007). The other group of the noted amino acid substitutions in Cu,Zn-SOD, like valine to methionine, glutamate to lysine, aspartate to tyrosine, glycine to arginine (Orrell, 2000) establishes direct change to more oxidizible side chain. So far, it is still not clear if oxidative modification of mutated SOD takes place in ALS development.

In this context, it would be relevant to mention other pathologies connected with accumulation of amyloid-like aggregates. They include Alzheimer (properly β-amyloid accumulation), Huntington (accumulation of mutated huntingtin particles), and Parkinson (accumulation of α-synuclein) diseases. Some proteins can also be components of the aggregates under all of aforementioned diseases, but they are out of scope of this chapter. Many links with oxidative stress have been discovered for these pathologies since time their molecular mechanisms were disclosed. However, most studies are concentrated mainly on the development of oxidative stress during the course of a disease. It is still unclear, if oxidative stress could be the cause of the pathology or, at least, be responsible for its progression and general symptoms. To date, there is no direct evidence if some kind of oxidative modifications of protein is involved in the aggregate formation. Nevertheless, some hints collected from different studies afford to assume that oxidative stress might be the cause of these diseases (Norris & Giasson, 2005).

In some cases, the brain trauma precedes the disease (McKee et al., 2009; Knight & Verkhratsky, 2010). This traumatic event may become a predisposition to further pathology, and triggering signal for oxidative stress, developed during inflammation. A chronic traumatic encephalopathy, found often in American football players, is the distinguishable example for this event (Omalu et al., 2010). This tauopathy is characterized by accumulation of tau protein aggregates. The similar causes, like initial traumatic event, are described for several cases of Parkinson disease (Uryu et al., 2003).

Human amyloid precursor protein and matured human β-amyloid possess rather high affinity to ions of transition metals (Kong et al., 2008). Moreover, this metal binding capacity confers it both, antioxidant and prooxidant, properties (Atwood et al., 2003) what depends on the intracellular environment and type of the metal ion bound. In the case of mutations or at certain cell milieu, affected by external factors, this protein can also be easily oxidized. The ability to bind metals dependently on conditions provide both, antioxidant and prooxidant, properties which was shown for α-synuclein (Zhu et al., 2006) and prion PrP (Brown et al., 2001; Nadal et al., 2007).

Notably, G6PDH and Cu,Zn-SOD provide examples of the enzymes which become unstable after single amino acid change throughout the whole primary sequence. In G6PDH case, single amino acid substitution may lead to severe loss of activity, while in Cu,Zn-SOD case amino acid substitution may not lead to the loss of activity, but frequently causes considerable alteration of the protein properties. It would be important to decipher not only the mechanisms for the development of particular pathology, but the reasons of high mutability of the genetic loci, coding G6PDH and Cu,Zn-SOD. There is also a sense to understand why these enzymes are so susceptible to mutations.

2.4 Polymorphism of Mn-SOD, extracellular SOD and glutathione peroxidase

Unlike Cu,Zn-SOD, less mutations were found in the gene coding human manganese-containing superoxide dismutase (SOD2). Substitution of alanine-16 to valine (so called "Ala variant") is the most known mutation (Lightfoot et al., 2006). This mutation has recently been associated with cancers of breast, prostate, ovaries and bladder, as well as non-Hodgkin lymphoma, mesothelioma and hepatic carcinoma (Lightfoot et al., 2006). A16V substitution affects N-terminus of the protein, particularly, leading sequence responsible for the mitochondrial targeting (Sutton et al., 2003, 2005). It was shown that mice homozygous in SOD2 knock-out died after birth due to lung damage (Halliwell, 2007). Heterozygotes, in turn, demonstrated increased appearance of malignant tumours developed with age.

Mammals possess also extracellular Cu,Zn-SOD (EC-SOD) encoded in humans by gene SOD3. The enzyme is a homotetramer presenting in plasma, lymph, and synovial fluid (Forsberg et al., 2001). Extracellular SOD is abundant particularly in the lung, blood vessels, and the heart. In blood vessels EC-SOD activity can reach up to 50% of total SOD activity (Gongora & Harrison, 2008). Consequently, polymorphism of SOD3 gene is associated with pulmonary and cardiovascular diseases. It was shown, that mice lacking EC-SOD were more susceptible to hyperoxia, had vascular dysfunctions and were predisposed to hypertension (Carlsson et al., 1995). The ability to bind extracellular matrix proteoglycans containing heparan and hyaluronan sulfates is a distinguishable property of EC-SOD (Dahl et al., 2008). Additionally, this enzyme can associate with collagen type I and fibrillin 5 (Gongora & Harrison, 2008). Due to this binding capacity, EC-SOD can operate locally on the surface of endothelial cells. The most known polymorphism came from C-to-G transversion in the second exon of SOD3 gene, leading to the substitution of arginine-213 by glycine (R213G) (Chu et al., 2005). The substitution is located closer to C-terminal end of the protein (whole enzyme consists of 251 amino acid residues) and affects heparin-binding domain. Hence, the mutated enzyme maintains the activity, but fails to attach the surfaces of endothelial cells and also possesses higher resistance to trypsin-type proteinases. The 8-10-fold increase in heterozygotes, and up to 30-fold increase in homozygotes in serum SOD activity are reported for the persons with the R213G polymorphism (Fukai et al., 2002). Studies *in vitro* showed that EC-SOD with R213G substitution lost approximately one third of capacity to bind type I collagen. Nevertheless, binding to the endothelial cell surface was dramatically decreased (Dahl et al., 2008), whereas only tissue-bound EC-SOD can be vasoprotective (Chu et al., 2005). Different studies found association between R213G substitution in EC-SOD with increased risk of ischemic cardiovascular and cerebrovascular diseases for diabetic patients or individuals with renal failure on hemodialysis (Nakamura et al., 2005).

Polymorphism of glutathione peroxidase (GPx) was found to be associated with some cancers. Four GPx isoforms have been described in humans. It was found that mutations in exon 1 of human GPx-1 gene lead to appearance of polyalanine tract at N-terminus of the protein (Forsberg et al., 2001). These tracts themselves are not connected with diminished enzyme activity. Another polymorphism, substitution of proline-198 to leucine, was found in Japanese diabetic patients and associated with intima-media thickness of carotid arteries (Hamanishi et al., 2004). The same substitution for adjacent proline-197 was associated with lung and breast cancers, as well as with cardiovascular diseases (Forsberg et al., 2001). Mice knockouted in GPx-1 and GPx-2 developed intestinal cancers (Halliwell, 2007).

2.5 Polymorphism of enzymes involved in reparation of oxidized molecules

Mutations may also affect enzymes involved in DNA reparation. The enzyme 8-hydroxy-2'-deoxyguanosine glycosylase (hOGG) encoded in human genome by the gene hOGG1 is probably the most known example. Recent studies associate mutations in hOGG1 with different cancer types, such as lung, stomach and bladder cancers (Sun et al., 2010). Most of the mutations in this gene affect exon 7 and cause serine-to-cysteine substitution. It was demonstrated that substitution S326C in hOGG1 protein confers susceptibility to oxidation and makes the enzyme prone to form disulfide bond between different polypeptide chains (Bravard et al. 2009). The possibility to form disulfide bonds in the mutated hOGG1 is additionally supported by the fact that serine-326 in the protein is flanked by positively charged amino acid residues, lysine and arginine, from N-terminus and arginine and histidine from C-terminus (Bravard et al. 2009). Such amino acid cluster is thought to increase possibility for disulfide bond formation in the case of serine-to-cysteine substitution. Some studies showed that hOGG1 overexpression lead to inhibition of H_2O_2-induced apoptosis in human fibroblasts (Youn et al., 2007).

Hydrolase MTH1 is other important enzyme preventing incorporation of oxidized purine nucleotide triphosphates in DNA (Nakabeppu et al., 2006). Knockout of this enzyme in mice resulted in increased frequency of lung, stomach and liver tumours with age (Halliwell, 2007).

Glutathione S-transferases (GSTs) are recognised as important antioxidant enzymes. However, they have broader function consisted in conjugation of different electrophilic compounds with glutathione (Hayes et al., 2005). Oxidatively modified compounds as well as lipid oxidation products, like 4-hydroxy-2-nonenal, are subjected to conjugation with glutathione. In general, GSTs are belong to xenobiotic-elimitating system. Some of them, namely GSTs of μ class, are known well by their ability to eliminate polycyclic aromatic hydrocarbons, oxidized previously by cytochrome P450 monooxygenases. To date, eight classes of GSTs have been described: α, κ, μ, σ, ζ, π, θ and ω. Cytosolic enzymes belong to classes α, μ, π and θ (Konig-Greger, 2004). The gene coding GSTM1 (GST of μ class, isoform 1) is appeared to be highly polymorphic and found inactivated in half of human population. Several studies associate polymorphism of GSTM1 with lung cancer (Ford et al., 2000; Forsberg et al., 2001; Mohr et al., 2003), although reports are controversial. For example, meta-analysis conducted by Benhamou *et al.* (2002) found no association of GSTM1 null genotype with lung cancers as well as with smoking. Other authors found such association and reported increased susceptibility to cancerogens among Caucasian and African-American populations (Cote et al., 2005). Polymorphism of GSTM1 was also found to be associated with head and neck carcinomas (Konig-Greger, 2004). The need in GSTM1 and its role in prevention of lung cancer are explained by the ability of the enzyme to detoxify constituents of cigarette smoke, such as mentioned above polycyclic aromatic hydrocarbons. Some studies also associate lung cancer with polymorphism of GSTT1 (GST of θ class) which participates in catabolism of tobacco smoke constituents, such as halomethanes and butadione (Cote et al., 2005). Similar association was found for GSTP1 (GST of π class) (Wenzlaff et al., 2005). Substitution of isoleucine-105 to valine, resulting from a single nucleotide polymorphism at exon 5 of GSTP1 gene, lead to considerably decreased activity of the enzyme. Moreover, it was shown earlier that polymorphism of glutathione S-transferase P1 confers susceptibility to chemotherapy-induced leukaemia (Allan et al., 2001).

Mechanism for regulation of GSTs by carcinogenic events was described recently (McIlwain et al., 2006), and involves GSTs of different classes in signalling pathways. It was shown that GSTPs can associate with c-Jun N-terminal kinase (JNK) preventing its phosphorylation. Oxidation of GSTPs under oxidative stress causes dissociation of the enzyme from JNK and allows phosphorylation of the latter. Thus, phosphorylated JNK triggers signalling pathways promoting either proliferation, or apoptosis (McIlwain et al., 2006). Similar mechanism was proposed for GSTM which can interact with apoptosis signalling kinase-1 (ASK1) preventing its autophosphorylation. In both cases, GSTPs and GSTMs, oxidative modification of the proteins plays a central role.

3. Role of oxidative modifications of antioxidant and related enzymes in disease progression

From examples, ascribed above, it was seen that sometimes genetic polymorphism of genes coding antioxidant enzymes may result in serious consequences to health, while in other cases, like ALS, it leads to lethality. It is very important to understand how oxidative stress is developed under full or partial loss of activity of certain antioxidant enzyme, how it can be replaced by cellular resources, and what leads to development of certain disease. It is important that in some cases antioxidant enzymes themselves can be targets for ROS attack. Moreover, operation of many antioxidant enzymes depends on the availability of cofactors and prosthetic groups listed in Table 1.

Enzyme	Prosthetic group or cofactor
Cu,Zn-Superoxide dismutase	Ions of copper and zinc
Mn-Superoxide dismutase	Manganese ions
Catalase	Haem, iron, NADPH
Glutathione peroxidase	Reduced glutathione
Glutathione reductase	Flavin adenine dinucleotide

Table 1. Requirements of antioxidant enzymes in cofactors and prosthetic groups

Many disorders related to the metabolism of transition metals, amino acids or low molecular mass reductants are known to be connected with activities of antioxidant enzymes. Particularly, impairement in selenium uptake or synthesis of selenocysteine needed for glutathione peroxidases may lead to GPx deficiency and subsequent disorders such as cardiovascular ones (Lubos et al., 2007). Disruption of iron-sulfur clusters by superoxide anion radicals or peroxynitrite leads frequently to impairment of many metabolic pathways. Indeed, aconitase, NADH-ubiquinone-oxidoreductase (complex I of mitochondrial electron transport chain), ubiquinol-cytochrome c oxidoreductase (complex III), ribonucleotide reductase, ferredoxins possess iron-sulfur clusters, susceptible to oxidation. Owing to this, aconitase is used as one of oxidative stress markers (Lushchak, 2010). On the other hand, iron is a component of haem, a prosthetic group in catalase holoenzyme. Susceptibility to oxidative modification is described for catalase, glutathione peroxidase, Cu,Zn-SOD (Pigeolet et al., 1990; Tabatabaie & Floyd, 1994; Avery, 2011), and G6PDH (Lushchak & Gospodaryov, 2005). The latter is believed to be one of the most susceptible to oxidation enzymes (Lushchak, 2010). Thus, oxidative stress induced by exogenous factors, like carcinogens, certain drugs, ions of transition metals, etc., or by metabolic disorders, like

diabetes, can be exacerbated by oxidative modification of antioxidant enzymes. These assumptions demonstrate the potential of antioxidant therapy in particular cases.

At some pathological states, whatever the cause of the disease, oxidative stress is seen to be a powerful exacerbating factor. Type II diabetes, cardiovascular diseases and neurodegenerative diseases, associated with protein aggregation are among such pathologies. Indeed, enhanced level of glucose results in higher probability of protein glycation (Wautier & Schmidt, 2004). Products of amino acid glycation, like N^{ε}-carboxymethyllysine, pyralline, pentosidine can activate receptors to advanced glycation end products (Huebschmann et al., 2006). In turn, these receptors can activate endothelial NADPH oxidase, the enzyme which produces superoxide radicals (Sangle et al., 2010). The ability of β-amyloid to produce reactive oxygen species has been described in many studies (reviewed in Sultana & Butterfield, 2010). Enhanced ROS production was also found in neurons at ALS (Liu et al., 1999). Loss or inhibition of mitochondrial respiratory chain complex I in dopaminergic neurons is described for Parkinson's disease (Marella et al., 2009). Such inhibition of complex I intensifies production of superoxide anion radical by mitochondrial respiratory chain (Adam-Vizi, 2005). It would be important to mention that most of the mitochondrial diseases are caused by loss of complex I subunits (Scacco et al., 2006). Thus, oxidative stress can be crucial factor promoting cell death at mitochondrial diseases.

4. Model organisms for study oxidative stress involvement in diseases

Model organisms, such as mice, fishes, fruit flies, nematodes, plants, cell cultures, budding yeast, or even bacteria, are broadly used to study different aspects concerning connection between oxidative stress and diseases. The necessity of these model studies is linked with relative rarity of some diseases, like ALS or Huntington disease, as well as with well known difficulties of studying humans. Clinical studies or case analyses give raw material for further investigation of mechanisms using lower organisms or cell cultures. Disclosing of function for protein encoded by the gene *TTC19* provides the example of such studies (Ghezzi et al., 2011). The works started from the analysis of several clinical cases, followed by studies with cell cultures and fruit flies to get mechanistic explanation for function of the mutated protein. Usage of mice or rats, as mammallian models, lower organisms and cell cultures is beneficial in view of relative easiness of specific knockout production. However several caveats exist regarding possible artifacts which could be obtained with model studies. Possible sources of artefacts resulting from cell culture studies were reviewed by Halliwell (2007). The main point here is that widely used conditions for cell culture growth may confer mild oxidative stress itself, because of high oxygen partial pressure and lack of antioxidants in cultural media. The examples described above, demonstrate relatively easy ways to produce transgenic mice with knockout on the whole gene. Nevertheless, some cases require expression of properly mutated gene than no expression at all, like in the case with mutated Cu,Zn-SOD at ALS. In the situation with ALS, only mutated gene induces pathology, while mice with Cu,Zn-SOD knockout are viable (Sentman et al. 2006). However, it is known that polymorphism of Mn-SOD in humans, e.g. A16V substitution, is not characterized by severe phenotype (Ma et al., 2010), while mice with Mn-SOD knockout are not viable (Halliwell, 2007). Fish, fruit flies, worms, plants and yeasts have even more disadvantages because of larger difference between their and human metabolism. However, usage of these model organisms affords to study particular mechanisms of the phenomena.

Whole genome sequences for most of the lower organisms were obtained during last two decades, what is also beneficial

Elucidation of molecular mechanisms of Friedreich's ataxia, a progressive hereditary neurological disorder (Kaplan, 1999; Knight et al., 1999), and discovery of chaperon for Cu,Zn-SOD (Culotta et al., 2006) are the most striking examples, proving merits of studies on model organisms. Moreover, all genes responsible for transport and utilization of iron in humans are mapped against yeast homologs (Rouault & Tong, 2008). It is also worth mentioning that details of copper transport, underlying Wilson and Menke diseases, were also discovered on the budding yeast model (Van Ho et al., 2002). This organism was used also for uncovering mechanisms of amyotrophic lateral sclerosis and allowed to find many details of posttranslational maturation of apo-Sod1 protein (Furukawa et al., 2004).

Budding yeasts were also used for advancement in understanding of redox-sensor capabilities for several regulatory proteins. One of them is AP-1 (activator protein 1) which in yeast is presented by YAP1 ortholog and is the central regulator of expression of the genes coding antioxidant enzymes (Lushchak, 2010). In yeast, but not in mammal cells, AP-1 operation is regulated by reversible oxidation of cysteine residues with subsequent translocation of the protein from cytoplasm into nucleus (Lushchak, 2011). It was also shown that reactive nitrogen species can activate YAP1 also (Lushchak et al., 2010). There are also several studies on budding yeasts revealed changes in gene expression and protein synthesis in response to oxidants (Godon et al., 1998; Thorpe et al., 2004; Temple et al., 2005). Experiments conducted in our laboratory with acatalatic budding yeast had shown that the loss of mitochondrial catalase is not crucial for yeast surviving, while loss of the cytosolic enzyme or both isoenzymes lead to serious growth retardation and is accompanied by inhibition of other enzymes (Lushchak & Gospodaryov, 2005). Interestingly, like in some cases with antioxidant enzyme deficiencies in humans, only special conditions revealed catalase deficiency in the yeast, e.g. ethanol consumption. The similar data were obtained with yeast strains deficient in either Cu,Zn-SOD or Mn-SOD, or both isoenzymes (Lushchak et al., 2005b). Glycerol as a carbon source was an exacerbating factor, which forced cells to perform respiratory metabolism instead more common for *Saccharomyces cerevisiae* fermentation. As in the case with catalase, loss of SOD lead to decreased activities of whole bunch of important enzymes, including antioxidant ones. Surprisingly, the cells deficient in Cu,Zn-SOD demonstrated more dramatic decrease in the activity of isocitrate dehydrogenase than the cells deficient in both SOD isoenzymes (Lushchak et al., 2005b). On the other hand, inhibition of Cu,Zn-SOD by *N,N'*-diethyldithiocarbamate increased the activity of NADPH-producing enzymes and glutathione reductase while decreased catalase activity (Lushchak et al., 2005a). The latter work clearly showed that the highest content of protein carbonyls was associated with moderate SOD activities. The situation is somewhat reminiscent to ALS pathology where oxidative damage results from the aggregates of mutated SOD.

Fruit fly *Drosophila melanogaster* is emerging model to study mechanisms of commonly known neurological diseases and diabetes (Pandey & Nichols, 2011) and diabetes (Kühnlein, 2010; Pandey & Nichols, 2011). However, despite advances in these fields done on *D. melanogaster*, it seems that fruit fly is the also good organism to investigate regulatory mechanisms underlying development of oxidative stress at neurological diseases. Fruit flies, unlike budding yeast, share more properties of signalling machinery with that for humans. Hence, Drosophila seems very convenient model organism to investigate ways of ROS

production connected with numerous signalling pathways, like those involved c-Jun N-terminal kinase or transcription regulators NF-κB, p53, FoxO, TOR or AP-1.

5. Conclusions and perspectives

Oxidative stress can be induced in different ways, some of which are connected with known pathologies. Deficiencies in antioxidant or related enzymes are the factors which can result in oxidative stress. Genetic polymorphism is the most common cause of these deficiencies. Despite importance of such enzymes as catalase and glucose-6-phosphate dehydrogenase, deficiencies in them are rarely characterized by severe phenotype and become obvious often under special conditions. For glucose-6-phosphate dehydrogenase deficiency, consumption of redox-active compounds with food or drug treatment are risk factors. Polymorphism of copper and zinc-containing superoxide dismutase may lead to the development of amyotrophic lateral sclerosis, a severe neurodegenerative disorder. Polymorphism of genes coding glutathione peroxidases and enzymes involved in repair of oxidative injuries are frequently associated with carcinogenesis. Many antioxidant and related enzymes are themselves susceptible to oxidative modification. At enhanced ROS steady-state levels in some pathological states, like diabetes and cardiovascular or neurodegenerative diseases, oxidative modification of antioxidant enzymes may exacerbate the disease. It was found that antioxidant enzymes like Cu,Zn-SOD, catalase, glutathione peroxidase and others are susceptible to oxidative modification. Thus, oxidative stress caused initially by environmental factors and conditions, like hypoxia, drug metabolism, metal ions (e.g., chromium, cobalt, nickel, high concentrations of iron and copper) in particular situations could be enhanced and exacerbated by the following deficiency of antioxidant enzyme activity. And, vice versa, deficiency in particular antioxidant enzyme can be revealed by specific environmental conditions like drug metabolism at G6PDH deficiency, hyperoxia in case of lack of mitochondrial superoxide dismutase, or tobacco smoke in case of glutathione S-transferase. Regardless, whether primary or secondary role oxidative stress plays in disease progression, antioxidant therapy looks promising approach especially in combination with specific drugs. Nevertheless, it should be applied cautiously taking into account that antioxidants may acquire prooxidant properties dependently on conditions. Moreover, excess of reductants like glutathione may lead to the development of reductive stress and make cells insensitive to apoptotic signals, consequently converting them into neoplastic, prone to form tumours.

To date, there is quite large collection of data indicating the association of polymorphism of certain enzymes with cancers, neurodegenerative, cardiovascular diseases, diabetes, etc. Unfortunately, the roles of antioxidant and related enzymes in disease progression are still poorly understood. The huge work has been done to disclose the role of Cu,Zn-SOD in amyotrophic lateral sclerosis, and although many aspects are still obscure, it seems to be the issue attracting high attention. On the other hand, association of Mn-SOD and GPx deficiencies with certain diseases remains controversial. In all cases, oxidative modification of essential compounds, like proteins, lipids, carbohydrates and nucleic acids, is underestimated. For instance, changes in susceptibility of amyloid precursor or tau protein to oxidation may be a cause of their aggregation, similarly to Cu,Zn-SOD. However, information on this issue is scarce up to now; simply because of investigations do not go in

this direction. More attention is paid to consequences, and reports on development of oxidative stress under cardiovascular diseases, diabetes, and neurodegeneration, are widely spread. It is difficult to catch conception of the disease; however properties of proteins and critical events leading to the disease could be modelled or studied *in vitro*. Despite significant limitations, a great impact from application of lower organism models, like budding yeast, nematodes and fruit flies, is appeared today. Turn to view on oxidative stress as a cause of the diseases due to damage of important molecules might considerably change therapeutic approaches. Such knowledge on oxidative stress as a trigger of the disease might also suggest necessary changes in human lifestyle and nutrition strategies for the prophylactics.

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Section 3

Cardiovascular Diseases

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3

Reactive Oxygen Species and Cardiovascular Diseases

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1. Introduction

Reduction or oxidation caused by addition or loss of any electron is responsible for alterations in functional and structural profile of molecules, hence, changing signaling mechanism. Reactive free radicals play a crucial role in different physiological mechanisms ranging from the immune defense to cell signaling and inflammation (Elahi & Matata, 2006). There is increasing evidence that irregular production of free radicals lead to enhanced stress on cellular structures and causes changes in molecular pathways that underpins the pathogenesis of several relevant human disorders, such as cancer, heart diseases, the process of physiological ageing and neurological diseases (Pacher & Szabo, 2008; Lushchak, 2011a; Lushchak, 2011b). Comprehending the involvement of free radical stress in the pathogenesis of disease will allow us to investigate the development of oxidative stress; a condition that occurs due to an imbalance between cellular production of oxidant molecules and the availability of appropriate antioxidants species that defend against them. It is hoped that this knowledge will subsequently lead to the development of effective therapeutic interventions against oxidative stress.

The main molecules that are involved in redox signaling are called as reactive oxygen species (ROS), in which we may include hydrogen peroxide (H_2O_2), nitric oxide (NO), hydroxyl radical, superoxide ($O^{2\bullet-}$) and peroxynitrite. Current redox signaling investigations indicate that all the vascular constituents, including vascular smooth muscle cells (VSMCs), endothelial and adventitial cells and macrophages, produce ROS (Papaharalambus & Griendling, 2007). ROS are involved in signal transduction which is related to relaxation and contraction of blood vessels, migration, growth and death of vascular cells, and also extracellular matrix (ECM) alterations.

It is known that vascular diseases such as peripheral vascular disease, coronary artery disease and cerebrovascular diseases are the largest cause of morbidity and mortality in industrialized countries. Some common risk factors for vascular disease, including diabetes and hypertension are still prevalent in Western and other populations, indicating that vascular disease will possible continue to impose a substantial burden on health care resources throughout the next generation. The earliest detectable changes in vascular

disease states are irregularities of the endothelium, resulting in loss of the endothelium's normal homeostatic functions that normally act to inhibit disease-related mechanisms such as thrombosis and inflammation. Particularly, it was previously demonstrated that nitric oxide (NO) produced by NO synthase (eNOS) in the vascular endothelium modulates blood flow and pressure and presents important antiatherogenic effects on platelets, vascular smooth muscle and endothelial cells (Umans and Levi, 1995).

Many previous studies have already demonstrated the effects of oxidative stress on the cardiovascular system. Superoxide dismutase (SOD), an enzyme that catalyze the dismutation of $O^{2\bullet-}$ into oxygen and H_2O_2, injected into brainstem areas involved in cardiovascular regulation decreased sympathetic nerve activity and decreased blood pressure in swine (Zanzinger and Czachurski, 2009). According to Campese et al. (2004) the lack of low-density lipoprotein (LDL) receptor-enhanced cholesterol blood levels enhanced ROS and impared baroceptor reflex function. Monahan et al (2004) indicated that oxidative stress collaborates to age-associated decreases in cardiovagal baroreflex sensitivity in healthy subjects. Conversely, it was indicated in male smokers that circulating antioxidants had no effect on baroceptor reflex, and minor effects on the cardiovascular system were seen following acute fat and vitamin ingestion (Wright et al, 2009). Overall, comprehending the process which redox signaling modulates cardiovascular system will provide further precise ROS regulation as a therapy for cardiovascular disorders. In this chapter we summarize concepts regarding oxidative stress related to cardiovascular disorders.

2. Models of ROS-induced cardiovascular diseases

Basic science applied in animal is indispensable to comprehend the pathogenesis, mechanisms involved in therapeutic agents, molecular process, and environmental or genetic factors that increases the risks of disease development. The species of animal studied are influenced by numerous aspects. Usually, animals with small size are preferred because they are more manageable and experiments are less expensive. According to the guide of the principles of animal research, we should use the lowest possible animal and, nowadays, permission would not be granted for using larger animals unless a similar experiment could not be performed on rodent. Nevertheless, a major criticism of using rodents is that they may not adequately correspond to the human situation and this fact occasionally justifies the use of larger animals such as pigs and monkeys (Rees & Alcolado, 2005). In this topic we described the main animal models used in the literature to investigate the mechanisms involved in ROS-induced cardiovascular disorders.

The relationship between enhanced ROS and hypertension is well established in many studies involving diet or endocrine-induced and surgically-induced hypertensive animals (Banday et al, 2007). A variety of evidence suggests that ROS collaborate to impaired endothelial function in several forms of hypertension and that there is enhanced ROS in the microvessels of spontaneously hypertensive rats (SHR) and Dahl salt-sensitive hypertensive rats (Manning et al, 2003). An interesting study by Lenda et al (2000) suggested that ROS can also collaborate to a decreased endothelium-dependent dilation in normotensive rats under an enhanced salt diet. Although the latent level of ROS in contributing to damaged endothelium-dependent vasodilation and decreased NO production during increased dietary salt intake, the nature and mechanisms of the impaired vascular relaxation with the

high-salt diet and the role of enhanced ROS in contributing to salt-induced changes in vascular function and hypertension are not completely understood (Cai & Harrison, 2000).

Hypertension is a result of enhanced ROS, however, data regarding the most potent cause of ROS-induced hypertension is controversial. In fact, oxidative stress does not elucidate the cause of every kind of hypertension, which develops through many processes. A small number of clinical investigations indicated the protective property of antioxidants (Ceriello et al, 1991; Galley et al, 1997). Nevertheless, it is known that not all animal models of hypertension are related to ROS (Rajagopalan et al, 1996). Additionally, clinical studies demonstrated negative correlation between arterial pressure and oxidative stress markers in subjects with mild to moderate hypertension (Cracowski et al, 2003). It is hard to find a cause–effect association between hypertension and oxidative stress in clinical studies, however, some studies indicated that increased ROS is a risk factor for human hypertension (Adbilla et al, 2007).

Animal models are important to support the link between hypertension and oxidative stress. Some procedures performed in animal models helped to comprehend the mechanisms involved in ROS-induced hypertension (Rajagopalan et al, 1996; Puzserova et al, 2010; Valenti et al, 2011a).

The group of Zucker and coworkers from Nebraska have used a model based on heart failure in rabbits (Mousa et al, 2008). According to their method, a platinum wire pacing electrode is sutured to the epicardium of the left ventricle in the rabbits. A ground electrode is secured to the left atrium. All wires are tunneled beneath the skin and exited in the midscapular area. The chest is closed and evacuated; in the same setting, a radiotelemetry unit is implanted into the right femoral artery with the tip of its catheter in the descending aorta to monitor blood pressure and heart rate in the conscious state. Rabbits were allowed to recover from surgery for two weeks before they were used in the study. They developed a rapid pacing model of chronic heart failure. After recovery from surgery, animals are paced at a rate of 360–380 beats/min with the use of a small, light-weight pacing unit of their own design. The pacing rate is adjusted and monitored by frequent echocardiograms. In general, each rabbit is paced at 360 beats/minute for the first week to determine whether it would tolerate this protocol. After the first week, the pacing rate is enhanced to 380 beats/minute and continued at this rate for the remainder of the protocol. The rabbits are continuously paced for 3 weeks. Cardiac dimensions (left ventricular end-diastolic diameter, left ventricular end-systolic diameter, fractional shortening, and ejection fraction) and other hemodynamic parameters are monitored on a weekly basis. Additionally, to left ventricular dimension changes, clinical signs of chronic heart failure such as ascites, pulmonary congestion, and cachexia are appreciated as symptoms of this chronic heart failure model. This model is well accepted in the literature and it was observed enhanced production of ROS in the heart and in the brainstem. Furthermore, they measured two of the three major SOD isoforms (Cu,Zn-SOD and Mn-SOD) and the catalytic subunit of NAD(P)H oxidase, gp91phox into the brainstem. They reported that protein expression of both CuZn SOD and Mn SOD was significantly downregulated in the chronic heart failure condition. The gp91phox protein was significantly enhanced in chronic heart failure rabbits (Gao et al, 2007). The more affected area was the rostroventrolateral area of the medulla oblongata.

The renovascular model of hypertension is a model which presented enhanced level of ROS (Campos et al, 2011). In this model, rats are anesthetized with ketamine and xylazine (40 mg and 10 mg/kg, respectively, ip); the left renal artery is exposed through an abdominal incision, and the renal artery and renal vein are dissected free from the adherent tissues. The left renal artery is partially obstructed with a silver clip of 0.2-mm width. No clip obstruction is applied to the sham-operated group (n = 15). Animals are submitted to the final experimental procedures 3 or 6 weeks after the surgical procedure. Systolic blood pressure is measured in conscious rats using a pneumatic tail-cuff method.

The role of ROS has been shown in this model of ROS-induced cardiovascular injury, in which the chronic administration of a SOD mimetic, tempol, to reduce ROS was shown to reduce blood pressure (Welch et al, 2003). Moreover, tempol was more effective than AT1 antagonist (candesartan) in reducing blood pressure and in improving renal function in renovascular hypertensive rats, suggesting that ROS plays an important role in mediating of renovascular hypertension (Palm et al, 2010). However, besides the actions of ROS on many tissues, the brain is one of the Ang II targets most affected by ROS. Even when an increase in plasma renin activity is modest after moderate renal artery stenosis, ROS remains increased and collaborates to hypertension (Lerman et al, 1991).Therefore, the involvement of Ang II is believed to decline, whereas ROS increases, during the progression of the 2K1C model. Our hypothesis is that even with a modest increase in circulating Ang II, this peptide acting in the CNS through AT1 receptors might collaborate to NADPH activation, which leads to an increase in local ROS production, causing sympathoexcitation and arterial hypertension.

The central regulation of the sympathetic nervous system (SNS) involved in cardiovascular regulation is complex, involving multiple reflex pathways and neural connections with a large number of neurotransmitters and neuromodulators acting in specific groups of neurons in the CNS involved in the tonic and reflex control of the cardiovascular system. In the CNS, Ang II is able to increase sympathetic vasomotor tone and blood pressure, and is involved in the pathogenesis of many experimental models of hypertension (Campos, 2009). Therefore, the close functional association between NADPH oxidase and the Ang II is of particular relevance in linking oxidative stress in the brain to sympathoexcitation and hypertension. For instance, intracerebroventricular infusion of NADPH oxidase inhibitor antagonizes the pressor response induced by centrally mediated Ang II actions (Gao et al, 2007). In the brain, the overexpression of SOD, an enzyme responsible for $O^{2\bullet-}$ breakdown, also abolishes the central pressor effect of the octapeptide, suggesting that in the CNS there is a positive correlation between the increase in ROS and the central pressor response mediated by Ang II (Zimmerman et al, 2004). Considering that the paraventricular nucleus of the hypothalamus (PVN) and the rostroventrolateral medulla (RVLM) contain critically important neurons involved in the control of sympathetic vasomotor tone and arterial pressure (Valenti et al, 2011a), in the studies reviewed in this article it was examined an increase in AT1 receptor expression and oxidative stress markers within these two nuclei in renovascular hypertension. NAD(P)H oxidase subunits (p47phox and gp91phox) and anti-oxidant enzyme CuZnSOD mRNA expression were quantified in the RVLM and PVN of renovascular hypertensive rats. It was hypothesized that the overactivity of NADPH oxidase-derived ROS associated with a reduction in the activity of CuZnSOD within the RVLM and PVN could collaborate to renovascular hypertension, particularly in the renin-dependent phase of hypertension.

In hypertensive renovascular rats, there is a significant increase in systemic ROS, estimated by the thiobarbituric acid reactive substance (TBARS) level in plasma, compared with control rats. Administration of tempol or Vitamin C systemically decreased blood pressure and RSNA only in renovascular hypertensive rats, indicating that the depressor effect in response to the anti-oxidant administration is mediated by a reduction in sympathetic vasomotor activity (Oliveira-Sales et al, 2008).

Some studies evaluated the effects of ROS on vascular properties in rat aorta (Toba et al, 2010; Olukman et al, 2010). Others tried to reveal the mechanisms involved in ROS-induced cardiovascular disease inside the brainstem (Zanzinger et al, 2009; Valenti et al, 2011a; Campos et al, 2011).

The SHR is a model which has been well investigated (He et al, 2011). SHR and stroke-prone SHR (SPSHR), genetic models that develop hypertension spontaneously, exhibit enhanced NAD(P)H driven $O^{2\bullet-}$ generation in resistance (mesenteric) and conduit (aortic) vessels (Rodriguez-Iturbe et al, 2003). This is associated with NAD(P)H oxidase subunit overexpression and enhanced oxidase activity (Kishi et al, 2004). Several polymorphisms in the promoter region of the p22phox gene have been identified in SHR (Zalba et al, 2001). This has clinical relevance because an association between a p22phox gene polymorphism and NAD(P)H oxidase–mediated $O^{2\bullet-}$ production in the vascular wall of patients with hypertension and atherosclerosis has been described (Moreno et al, 2003).

Enhanced expression of p47phox has been reported in the renal vasculature, macula densa, and distal nephron from young SHR, suggesting that renal NAD(P)H oxidase upregulation precedes development of hypertension (Kishi et al, 2004). Diminished nitric oxide bioavailability as a consequence of enhanced vascular $O^{2\bullet-}$ generation and downregulation of the thioredoxin system may also collaborate to oxidative stress in SHR and SPSHR (Touyz 2003). Treatment with antioxidant vitamins, NAD(P)H oxidase inhibitors, SOD mimetics, and BH_4 and Ang II type-1 (AT1) receptor blockers decrease vascular $O^{2\bullet-}$ production and attenuate development of hypertension in these models (Rodriguez-Iturbe et al, 2003; Shokoji et al, 2003). Taken together, these findings suggest that oxidative stress in genetic hypertension involves enhanced NAD(P)H oxidase activity and dysfunctional endothelial nitric oxide synthase (uncoupled NOS) and is regulated, in part, by AT1 receptors. Figure 1 presents a surgical procedure to record mean arterial pressure and heart rate, while Figure 2 shows recordings from one normotensive Wistar Kyoto and one SHR rat illustrating reflex bradycardia (top) in response to blood pressure increases. In this Figure we may observe the enhanced mean arterial pressure of the SHR compared to the control animal.

In animal models of diabetes, several functional and structural alterations of the heart or in cardiac muscle have been documented (Russel et al, 2006). In most studies of type 1 diabetes mellitus, diabetes are induced after administration of the pancreatic beta-cell toxin streptozotocin, and most studies of type 2 diabetes mellitus have been performed in genetic models of obesity and insulin resistance such as the Zucker fatty rat or db/db mice, both of which have mutations that impair leptin receptor signaling, or ob/ob mice, which lacks leptin. Furthermore, because diabetes mellitus develops at varying tempos in these models, it is important to bear in mind that studies performed in animals before the onset of diabetes may reflect changes that are secondary to the underlying obesity and insulin resistance, and

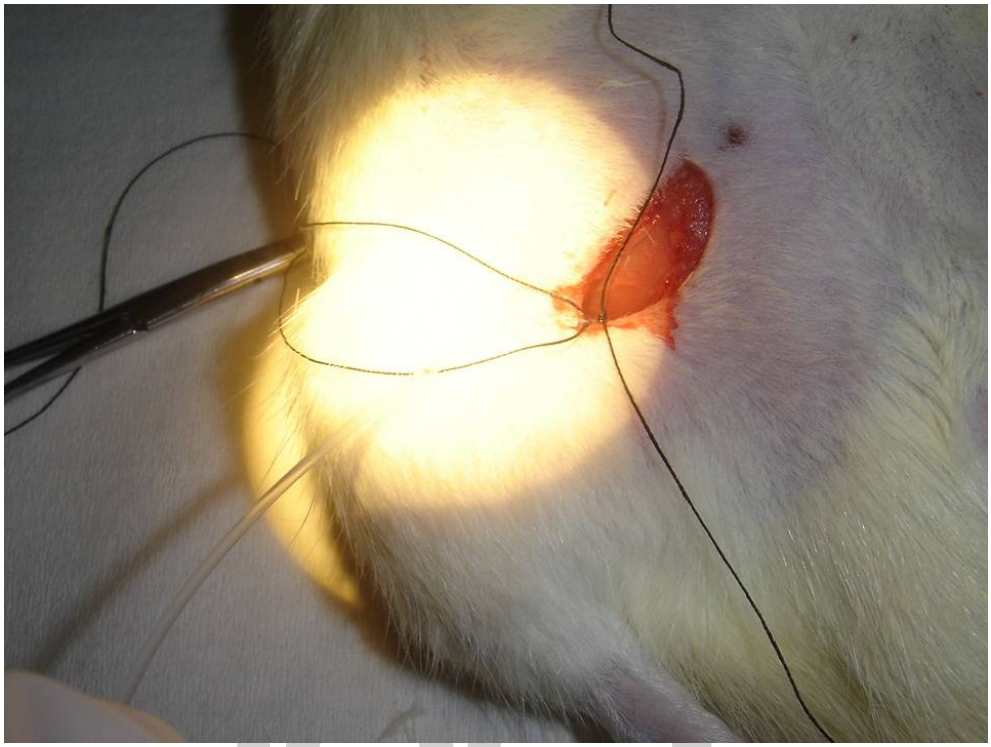

Fig. 1. Surgical procedure to record basal mean arterial pressure and heart rate in one spontaneously hypertensive rat.

studies performed after the onset of diabetes may reflect the added effects of hyperglycemia of various durations. Most studies have been performed in isolated perfused hearts and reveal depressed cardiac function (Aasum et al, 2002; Aasum et al, 2003). In vivo studies in these rodent models have provided evidence for systolic and diastolic dysfunction by echocardiography (Christoffersen et al, 2003) but in some studies using invasive left ventricle catheterization in mouse models of obesity and diabetes mellitus, left ventricle contractility as determined by developed pressure/developed tension was initially enhanced and may reflect the impact of the enhanced plasma volume and perhaps sympathetic activation associated in part with the underlying obesity (Buchanan et al, 2005). These first observations were additionally clarified later (Van den Bergh et al, 2006). It was assessed the hemodynamic changes in db/db mouse hearts in vivo using a pressure–volume instrument. It was reported decreased contractility using load-independent variables such as preload recruitable stroke work, but steady-state measurements of cardiac output and other load-dependent parameters were increased in db/db mice compared with control mice because of favorable loading conditions, specifically enhanced preload and decreased afterload.

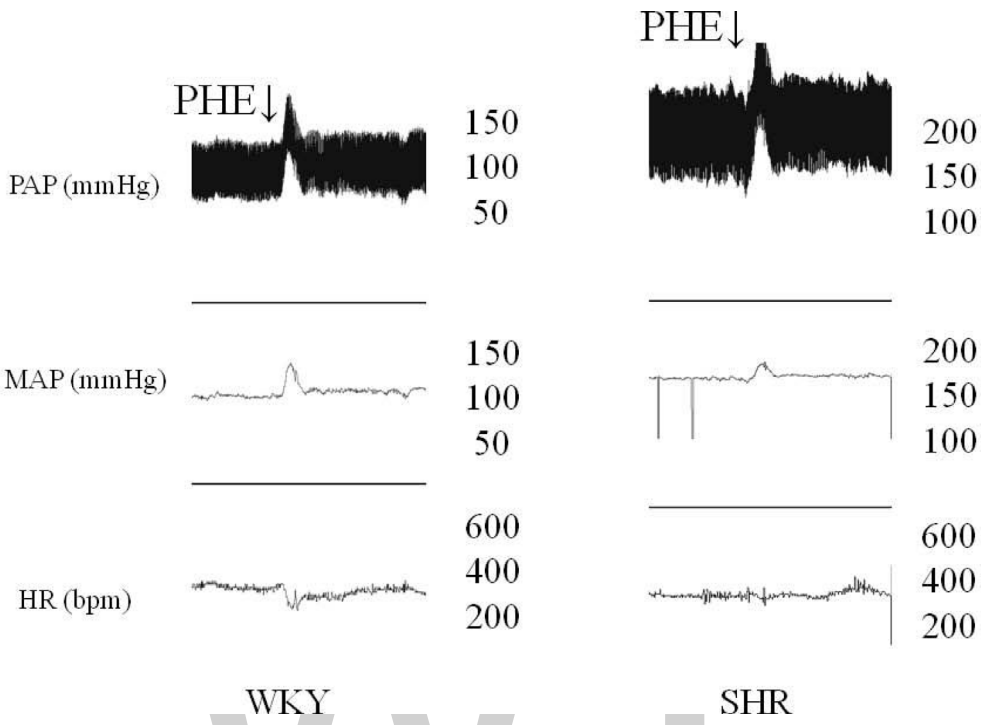

Fig. 2. Recordings from one Wistar Kyoto control rat and one spontaneously hypertensive rat illustrating reflex bradycardia (top) in response to blood pressure increases. Infusions were given in bolus. MAP: mean arterial pressure; PAP: pulsatile arterial pressure; HR: heart rate; PHE: phenylephrine.

Enhanced ROS production in the diabetic heart is a contributing matter to the progression and the development of diabetic cardiomyopathy (Cai et al, 2006). Cumulative superoxide-mediated damage or cellular dysfunction results when an imbalance exists in ROS generation and ROS-degrading pathways. Enhanced ROS generation and impaired antioxidant defenses could both collaborate to oxidative stress in diabetic hearts. Several groups have shown that ROS is overproduced in both type 1 and type 2 diabetes (Cai et al, 2006). Under physiological states, most of the ROS generated within cells arises from mitochondria. Whereas enhanced mitochondrial ROS generation has been shown in various tissues such as endothelial cells that are exposed to hyperglycemia (Brownlee, 1995), relatively few studies to date have directly measured mitochondrial ROS production in mitochondria obtained from diabetic hearts. However, overexpression of mitochondrial superoxide dismutase (SOD2) in the heart of a mouse model of type 1 diabetes mellitus reversed altered mitochondrial morphology and function and maintained cardiomyocyte function (Shen et al, 2006). Evidence also exists for enhanced production of ROS from non mitochondrial sources such as NADPH oxidase or decreased neuronal nitric oxide synthase (NOS1) activity coupled with enhanced activation of xanthine oxidoreductase (Saraiva et al, 2006). Whereas evidence for enhanced ROS production in diabetes mellitus is reasonably

strong, the effect of diabetes on antioxidant defenses in the heart is controversial. Thus, the activities/expression levels of glutathione peroxidase, copper/zinc SOD, or catalase were either enhanced (Li et al, 2006) or decreased (Matkovics et al, 1997). Enhanced ROS generation may activate maladaptive signaling pathways, which may lead to cell death, which could collaborate to the pathogenesis of diabetic cardiomyopathy. Enhanced ROS production was associated with enhanced apoptosis, as evidenced by enhanced in situ nick end-labeling (TUNEL) staining and caspase 3 activation in ob/ob and db/db hearts (Barouch et al, 2003). In the same study, enhanced ROS was also associated with enhanced DNA impairment and loss of activity of DNA repair pathways that declined more rapidly with age in diabetic versus control animals.

Therefore, enhanced ROS-modulated cell death is able to promote irregular cardiac remodeling, which ultimately may collaborate to the morphological characteristic and functional abnormalities that are associated with diabetic cardiomyopathy. In addition to causing cellular injury, enhanced ROS production might lead to cardiac dysfunction via other mechanisms. For instance, enhanced ROS has been proposed to amplify hyperglycemia-induced activation of protein kinase C isoforms, enhanced formation of glucose-derived advanced glycation end products, and enhanced glucose flux through the aldose reductase pathways (Brownlee et al, 1995), which may all collaborate to various ways to the development of cardiac complications in diabetes mellitus. Enhanced ROS also might collaborate to mitochondrial uncoupling, which could impair myocardial energetic metabolism in diabetes.

Strategies that enhance mitochondrial ROS scavenging systems have been demonstrate to be effective in decreasing diabetes-induced cardiac dysfunction. Overexpression of metallothionein, catalase, and manganese SOD (Shen et al, 2006) in the heart reversed diabetic cardiomyopathy in animal models of both type 1 and type 2 diabetes. Therefore, strategies that either reduce ROS or augment myocardial antioxidant defense mechanisms might have therapeutic efficacy in improving myocardial function in diabetes mellitus.

In summary, experimental protocols applied in animals are necessary to understand the pathological events involved in cardiovascular diseases development. However, there are differences between human and animals like rat and mouse. Thus, we should be careful when interpreting data aiming to apply in humans regarding processes related to therapeutic agents, molecular process, and environmental or genetic factors that enhances the risks for cardiovascular disorders development.

3. Sources of ROS in cells

The literature indicated that vascular cells, as well as cardiomyocytes and neurons, produce ROS, contributing to the development of disorders related to the cardiovascular system. Although several enzyme systems produce ROS, many of them are prevalent in pathologic processes. Among the main ROS generators we may include cytochrome P450, the mitochondrial respiratory chain, xanthine oxidase (XO), uncoupled endothelial nitric oxide synthase (eNOS), heme oxygenase, myeloperoxidase, lipoxygenase, cyclooxygenase and NADPH oxidases. Some of these systems have been proven to be relevant to hypertension (Lee & Griendling, 2008).

Others mechanisms involved in ROS production are better described in the chapters from this book. Thus, we will only briefly describe the main sources in this topic.

Based on the literature, the NOX family includes seven members, which are NOX1-5 and DUOX1-2. NOX2 NADPH oxidase is the predominant source of ROS production in humans (Nauseef, 2004). The main sources of ROS are phagocytic cells—neutrophils and macrophages. NOX2-NADPH oxidase is formed by functional transmembrane heterodimers, gp91 phox and p22 phox (also known collectively as the cytochrome b558), and four regulatory cytosolic subunits—p40 phox , p47 phox , p67 phox , and the small GTPase, Rac2. In the dormant state, cytochrome b558 resides in intracellular vesicles, while cytosolic Rac2 remains inactive in the guanosine diphosphate (GDP) bound state via interaction with RhoGDI (Ando et al, 1992). Upon the initiation of phagocytosis, GDP-Rac2 is converted to GTP-Rac2 through the activity of a Rac guanine nucleotide exchange factor. This allows for Rac2 translocation to the plasma or phagosomal membrane, thereby allowing the subsequent transit of cytochrome b558 from the vesicle to the membrane (Diebold and Bokoch, 2001). Concurrently, p47 phox is phosphorylated and undergoes a conformational change that now exposes two SRC-homology 3 regions to interact with the proline rich motif on p22 phox (Dusi et al, 1993). Furthermore, Phox homology domains on p47 phox allow for binding to phosphatidylinositol 3-phosphate (PI(3)P) and PI(3,4)P2, transient phosphoinositides that are generated only at the plasma membrane upon phagocytosis, thus, further stabilizing p47 phox localization to cytochrome b558 (Nauseef, 2004).

Among ROS sources, the NADPH oxidases are considered unique because from those components it is generated ROS in a highly regulated mode whereas ROS are generated as a by-product of enzymatic activity for all the other sources (Cave et al, 2006). Moreover, NADPH oxidases can stimulate further ROS production from one or more of the above enzymes, thereby being able to act as initiating sources of ROS. $O^{2\bullet-}$radical is the first moiety that is generated by NADPH oxidases (or most of the other sources) and can be rapidly dismutated to H_2O_2. The biological effects of ROS are expected to depend on the specific moiety generated, its localization and the relative balance between levels generated and the activity of antioxidant mechanisms; most signaling effects of ROS are considered to be mediated by H_2O_2 which is more stable and diffusible than $O^{2\bullet-}$.

XO is another potential source for ROS in vasculature. XO is a form of xanthine oxidoreductase that occurs in two different forms. The predominant form, xanthine dehydrogenase (XDH), can be converted into XO reversibly by direct oxidation of critical cysteine residues or irreversibly by proteolysis (Harris et al, 1999). XO is expressed mainly in the endothelium, and its expression and activity are enhanced by Ang II or oscillatory shear in a NADPH oxidase-dependent manner (Landmesser et al, 2007).

Mitochondrial electron transport generates $SO^{2\bullet-}$ as a side product of electron transport during oxidative phosphorylation. Most superoxide never escapes the highly reducing state of the mitochondrial matrix. If the $SO^{2\bullet-}$ generation is excessive, however, superoxide can escape to the intermembraneous space and cytosol via anion channels (Aon et al, 2004).

In summary, the investigation of the sources of reactive oxygen species is essential to describe new pathways involved in the pathogenesis of cardiovascular diseases as well as to develop new therapies to treat those disorders.

4. ROS in the heart

It is already known in the literature some mechanisms regarding the role of antioxidants during oxidative stress caused by ROS in the heart tissue. Among the enzymatic and non-enzymatic antioxidants involved in ROS-induced heart tissue injury we may include catalase, glutathione, SOD, ascorbid acid, melatonin, Vitamin C and E, among others. The antioxidant system in SHR cardiomyopathic hearts is induced, possibly due to events of enhanced ROS. This conditioning of the antioxidant system may help to overcome acute stress situations caused by ROS in the failing myocardium (Takimoto & Kass, 2007).

There are several potential sources of ROS in the heart with chronic heart failure (CHF). Excessive ROS derived from mitochondria have been shown in cardiomyocytes from experimental models of myocardial infarction and rapid pacing-induced heart failure (Ide et al, 2001). The enzyme xanthine oxidase produces $O^{2\bullet-}$ as a byproduct of the terminal steps of purine catabolism and recent studies suggest that it collaborates to oxidative stress in CHF. Xanthine oxidase expression and activity are enhanced in experimental models of CHF as well as in human end-stage CHF.

Nitric oxide synthase enzymes normally generate nitric oxide, but may instead generate $O^{2\bullet-}$ if this molecule becomes "uncoupled", a state that is mainly observed likely in the setting of lack of the BH4, which is a NOS cofactor or the NOS substrate l-arginine. NOS uncoupling and subsequent $O^{2\bullet-}$ production are implicated in the genesis of vascular endothelial dysfunction in patients with heart failure (Dixon et al, 2003).

In this context, infiltrating inflammatory cells are also an important source of ROS, mainly in conditions such as myocarditis and in the early stages after myocardial infarction. Recent evidence suggests that complex enzymes called NADPH oxidases are mainly important with regard to redox signalling in CHF and its antecedent conditions (Li et al, 2002). These enzymes catalyse electron transfer from NADPH to molecular oxygen, resulting in the formation of $O^{2\bullet-}$. NADPH oxidase activity has been found to be enhanced in experimental models of left ventricle hypertrophy and CHF as well as in end-stage failing human myocardium (Li et al, 2002).

Interestingly, ROS produced by NADPH oxidases can promote ROS generation by other sources, thereby increasing total levels of ROS. For instance, $O^{2\bullet-}$ from NADPH oxidase may oxidize and degrade BH4, thereby leading to NOS uncoupling, and this mechanism has been shown in diabetes and experimental hypertension (Verhaar et al, 2004). Similarly, NADPH oxidase-derived ROS may also activate xanthine oxidase (Li and Shah, 2004).

Previous studies have already investigated the relationship between the regulation of myocardial growth and death by NADPH oxidase. The classical phagocyte oxidase (gp91phox or Nox2) is also expressed in non-phagocytic cells in the heart, such as cardiomyocytes and fibroblasts (Bendall et al, 2002; Zhang et al, 2006). Activation of Nox2 requires stimulus-induced membrane translocation of cytosolic regulatory subunits, including p47phox, p67phox, p40phox, and Rac1, a small GTPase (Uhlinger et al, 1994). In resting cells, p47phox, p67phox, and p40phox form a ternary complex in the cytoplasm, whereas Rac associates with Rho-GDP dissociation inhibitor. When cells are stimulated with agonists for G protein-coupled receptors, such as angiotensin II (Ang II) type 1 receptors, p47phox is phosphorylated by protein kinase C, which in turn undergoes conformational

changes and allows the phox homology (PX) domain and the SH3 domain in p47phox to interact with phosphoinositides and p22phox in the membrane, respectively (Ago et al, 2004). As p67phox and p40phox interact with p47phox, this process leads to membrane translocation of p67phox and p40phox. Rac1 translocates to the membrane independently of p47phox and p67phox, where they form a functional complex with the Nox2-p22phox heterodimer, followed by a transfer of electrons to molecular oxygen (Quinn et al, 1993). Therefore, the activity of Nox2 is subjected to regulation through multiple mechanisms.

In relation to myocardial damage and NADPH oxidase, the loss of cardiomyocytes through apoptosis or necrosis causes impairment in cardiac function in the heart submitted to chronic myocardial infarction (Wencker et al, 2003). Oxidative stress is involved in the pathogenesis of apoptosis through various pathways, in which it is included activation of enzymes involved in pro-apoptotic signaling, for example, JNK, p38, ASK-1, and CaMKII (Matsuzawa and Ichijo, 2005), effects on the cellular anti-apoptotic signaling and direct effects of ROS on mitochondria, leading to cytochrome-c release.

Although excessive production of ROS by Noxs is detrimental, local and modest production of H_2O_2 and $O^{2\bullet-}$ by Noxs allows those component to function as signaling molecules, thereby mediating physiological responses. For instance, since Noxs are functional at low pO_2, Noxs may function as a sensor, and ROS generated by Noxs as a transducer, for hypoxia (Shiose et al, 2001). Erythropoietin (EPO) synthesis occurs in the renal tubular cells, where Nox4 is abundantly expressed (Lacombe et al., 1988). Since DPI, an antioxidant drug, not only blocks oxygen sensing but also inhibits Nox4 in renal tubular cells, it has been proposed that Nox4 is an O_2 sensor in the kidney and may regulate EPO production. The causative role of Nox4 in mediating EPO synthesis through its function as an O_2 sensor remains to be shown. Recently, a role of Nox4 in mediating angiogenesis during cardiac hypertrophy was reported. Pathological hypertrophy induces upregulation/activation of Nox4, which in turn causes stabilization of HIF-1α, upregulation of VEGF, and increases in angiogenesis (Zhang et al, 2006). It appears that the protective effect of Nox4 prevails under the authors' experimental conditions. It remains unknown, however, whether such a mechanism is sufficient to overcome increases in cell death and mitochondrial dysfunction directly caused by upregulation of Nox4 in response to hypertrophic stimuli (Ago et al, 2003).

In addition, the regulation of Ca^{2+} level in cardiac myocytes is centrally important not only in excitation–contraction coupling but also in many other processes such as the regulation of gene expression and cellular energetics. ROS are recognized to be capable of influencing cellular Ca^{2+} regulation at several levels, notably via redox alterations of key amino acid residues involved in the function and gating properties of intracellular and plasma membrane ion channels and transporters — e.g., L-type channels, the Na^+/Ca^{2+} exchanger, the sarcoplasmic reticulum (SR) ATPase (SERCA) and the ryanodine receptor (Hool and Corry, 2007). Recent studies have started to address the role of NADPH oxidase-derived ROS in these effects.

It has been reported that ryanodine receptor-mediated Ca^{2+}-induced Ca^{2+} release in rat cardiac myocytes is inhibited by an endogenous NADH oxidase activity in the SR, although the molecular nature of this oxidase was not established (Cherednichenko et al, 2004). In contrast to this study, Sanchez et al. (2008) reported the presence of Nox2 NADPH oxidase activity in canine cardiac SR and showed that oxidase activation enhanced S-

glutathionylation of ryanodine receptors and hence SR Ca^{2+} release — effects which were abrogated by apocynin (a purported Nox inhibitor, but which may act as a non-selective antioxidant). The same group also showed that oxidase activity and the effects on SR Ca^{2+} release were augmented by tachycardia (Sanchez et al, 2008). $O^{2\bullet-}$radical production by NADPH oxidase on the SR of bovine coronary artery smooth muscle cells has also been shown to regulate calcium-induced calcium release (Yi et al, 2006). In isolated cardiac myocytes, plasma L-type Ca^{2+} channel open-state probability was reportedly enhanced by endothelin-1 together with enhanced NADPH oxidase activity, effects which were abolished in cardiomyocytes pre-treated with a specific NADPH oxidase inhibitor, gp91ds-tat (Zeng et al, 2008). These studies suggest that NADPH oxidases may acutely regulate at least two channels directly involved in intracellular Ca^{2+} homeostasis, i.e. the L-type Ca^{2+} channel and the ryanodine receptor.

In summary, Figure 3 presents the main mechanisms involved in ROS-induced cardiovascular disorders. Many studies implicate ROS-generating NADPH oxidases in redox signaling in cardiovascular cells and involvement in pathological processes such as cardiac hypertrophy, fibrosis, apoptosis and ventricular remodeling.

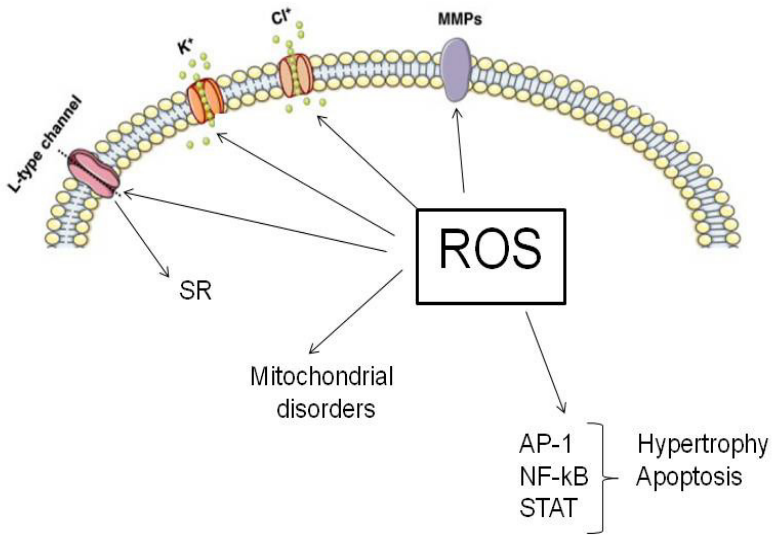

Fig. 3. Main mechanisms involved in the potential effects of NADPH oxidase-derived ROS in the cardiac myocyte. SR: sarcoplasmic reticulum; MMPs: matrix metalloproteinases; CICR: calcium-induced calcium release.

5. ROS-Induced vascular damage

ROS are involved in pathological and physiological processes in the vasculature. Enhanced arterial pressure is partially caused by enhanced total peripheral vascular resistance, which is due to disorders of structural remodeling of blood vessels and vasomotor function. Vascular diseases including coronary artery disease, cerebrovascular and peripheral

vascular diseases are the largest cause of mortality and morbidity in industrialized countries. Many common risk factors for vascular disease, such as hypertension and diabetes, remain prevalent in Western and other populations, suggesting that vascular disease will continue to impose a substantial burden on health care resources throughout the next generation. The earliest detectable changes in vascular disease states are irregularities of the endothelium, resulting in loss of the endothelium normal homeostatic functions that normally act to inhibit disease-related processes such as inflammation and thrombosis. In particular, nitric oxide (NO) produced by NO synthase (eNOS) in the vascular endothelium modulates blood flow and pressure (Umans & Levi, 1995) and has important antiatherogenic effects on platelets, vascular smooth muscle and endothelial cells.

It is known that ROS causes vascular tone increase, because it influences endothelium regulatory role and also due to its effects on vascular smooth muscle contractility. By influencing phenotype regulation of vascular smooth muscle cells, death of vascular cells, cell migration, atypical growth, and extracellular matrix (ECM) reorganization, ROS collaborate to vascular remodeling (Lee & Griendling, 2008).

The fact that nitric oxide (NO) is scavenged by superoxide suggests that superoxide production may in part underlie endothelial dysfunction in human atherosclerosis, as it does in some experimental models of vascular disease.

In vitro (Lambeth et al, 2000) and in vivo (Vita et al, 1990) studies indicate that ACh-mediated vasorelaxations in human vessels are inversely related to the number of atherosclerotic risks factors present. Nonetheless, functional studies of human vascular superoxide production have been more limited (Lambeth et al, 2000). It was found large variability in both NO mediated vascular relaxations and basal superoxide production in internal mammary arteries (Huraux et al. (1999), however, there was no consistent associations between these two parameters or with clinical risk factors (Lambeth et al, 2000).

It was investigated superoxide production by NAD(P)H oxidase in human vessels and the relationships between superoxide production, atherosclerotic risk factor profile and endothelial dysfunction. It was reported the expected inverse correlation between risk factor profile and NO-mediated endothelium-dependent relaxations in vessel ring isometric tension studies. However, it was also found that superoxide production by NAD(P)H oxidases progressively enhanced with increasing risk factor profile (Guzik et al, 2000). Furthermore, NAD(P)H oxidase-mediated superoxide production was inversely correlated with NO-mediated vasorelaxations in individual patients, such that patients with the highest superoxide production had the most deficient endothelial function.

The association between enhanced vascular NAD(P)H oxidase activity and impaired endothelial vasorelaxations may be due to direct scavenging of NO by superoxide, as has been demonstrated in animal model systems. However, the both could result independently from increasing exposure of endothelium, media and adventitia to factors acting through different signaling pathways. Alternatively, superoxide may directly modulate NO-mediated vascular signaling, for instance by peroxynitrite-induced nitration of G proteins or other membrane components (Feron et al, 1999). Previous data suggest that G protein-coupled receptor function is deficient in atherosclerosis (Liao & Clark, 1995). Previous observation that vasorelaxations to ACh were significantly lower than maximal relaxations

to the calcium ionophore A23187 is consistent with this hypothesis, and with observations in human internal mammary arteries (Hurax et al, 1999). Nevertheless, the significant correlation between ACh and A23187 - induced relaxations, and the association of NADH-dependent superoxide production with both ACh and A231287- stimulated vasorelaxations suggest that a change in G protein-coupled receptor signaling is unlikely to be the sole mechanism underlying decreased NO-mediated vasorelaxations, as A23187 activates endothelial NO synthase independently of any receptor mediated pathway. Alternatively, superoxide may impair endothelial function by direct effects on endothelial NO synthase activity, (Peterson et al, 1999), possibly mediated through oxidation of the NOS cofactor, tetrahydrobiopterin (BH4).

Many studies have focused on the potential role of BH4 oxidation different oxidized biopterin species in reducing BH4 bioavailability for eNOS (Figure 4).

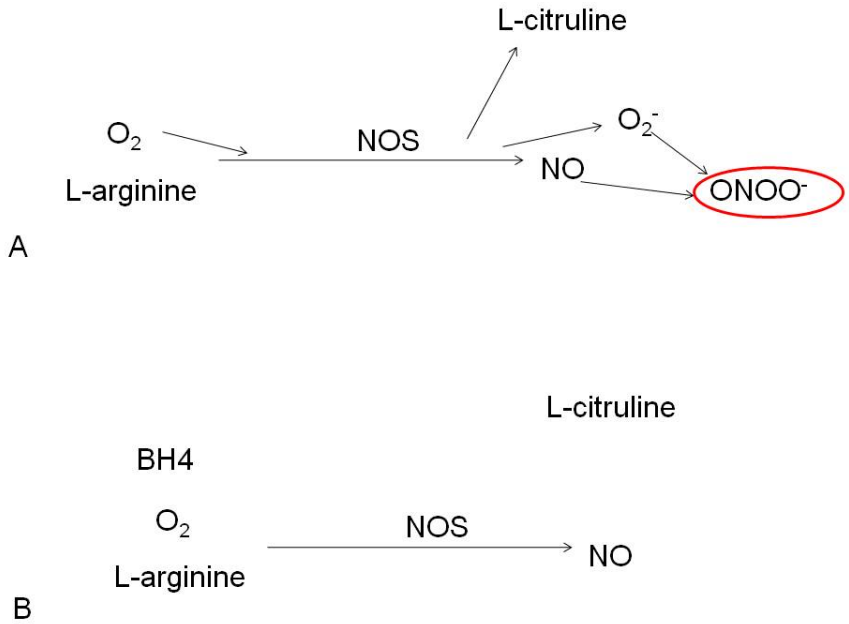

Fig. 4. Schematic representation of nitric oxide synthase (NO synthase) reaction leading to l-citrulline and nitric oxide (NO) from l-arginine and oxygen (O_2) without (A) and with BH4 (B). It is important to note that this reaction without BH4 increases ROS.

Although superoxide can indeed react directly with BH4, the rate constant of this reaction is many orders of magnitude lower than that for NO with superoxide (Vasquez-Vivar et al, 2002). A more likely mechanism for BH4 oxidation is the interaction with peroxynitrite (generated from the interaction between NO and superoxide). It was indicated that peroxynitrite can oxidize BH4 within minutes at physiologically relevant concentrations (Crabtree et al, 2011). EPR (electron paramagnetic resonance) spectroscopy experiments

have demonstrated that peroxynitrite oxidizes BH4 to the (non-protonated) BH3 (trihydrobiopterin) radical, and then to BH2, with a rate constant estimated to be 6 × 103 M−1 s−1, several-fold higher than reactions between peroxynitrite and ascorbate, glutathione or thiol groups (Gao et al, 2009). Oxidation not only directly reduces BH4 bioavailability, but the oxidation products themselves (such as BH2), which have no cofactor activity, may compete with BH4 for binding to endothelial NOS (eNOS) (Crabtree et al, 2008).

ROS are also involved in vascular remodeling. Vascular remodeling is defined as alteration of structure leading to alteration in wall thickness and lumen diameter. It can be induced through passive adaptation to chronic changes in hemodynamics and/or through neurohumoral factors including Ang II and ROS. The progression of hypertension involves two different types of vascular remodeling: inward eutrophic remodeling and hypertrophic remodeling (Schiffrin, 2004). Eutrophic remodeling is characterized by decreased lumen size, thickening of the media, enhanced media:lumen ratio and, usually, little change in medial cross-sectional area. In this case, the change in vascular smooth muscle (VSMC) size is negligible (Korsgaard et al, 1993), and medial growth toward the lumen is mainly mediated by reorganization of cellular and non cellular material of the existing vascular wall, accompanied by enhanced apoptosis in the periphery of the blood vessel (166). This is common in small resistance arteries of essential hypertensive patients and SHR (Korsgaard et al, 1993).

On the other hand, hypertrophic remodeling characterized by an increase in wall cross-sectional area predominates in conduit arteries of secondary hypertension, such as those of renovascular hypertensive patients or Ang II-infused hypertensive rats. An increase in cell size and enhanced accumulation of ECM proteins such as collagen and fibronectin are specific features of hypertrophic remodeling (Rizzoni et al, 2000). Hence, VSMC hypertrophy and ECM synthesis are required for hypertrophic remodeling. Both mechanical wall stress and humoral mediators such as Ang II collaborate to hypertrophic remodeling (Rizzoni et al, 2000).

The both forms of remodeling frequently coexist in different vascular beds and at different stages of hypertension, which occurs even in the same subject. Even though the determinants of each type of remodeling have not been clearly described, the reorganization of media mediated by phenotype modulation of VSMCs, migration, cellular growth, apoptosis, and ECM production and rearrangement is thought to be common to both processes. These events occur cooperatively and simultaneously.

Thus, it is not easy to distinguish contributions of each component in vivo. Vascular remodeling is improved by treatment with tempol, antioxidant vitamins (Chen et al, 2011), Ang II receptor antagonists, or NADPH oxidase inhibitors in animal experimental models, as well as in clinical trials (Zhou et al, 2005), emphasizing the role of ROS.

Recent studies with improved forms of ROS scavenging enzymes, specific inhibitors for different ROS generating enzymes, and redox signaling pathway blocking agents allow subtle modulation of redox signaling and may overcome the redundancy of general antioxidant treatments. Therefore, the spatial and temporal aspects of redox signaling in the vasculature are of much importance to understand the etiological role of ROS and to develop better strategies to treat hypertension.

6. Oxidative stress in the kidney

Renal artery obstruction can cause arterial hypertension, which is followed by impaired renal function and renal atrophy. Cardiovascular disorders caused by kidney injury are in part regulated by renin release from the stenotic kidney, with a subsequent increase in angiotensin II (Ang II) synthesis (Trinquart et al, 2010). Ang II results in the activation of $O^{2\bullet-}$ generation through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a multi-subunit enzyme, which is one of the enzymatic sources of $O^{2\bullet-}$ (Chabrashvili et al, 2002).

The mechanism(s) by which ANG II produces superoxide is not entirely elucidated yet. However, Mollnau et al. (2002) found that ANG II infusion during seven days enhanced expression of nox 1, gp91(phox), and p22(phox) subunits of NADPH oxidase via a PKC system. The mechanism which involves ANG II-mediated increase in the release of superoxide locates into activity a series of events that may play relevant functions in increased blood pressure.

Previous studies were conducted in attempt to clarify the specific components of ROS that are involved in the development of ANG II-induced hypertension. Haas et al. (1999) are among the first to demonstrate that the slow hypertensive response to ANG II was accompanied by a significant elevation of ROS as estimated indirectly by increases in plasma F2-isoprostanes, an oxidative metabolite of arachidonic acid (Morrow et al, 1990). Nishiyama et al. (2002) also demonstrated that a prolonged infusion of ANG II in rats stimulates ROS production. In this study, the administration of tempol, a SOD mimetic, reversed the vasoconstriction and produced vasodilation via an NO-dependent mechanism.

Ortiz et al. (2011) found in rats that the development of slow pressor responses to ANG II could be inhibited by the administration by antioxidants such as tempol and vitamin E. As a result of antioxidant treatment, there was a fall in renal blood flow and glomerular flow rate, whereas the indexes of oxidative stress, TBARS, and isoprostanes were found to be decreased in peripheral circulation as well as the renal vein.

Some investigations suggest that the decrease in the NO concentration due to interaction with superoxide anon radical constitutes a major component in the development of the observed vasoconstriction. Supporting the assumption that inhibition of NO synthesis enhances the vasoconstrictor effect of ANG II are the studies of Kitamoto et al. (2000). These investigators found that the continuous administration of l-NAME (a NOS inhibitor) to Sprague-Dawley rats for seven days induced ROS production, which was dependent on ANG II, because the effect was blocked by the administration of ANG II receptor blockers. Relevant to these findings are the studies of Usui et al. (1999), who also found an increase in ROS produced byl-NAME, which was blocked by the administration of antioxidants. In this study, l-NAME blockade was associated with an increase in angiotensin converting enzyme activity in the aorta. The studies of Kitamoto et al. (2000) and Usui et al. (199) reveal an interesting aspect of ANG II, NO, and oxidative stress. They suggest that a simple decrease in NO synthesis leaves unbalanced ANG II, which induces ROS release. This situation will be further stimulated by the increase in converting enzyme activity, which can accelerate the production of ANG II causing a positive feedback for oxidative stress.

The question of whether NO inhibition alone can cause ROS production without the participation of ANG II should be further explored. As mentioned previously, NOS can

produce superoxide when BH4 is oxidized to BH4, leading to an increase in ROS and a reduction in NO (Figure 4). This could set up a vicious cycle that accentuates NO dysfunction and tends to perpetuate oxidative stress. These alterations may constitute an important mechanism of dysregulation that produces hypertension and renal dysfunction (see later). An example of this alteration is the SHR in which blood pressure can be normalized by the administration of BH4 (McIntyre et al, 1997).

Researches performed in the renovascular model of hypertension in rats have shown relevant data regarding the relationship between oxidative stress and hypertension. The central regulation of the sympathetic nervous system (SNS)

involved in cardiovascular regulation is complex, involving multiple reflex pathways and neural connections with a large number of neurotransmitters and neuromodulators acting in specific groups of neurons in the central nervous sysem (CNS) involved in the tonic and reflex control of the cardiovascular system. In the CNS, Ang II is able to increase sympathetic vasomotor tone and blood pressure, and is involved in the pathogenesis of many experimental models of hypertension. Thus, the close functional association between NADPH oxidase and the Ang II is of particular relevance in linking oxidative stress in the brain to sympathoexcitation and hypertension (Campos, 2009). For instance, intracerebroventricular infusion of NADPH oxidase inhibitor antagonizes the pressor response induced by centrally mediated Ang II actions (Gao et al, 2004).

In the brain, the overexpression of SOD, an enzyme responsible for O_2 breakdown, also abolishes the central pressor effect of the octapeptide (Zimmerman et al, 2004) suggesting that in the CNS there is a positive correlation between the increase in ROS and the central pressor response mediated by Ang II. Considering that the paraventricular nucleus of the hypothalamus (PVN) and the rostroventrolateral medulla (RVLM) contain critically important neurons involved in the control of sympathetic vasomotor tone and arterial pressure (Colombari et al, 2001).

Previous studies reviewed and examined whether there was an increase in AT1 receptor expression and oxidative stress markers within these two nuclei in 2K1C hypertension. NAD(P)H oxidase subunits (p47phox and gp91phox) and antioxidant enzyme CuZnSOD mRNA expression were quantified in the RVLM and PVN of 2K1C hypertensive rats. It was hypothesized that the overactivity of NADPH oxidase-derived ROS associated with a reduction in the activity of CuZnSOD within the RVLM and PVN could collaborate to 2K1C hypertension, particularly in the renin-dependent phase of hypertension.

In summary, the recent studies support the idea that an increase in ROS in the kidney is involved in the development of cardiovascular disorders, playing a major role in maintaining high arterial pressure and sympathetic drive under conditions of renovascular hypertension.

7. The involvement of the nervous system in ROS-induced cardiovascular disease

Neurons in the brain present increased density of polyunsaturated fatty acids in its cell membranes. Fatty acids are targets of free radicals. An indirect marker of ROS, TBARS is enhanced in the brainstem of SPSHR compared to age-matched control (Hirooka, 2008). Others reported enhanced ROS in the brainstem of rabbits with heart failure (Gao et al, 2007).

The activity of sympathetic and parasympathetic systems, which are both involved in cardiopulmonary reflex, as well as the cardiovascular regulation, is under the control of a medullary circuitry comprising the nucleus of the solitary tract (NTS), rostral (RVLM) and caudal ventrolateral medulla (CVLM) and the nucleus ambiguous. Drugs injection into the fourth cerebral ventricle (4th V) may easily reach structures surrounding the ventricular system like the area postrema and the dorsal motor nucleus of the vagus (Colombari et al, 2001) (Figure 5). Those areas are also involved in cardiovascular reflex responses, in which we may include baroreflex (Valenti et al, 2009a; Valenti et al, 2009b; Cisternas et al, 2010).

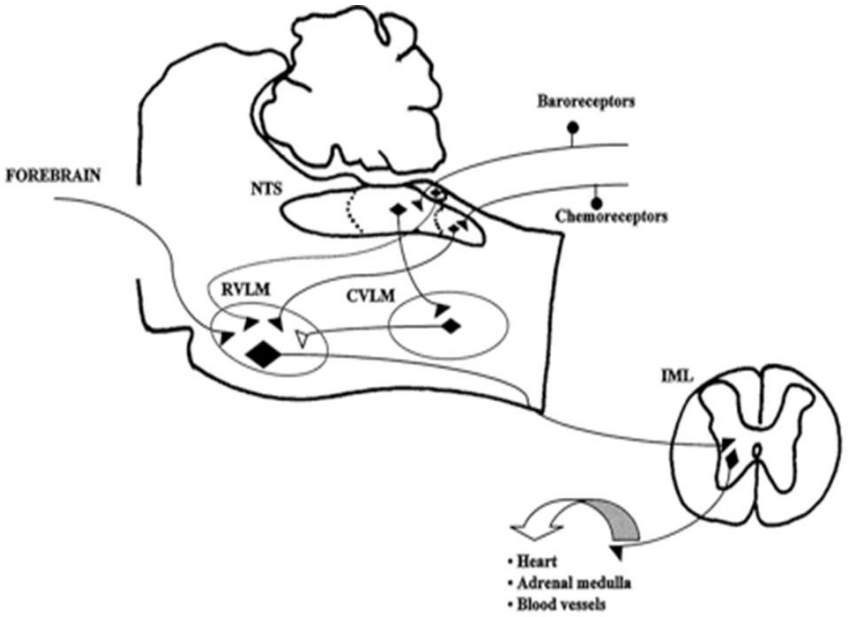

Fig. 5. Schematic sagittal view of the medulla oblongata showing brain pathways implicated in neurogenic hypertension. Premotor neurons from the RVLM send excitatory synapses to preganglionic neurons situated in the intermediolateral cell column (IML), providing sympathetic efference to target organs. The RVLM is the group of neurons that receive excitatory afference from the commissural nucleus of the solitary tract (NTS) and area postrema. It also receives inhibitory afferences from the caudoventrolateral medulla (CVLM). Adapted from Valenti et al, 2007.

A previous investigation suggested that brain ROS is associated with enhanced sympathetic activity (Gao et al, 2007) and systemic ROS is also related to impaired baroreflex (Bertagnolli et al, 2006). In addition, it was reported increase of NAD(P)H oxidase activity and expression into the RVLM, the primary central site for the maintenance of sympathetic nerve activity, in CHF rabbits. In the same sequence of procedures, the same authors observed that a reduction of brain $O^{2\bullet-}$ by tempol, a SOD mimetic, decreased the sympathetic outflow in chronic heart failure rabbits. Conversely, an increase of central $O^{2\bullet-}$ due to administration of the SOD inhibitor diethyldithiocarbamic acid enhanced the

sympathetic outflow in both normal and chronic heart failure rabbits (Gao et al, 2007). Taken together, those data suggest that antioxidant enzymes, i.e., SOD and catalase, into the brainstem are involved in baroceptor reflex regulation, since baroreflex is modulated by sympathetic and parasympathetic activity (Valenti et al, 2009a).

Recent studies from our laboratory have investigated the effects of ROS into the fourth cerebral ventricle (4th V) on cardiovascular responses.

In one study (Valenti et al, 2011a) it was evaluated the effects of 3-amino-1,2,4-triazole (ATZ), a catalase inhibitor, into the 4th V on baroreflex components in conscious rats. It was revealed that this drug significantly attenuated bradycardic and tachycardic reflex, bradycardic peak and it also decreased heart rate range 30 minutes after its injection. While in Wistar rats treated with vehicle (saline 0.9%) there were no significant changes regarding baseline mean arterial pressure (MAP) and heart rate (HR) and baroreflex components. Considering that the tachycardia (tachycardic reflex) in response to SNP is mediated by both sympathetic and parasympathetic activity (Stornetta et al, 1987) and that we reported reduction in the maximal parasympathetic responses to elevation in mean arterial pressure, while there were no changes in tachycardic peak response to decrease in mean arterial pressure (highest sympathetic response), we suggest that ATZ into the 4th V is acutely involved with parasympathetic activity but is not involved in baroreflex changes. The lack of any change in the vehicles groups is consistent with this assumption. In view of the anatomical scope of the 4th V, an action on an only one neuronal cluster is not an easy accomplishment. However, prior researches indicated a preference for parasympathetic system which modulates HR, such as the dorsal motor nucleus of the vagus and nucleus ambiguous, which receive glutamathergic projections from the nucleus of the solitary tract (Colombari et al, 2001).

In another study (Valenti et al, 2011b), it was evaluated the effects of catalase inhibition into the 4th V on cardiopulmonary reflex in conscious Wistar rats. In this method, we used male Wistar rats, which were implanted with a stainless steel guide cannula in the 4th V. The femoral artery and vein were cannulated for MAP and HR measurement and for drug infusion, respectively. After basal mean arterial pressure and heart rate recordings, the cardiopulmonary reflex was tested with a dose of phenylbiguanide (PBG, 8 µg/kg, bolus). Cardiopulmonary reflex was evaluated before and 15 minutes after 1 µl of ATZ (0.01 g/100 µl) injection into the 4th V. Vehicle treatment did not change cardiopulmonary reflex responses. ATZ injected into the 4th V significantly enhanced hypotensive responses without influencing the bradycardic reflex. Taken together, those data suggested that ATZ injected into the 4th V increases sympathetic inhibition but does not change the parasympathetic component of the cardiopulmonary reflex in conscious Wistar rats.

Nevertheless, opposite findings were found in SHR. Another study (Valenti et al, 2011c) was undertaken to evaluate the acute effects of central n-acetylcysteine, an antioxidant drug, on baroreflex in juvenile SHR and age-matched Wistar Kyoto (WKY) rats. It was observed that n-acetylcysteine injection into the 4th V did not significantly change baroreflex gain, bradycardic and tachycardic reflex, bradycardic and tachycardic peak in SHR and WKY rats. Interestingly, n-acetylcysteine caused slight but significant increase in basal heart rate 15 minutes after its injection in conscious WKY rats.

Many previous studies have already demonstrated the effects of oxidative stress in cardiovascular reflex. Zanzinger and

Czachurski (2009) demonstrated that SOD injected into the RVLM decreased sympathetic nerve activity in swine. Several groups have now shown that ROSs stimulate sympathetic outflow (Campese et al, 2004). Campos et al (2011) evidenced that the lack of LDL receptor enhanced cholesterol blood levels, enhanced ROS and impaired baroreflex sentivity. Monahan et al (2007) supported the hypothesis that oxidative stress collaborates to age-associated decreases in cardiovagal baroreflex sensitivity in healthy men. On the other hand, Wright et al (2009) indicated that in male smokers, circulating antioxidants had no effect on baroceptor reflex function and minor effects on the cardiovascular system were seen following acute fat and vitamin ingestion.

In the central nervous system, the complex regulating blood pressure is contained within topographically selective networks characterized at all levels of the neuraxis. Adjustments in this modulation network may lead to labile changes in autonomic function. The development of neurogenic hypertension may involve improper alterations in synaptic function within these networks. Thus, investigations regarding the relationship between the nervous system and the cardiovascular system and its importance to regulation of the physiologic homeostasis are always welcome in the basic and clinical research.

8. Perspectives

At the moment, relevant milestones were achieved with the availability of more overt facts that demonstrates that cardiovascular disorders mechanisms are linked to ROS increase and dysregulation of oxidant-antioxidants systems. The oxidation and nitration of cellular lipids, proteins and nucleic acids, and formation of aggregates of oxidized molecules underlie the loss of cellular function, cellular ageing and the inability of cells to withstand physiological stresses. Moreover, ROS regulate energy metabolism and signal transduction mechanisms in response to situations of nitrosativeor or oxidative stress. Sources of ROS, physiological and pathophysiological conditions, and cellular oxidant targets determine the profile nature of a disease process and resultant outcomes.

In summary, the data presented in this chapter is significant to the literature, because progress in redox signaling provides insight into the function of ROS in the pathological and physiological mechanisms involved in cardiovascular disorders. Nonetheless, the literature raises more questions. Regarding hypertension treatment, there are a few points to be underscored. ROS act as signaling molecules associated with diverse physiological mechanism which are indispensable for normal function of the brainstem. Inappropriate modulation of ROS impairs redox signaling, which is assumed to stimulate pathologic situations, in which we may include hypertension. Moreover, our study reinforces the importance to investigate the integrative neuroscience.

9. Concluding remarks

Advances in ROS signaling provide insight into the role of ROS in the pathological and physiological mechanisms related to cardiovascular disorders. The comprehension of how redox state regulates the cardiovascular system is a relevant step for a best explanation for

the mechanism involved in antioxidant species applied in clinical therapies. Therefore, the presentation of data regarding the systems different from the cardiovascular system implicated in ROS-induced cardiovascular diseases is relevant to the integrative physiology and more particularly to control physiology and clinical therapies that aim to prevent cardiovascular disorders such as hypertension and heart failure.

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4

Adipocytokines, Oxidative Stress and Impaired Cardiovascular Functions

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1. Introduction

In spite of the considerable progress in their diagnosis, prevention and treatment, cardiovascular diseases remain the number one cause of death worldwide. This is partially due to the rapidly growing incidence of obesity, which is a well-known independent risk factor for insulin resistance, diabetes, dyslipidaemia, high blood pressure and thrombosis (Lopaschuck et al., 2007).

The metabolic complications of obesity, often referred to as the metabolic syndrome, characterized by a heterogenic complex of symptoms and consist of glucose intolerance, central obesity, dyslipidemia (hypertriglyceridemia, elevated nonesterified fatty acids (NEFAs), and decreased high-density lipoprotein (HDL) cholesterol), and hypertension. These, often culminating in β-cell failure, impaired glucose tolerance and type 2 diabetes (T2D). In addition, dyslipidaemia, coronary heart disease (CHD), systemic hypertension and premature heart failure are pathologies related (Hubert et al., 1983). Abdominal obesity, ectopic lipid accumulation, hepatic steatosis, and sleep apnea can also be included in the metabolic complications of obesity (Parati et al., 2007).

On the other hand, obesity leads to an alteration in the profile of hormones secreted by adipose tissue (adipokines). Secretion of adipocytokines has been shown particularly for visceral fat (Dusserre et al., 2000; Fontana et al., 2007; Yang & Smith, 2007). It is evident that many of these adipokines have the ability to influence other tissues such as the liver, muscle and brain, e.g. the adipokine leptin affects appetite regulation, others have an important impact on the consequences of adipose tissue inflammation (e.g. interleukin 6 (IL), PAI-1, monocyte chemoattractant protein 1 [MCP-1]) and vascular biology (e.g. serum amyloid A [SAA]) (Bastard et al., 2002; Mutch et al., 2001; Sartipy et al., 2003; Stofkova, 2009; Yang et al., 2006). In addition, increased tumor necrosis factor (TNF) and IL-6 expression and

secretion from adipose tissue are involved in both whole-body and local insulin resistance at different tissue sites.

The principal purpose of this chapter is to describe how the adipocytokines and oxidative stress interact with insulin signaling in the context of low-grade inflammation related to obesity in order to promote cardiovascular complications.

2. Pathophysiology of cardiovascular morbidity

2.1 Introduction

The pathophysiology of cardiovascular morbidity is complex and multifactorial. Oxidative stress is an important contributory factor to the etiology of many cardiovascular diseases, including atherosclerosis, coronary heart disease (heart attack), cerebrovascular disease (stroke), cardiomyopathies, peripheral vascular disease, diabetes, heart failure, and hypertension (Dusting & Triggle, 2005). Ischemic heart disease and hypertension are the two most important causes of heart failure in the Western world. Other common causes include valvular heart disease (especially aortic stenosis and mitral regurgitation).

Arterial hypertension is the most prevalent cardiovascular risk factor and the leading cause of morbidity and mortality from cardiovascular disease (CVD) worldwide (Gómez-Marcos et al., 2009). Heart failure (HF) is a complex clinical syndrome caused by impaired ventricular performance. It is the final common pathway for a variety of cardiovascular disease processes, leading to potentially disabling symptoms and shortened life expectancy. Currently, 1% of the population aged 50–59 yr, and 10% of those over 80 yr, have HF; is the only major cardiovascular condition that is increasing in prevalence, because of an ageing population and improved survival from other CVD (Kotzé & Howell, 2008). Understanding these profound mechanisms of disease can help clinicians identify and treat CVD, as well as help patients prevent these potentially devastating complications.

2.2 Epidemiology

Cardiovascular diseases are the world's largest killers, claiming 17.1 million lives a year, CVD contributed to a third of global deaths. An estimated 79 400 000 American adults (1 in 3) have 1 or more types of CVD. Of these, 37 500 000 are estimated to be age 65 or older (Rosamond et al., 2007).

Extensive epidemiological research has established diabetes, hyperlipidemia, hypertension, and cigarette smoking, as independent risk factors for CHD. The risk increases 2–3 folds with tobacco smoking, with age and is greater for women than for men. In contrast, cardiac events fall 50% in people who stop smoking and the risk of CVDs, also decreases significantly over the first two years after stopping smoking (Khot, et al., 2003).

The health interview part of the National Health and Nutrition Examination Survey (NHANES) III was used to categorize adults over 50 years of age by presence of metabolic syndrome (National Cholesterol Education Program [NCEP] definition) with or without diabetes. The prevalence of CHD for each group was then determined. Metabolic syndrome (MetS) is very common, with ~44% of the U.S. population over 50 years of age meeting the NCEP criteria. In contrast, diabetes without MetS is uncommon (13% of those with

diabetes). Older Americans over 50 years of age without MetS regardless of diabetes status had the lowest CHD prevalence (8.7% without diabetes, 7.5% with diabetes). Those with MetS without diabetes had higher CHD prevalence (13.9%) and, those with both MetS and diabetes had the highest prevalence of CHD (19.2%) compared with those with neither (Alexander et al., 2003).

The Systematic Coronary Risk Evaluation (SCORE) data set comprises data from 12 European cohort studies. The SCORE population was also divided into gender and age strata: under 40, 40–49, 50–59, and over 60. The rate of CVD mortality in each body mass index (BMI) category was calculated, each 5-unit increase in BMI was associated with an increase in CVD mortality of 34% in men and 29% in women. This increases the public health importance of BMI as both a simple indicator and mediator of CVD risk (Dudina et al., 2011).

2.3 Pathologies associated to cardiovascular morbidity

Impaired myocardial diastolic relaxation (e.g., diastolic dysfunction) is the earliest myocardial contractility observed in metabolic conditions such as obesity, insulin resistance, and hypertension. Diastolic dysfunction manifests as a reduction in velocity of myocardial relaxation, as well as decreasing myocardial compliance. Mechanisms that contribute to this selective cardiac dysfunction include decreases in energy production due to reductions in mitochondrial respiration, increased oxidative stress, and defective contractile and intracellular "Ca2+" regulatory proteins. Abnormalities in "Ca2+" signaling/flux and myofilament function contribute to the cardiomyopathic alterations observed in the metabolic syndrome (Ren et al., 2010). Reductions in the oxidative capacity of the mitochondrial electron transport chain are manifested in obese, insulin-resistant persons as well as diabetic patients. Mitochondria in endothelial cells are thought to play an important role in cellular signaling as sensors for local oxygen concentration and regulations of nitric oxide (NO) production. Renin-angiotensin-aldosterone system (RAAS)-mediated increases in nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase activity and generation of reactive oxygen species (ROS) may result in mitochondrial damage and associated decreases in oxidative phosphorylation, Adenosine triphosphate (ATP) production and bioavailable NO (Ren et al., 2010).

The mechanisms underlying ventricular dysfunction are dysfunction of cardiac myocytes and longstanding pressure or volume overload. As myocardial contractility decreases, the stroke volume drops and the end-diastolic volume and pressure increase. If sustained in the long-term, this volume increase leads to what is termed cardiac remodelling. This involves myocardial hypertrophy, chamber enlargement and an increase in ventricular wall stress, and increases oxygen demand. An increase in ventricular stiffness also occurs due to increased collagen deposition in the heart, which impairs filling and exacerbates the situation (Kotzé & Howell, 2008).

The new paradigm of atherosclerosis links oxidative stress, inflammation, thrombosis, and endothelial dysfunction. Growing evidence indicates that chronic and acute overproduction of ROS under pathophysiologic conditions is integral in the development of CVD (Madamanchi et al., 2005). Coronary artery disease (CAD) is one of the most frequent causes of death and disabling symptoms worldwide. Epidemiological studies have indicated the

rising prevalence of atherosclerosis globally (Tedgui & Mallat, 2006). Formation of atheromatous plaques in the arteries obstructs the supply of oxygen and nutrients to the myocardium, resulting in CHD (Woods et al., 2000).

2.3.1 Diabetes

Diabetes is a prime risk factor for CVD, the link between diabetes and CVD is complex and multifactorial. The presence of insulin resistance, impaired glucose tolerance, and overt diabetes, are associated with an increased risk of CVD, these conditions are also accompanied by the presence of oxidative stress (Woods et al., 2000). Vascular disorders include retinopathy and nephropathy, peripheral vascular disease (PVD), stroke, and CAD. Diabetes also affects the heart muscle, causing both systolic and diastolic heart failure.

The etiology of this excess cardiovascular morbidity and mortality is not completely clear (Dokken, 2008). Evidence suggests that although hyperglycemia, the hallmark of diabetes, contributes to myocardial damage after ischemic events, it is clearly not the only factor, because both pre-diabetes and the presence of the MetS, even in normoglycemic patients, increase the risk of most types of CVD (Alexander, 2003; Dokken, 2008). In diabetes, where CVD is of particular concern, there are multiple sources of ROS including the auto-oxidation of glucose, increased substrate flux, and decreased levels of NADPH through the polyol pathway. Formation of advanced glycation end products (AGEs) and their interaction with cellular targets, such as endothelial cells, may lead to oxidative stress and promote formation of oxidized LDL (ox-LDL) (Ceriello & Motz, 2004).

The recent explosion of the worldwide epidemic of MetS combining disturbances in glucose and insulin metabolism, excess predominantly abdominally distributed weight, mild dyslipidemia, and hypertension, with the subsequent development of obesity, T2D and CVD, compromises progress made in reducing the morbidity and mortality of CVD in recent years. Cardiovascular risk increases in parallel to insulin resistance (as estimated by the homeostasis model assessment index (HOMA) both patients with diabetes and nondiabetic (Saely et al., 2005).

2.3.2 Metabolic syndrome

The incidence of CVD, coronary heart disease, and T2D has not been well defined in persons with the MetS. Conclusions were that MetS is common and is associated with an increased risk for CVD and T2D in both sexes, according to metabolic syndrome traits. MetS accounts for up to one third of CVD in men and approximately half of new T2D over 8 years of follow-up (Wilson et al., 2005).

A large family study of T2D in Finland and Sweden (the Botnia study) were included in the analysis of cardiovascular risk associated with the MetS. The aim of the study was to assess the prevalence of cardiovascular morbidity and mortality associated with the MetS by applying the WHO definition. In women and men, respectively, the MetS was seen in 10 and 15% of subjects with normal glucose tolerance, 42 and 64% of those with impaired fasting glucose (IFG)/impaired glucose tolerance (IGT), and 78 and 84% of those with T2D. Cardiovascular mortality was markedly increased in subjects with the MetS (12.0 vs. 2.2%, $p< 0.001$). Of the individual components of the MetS, microalbuminuria conferred the strongest risk of cardiovascular death (RR 2.80; $p< 0.002$) (Isomaa et al., 2001).

2.3.3 Obesity

Obesity has been increasing in epidemic proportions in both adults and children. In adults, overweight is defined as a BMI 25 to 29.9 Kg/m² and obesity as BMI ≥30 Kg/m². Other indexes that have been used less commonly but possibly with more predictive power include body fatness, waist circumference (WC), waist-to-hip ratio (WHR), and weight-to-height ratio. A recent study of nearly 360,000 participants from 9 European countries showed that both general obesity and abdominal adiposity are associated with risk of death and support the importance of WC or WHR in addition to BMI for assessing mortality risk. Obesity has many adverse effects on hemodynamics and CV structure and function (Lavie et al., 2009).

Elevated BMI predisposes to congestive heart failure (CHF) by promoting increased blood pressure, diabetes, and CHD. Factors related to obesity and hypertension, include: endothelial dysfunction, insulin resistance, sympathetic nervous system, substances released from adipocytes (IL-6, TNF-α, etc.), and sleep apnea (Poirier et al., 2006).

The role of obesity in the initiation and acceleration of tissue inflammation has been well studied. Excess adipose tissue can contribute to inflammation in two ways: (a) ectopic fat storage induces lipotoxicity, promoting an intracellular inflammatory response and (b) altered adipokine production in obesity contributes to the inflammatory response. It is now recognized that adiponectin has a role in both of these processes. Related to demonstrated association between hypo-adiponectinaemia and metabolic dysfunction (Cnop et al., 2003) the proposal provides that a replacement of adiponectin may function as pharmacological therapy (Chandran et al., 2003).

2.3.4 Endothelial dysfunction

Healthy endothelium regulates blood vessel tone, platelet activation, leukocyte adhesion, thrombogenesis, and inflammation. The net effect of healthy endothelium is vasodilatory, anti-atherogenic, and anti-inflammatory (Dokken, 2008). Endothelial dysfunction has been observed in patients with established coronary artery disease or coronary risk factors, both in the coronary and peripheral vasculature (Heitzer et al., 2001).

As shown in figure 1, endothelial dysfunction a key factor in atherogenesis, is associated with an increased risk of cardiovascular events and highest risk for vascular morbidity and mortality. A major risk for atherosclerotic plaque rupture is aging. One possible mechanism is that aging is associated with endothelial cell senescence, which is a risk factor for endothelial apoptosis and endothelial denudation, rendering the atherosclerotic plaque prone to rupture (Hulsmans et al., 2011).

A primary event in atherogenesis is the infiltration of activated inflammatory cells into the arterial wall. ROS can be produced from both endogenous and exogenous substances. Potential endogenous sources include mitochondria, cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation. In the vascular wall, ROS are generated by several mechanisms, including NADPH oxidases, xanthine oxidase, the mitochondrial respiratory chain, lipoxygenases, and nitric oxide synthases. ROS formation can be stimulated by mechanical forces (e.g., stretch, pressure, shear stress), environmental factors (such as hypoxia), secreted factors coupled to tyrosine kinase receptors (e.g., platelet derived

growth factor, PDGF), and secreted factors coupled to G protein-coupled receptors such as angiotensin II (Lehoux et al., 2006; Dokken, 2008; Hulsmans et al., 2011).

The general process of lipid peroxidation consists of three stages: initiation, propagation, and termination (Catalá, 2006). The initiation phase of lipid peroxidation includes hydrogen atom abstraction. Several species can abstract the first hydrogen atom and include the radicals: hydroxyl (−OH), alkoxyl (RO−), peroxyl (ROO−), and possibly HO_2− but not H_2O_2 or O_2−. The membrane lipids, mainly phospholipids, containing polyunsaturated fatty acids are predominantly susceptible to peroxidation because abstraction from a methylene (CH2-) group of a hydrogen atom, which contains only one electron, leaves at the back an unpaired electron on the carbon, CH-. The presence of a double bond in the fatty acid weakens the C–H bonds on the carbon atom nearby to the double bond and thus facilitates H- subtraction. The initial reaction of -OH with polyunsaturated fatty acids produces a lipid radical (L-), which in turn reacts with molecular oxygen to form a lipid peroxyl radical (LOO−).There they secrete ROS and oxidize lipoproteins, inducing foam cell formation and endothelial cell apoptosis, which in turn lead to plaque growth, erosion, and rupture (Hulsmans et al., 2011).

It is now widely recognized that chronic low-grade inflammation and oxidative stress play a key role in the initiation, propagation, and development of metabolic disorders. The aim of Hulsmans et al., (2011), was to review the functional roles of various microRNAs (miRs) in regulating oxidative stress and inflammation in adipose and vascular tissues leading to obesity and atherosclerosis, in order to analyze how these processes can be linked through communication between cells even at a remarkable distance, thus highlighting the communication between inflammatory and endothelial cells. The work of Targonski et al., was performed to evaluate the magnitude of the association between coronary endothelial dysfunction (CED) and cerebrovascular events. Kaplan-Meier analysis indicated that patients with CED had a significantly higher cumulative cerebrovascular event rate than those without CED (P=0.04). Presence of CED in patients without obstructive CAD is independently associated with an increased risk of cerebrovascular events (Targonski et al., 2003).

2.3.5 Dyslipidemia

The major threat to the macrovasculature for patients with and without diabetes is atherosclerosis, and dyslipidemia is highly correlated with atherosclerosis, up to 97% of patients with diabetes are dyslipidemic (Dokken, 2008). Insulin deficiency and insulin resistance promote dyslipidemia accompanied by increased oxidation, glycosylation, and triglyceride enrichment of lipoproteins.

Nonenzymatic glycosylation of HDL shortens its half-life and renders it less protective against atherosclerosis (Duell, 1991). The study of Marsuki et al., was undertaken to evaluate the effect, on macrophage cholesterol efflux, of functional modification of HDL by its glycation. They also investigated the effects of the glycation-inhibitors, metformin (MF) and aminoguanidine (AG), on glycated HDL-mediated cholesterol efflux. The conclusion was that glycated HDL particles are ineffective as acceptors of ATP-binding cassette transporter (ABCG1) mediated cholesterol efflux; and this may explain, at least in part, accelerated atherosclerosis in diabetic patients. Metformin serves as a possible candidate to restore impaired cholesterol efflux and reverse cholesterol transport (Matsuki et al., 2009).

Hypertriglyceridemia can lead to increased production of the small, dense form of LDL and to decrease HDL transport of cholesterol back to the liver (Poirier et al., 2006). In addition to the characteristic pattern of increased triglycerides and decreased HDL cholesterol found in the plasma of patients with diabetes, abnormalities are seen in the structure of the lipoprotein particles, where the predominant form of LDL cholesterol is the small, dense form. Small LDL particles are more atherogenic than large LDL particles because they can more easily penetrate and form stronger attachments to the arterial wall, and they are more susceptible to oxidation (Stocker & Keaney, 2004). In diabetic patients, LDL particles can also become glycated, in a process similar to the glycation of hemoglobin. Glycation of LDL lengthens its half-life and therefore increases the ability of the LDL to promote atherogenesis (Dokken, 2008).

2.3.6 Atherosclerosis

Atherosclerosis is no longer considered a pure lipid disorder. It has become increasingly clear that inflammation is at the root of atherosclerosis and its complications. In addition to playing a causal role in lesion formation, inflammation can yield predictive and prognostic information of considerable clinical utility. In addition to serving as biomarkers of atherosclerotic events, inflammatory mediators directly participate in lesion formation, propagation, and eventual rupture and in this fashion may represent a powerful tool to assess endothelial cell activation. Clearly, understanding the mechanisms and mediators of endothelial dysregulation and inflammation may yield new targets to predict, prevent, and treat cardiovascular disease (Szmitko, 2003).

Many common conditions predisposing to atherosclerosis, such as hypercholesterolemia, hypertension, diabetes, and smoking, are associated with a reduced vascular availability of NO, a free radical that not only produces vasodilation but also has potent antiatherogenic properties, such as inhibition of platelet aggregation, prevention of smooth muscle cell proliferation, reduction of lipid peroxidation, and inhibition of adhesion molecule expression (Landmesser & Harrison, 2001). Impaired endothelium-dependent vasodilation, a surrogate for NO bioavailability, may predict cardiovascular events. Thus, the loss of NO not only alters vascular tone but also may explaining in part why these conditions are risk factors for atherosclerosis.

3. Reactive oxygen species and oxidative stress in cardiovascular diseases

Oxidative stress (OS) is an imbalance between production and degradation of ROS in cells, leading eventually to enhanced oxidative modification of biomolecules. Therefore, is a phenomenon associated with pathogenetic mechanisms of several diseases including atherosclerosis, cancer, diabetes mellitus, heart failure, hypertension, inflammatory diseases, as well as psychological diseases or aging processes (Naito et al., 2010). An increase in ROS and/or a weakening in the antioxidant defense mechanisms can cause OS. Accumulating evidence suggests that OS increases with age, and that therapeutic and life style approaches that reduce oxidative stress likely slow the development of atherosclerotic cardiovascular disease. Increased cellular ROS is an important contributor to the pathophysiology of vascular diseases, including atherosclerosis, restenosis, myocardial infarction and stroke. Additionally, some ROS act as intracellular messengers, and ROS accumulation activates

proinflammatory signaling pathways with an increased propensity for the formation of atherosclerotic lesions within the vessel wall (Runge et al., 2010).

The antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) serve as primary line of defense in destroying free radicals. However, there are several human antioxidant genes, classified according to genes whose products are defined as "antioxidant enzymes" the first two groups, and genes whose products are not enzymes, but also deal directly with reactive species. Also, are subclassified into 3 functional groups: peroxidases: catalase, ceruloplasmin (ferroxidase), glutathione peroxidase 1-7, lactoperoxidase, myeloperoxidase, peroxiredoxin 1-6; superoxide dismutases: copper chaperone for superoxide dismutase, superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult), superoxide dismutase 2, mitochondrial, superoxide dismutase 3, extracellular and thiol redox proteins: glutaredoxin (thioltransferase), glutaredoxin 2,3,5, glutathione reductase,methionine sulfoxide reductase A, metallothionein 1A, 1B, 1E,1F,1G,1H,1M,1X,2A, protein disulfide isomerase family A, member 6, selenoprotein P, plasma, 1, sulfiredoxin 1 homolog (S. cerevisiae), thioredoxin, thioredoxin 2, thioredoxin domain containing 1,2,3,4,5,6,8,9,10,11,12,13,14,17, thioredoxin interacting protein, thioredoxin-like 1, 4A, 4B, thioredoxin reductase 1,2,3 (Dusting & Triggle, 2005).

An elevation of ROS may cause CVD due the overproduction of superoxide anion (O_2•-). This overproduction is detrimental, because of the rapid interaction of O_2•- with NO, which leads to the loss of NO bioavailability and increase in the production of peroxynitrite (ONOO−). A subsequent reduction in the vascular effects of NO, as well as a reduction in the antiatherogenic effects of NO, as a consequence will compromise cardiovascular function. An elevation of O_2•- will also lead to the oxidation of the important co-factor in the regulation of nitric oxide synthase, tetrahydrobiopterin (BH_4), and this will lead to an "uncoupled eNOS", which will then synthesize O_2•- rather than NO (Dusting & Triggle, 2005).

The term ROS refers to a subset of molecules called "free radicals", however there are some ROS which are not free radicals, such as hydrogen peroxide. This term refers to any molecule that contains an unpaired electron in the outer orbital. This unpaired electron makes the molecule highly reactive that leads to the formation of bonds between the ROS and other compounds (Dokken, 2008). Unpaired electron makes the molecule highly reactive, seeking to either donate an electron to another compound or take up protons from another compound to obtain a stable electron pair. These free radicals include superoxide anion (O_2•-), hydroxyl radical (•OH), and the free radical form of nitric oxide (•NO). Other members of the ROS family include hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) (Dusting & Triggle, 2005). On the other hand, several enzyme systems are known to be sources of ROS including the mitochondrial respiratory chain, xanthine oxidase, NADPH oxidase, cyclooxygenase, cytochrome P450, and uncoupled eNOS. Mitochondria are the source of ROS. There is also growing evidence that NADPH-oxidase is a major source of vascular superoxide production.

The high reactivity of free radicals leads to the formation of bonds between the ROS and other compounds, altering the structure and function of the tissue. Because of the reactive propensity of these molecules, ROS can directly damage a number of cell components, such as plasma membranes and organelles (Dokken, 2008). In diabetes, where cardiovascular disease is of particular concern, there are multiple sources of ROS including the auto-

oxidation of glucose, increased substrate flux, and decreased levels of NADPH through the polyol pathway. Formation of AGEs products and their interaction with cellular targets, such as endothelial cells, may lead to oxidative stress and promote formation of oxidized LDL (Ceriello & Motz, 2004).

Increased production of oxygen-derived free radicals such as the superoxide anion has been linked to impaired endothelial vasomotor function in experimental models of atherosclerosis. Accordingly, treatment with antioxidants has been shown to improve coronary and peripheral endothelial function in patients with CAD or coronary risk factors (Heitzer et al., 2001). Mechanisms that contribute to this selective cardiac dysfunction include decreases in energy production due to reductions in mitochondrial respiration, increased oxidative stress, and defective contractile and intracellular "Ca^{2+}" regulatory proteins. Changes in mitochondrial biogenesis and function have been documented in the metabolic syndrome and diabetes. Alterations in mitochondrial biogenesis as well as mitochondrial content and function provoke a heterogeneous group of CVD risk factors that constitute the metabolic syndrome. (Ren et al., 2010). It is increasingly recognized that important aspects of mitochondrial dysfunction that contribute to CVDs are induction of apoptosis and changes in mitochondrial morphology under the influence of oxidative stress. Finally, inefficient mitochondrial oxidative phosphorylation/biogenesis and increases in oxidative stress appear to be overarching abnormalities contributing to cardiac diastolic function, the hallmark of metabolic cardiomyopathy (Ren et al., 2010).

4. Adipocytokines and the metabolic complications of obesity

4.1 Fat depots, adipocitokines and their relation to the human metabolic syndrome

Adipose tissue is composed of adipocytes embedded in a loose connective tissue meshwork containing adipocyte precursors, fibroblasts, immune cells, and various other cell types. Adipose tissue was traditionally considered an energy storage depot with few interesting attributes. However, adipocytes express and secrete a variety of products known as 'adipokines', including leptin, adiponectin, resistin and visfatin, as well as cytokines and chemokines such as TNF-α, IL-6 and monocyte chemoattractant protein (Antuna-Puente et al., 2008) and due to the dramatic rise in obesity and its metabolic sequelae during the past decades, adipose tissue gained tremendous scientific interest. It is now regarded as an active endocrine organ that, in addition to regulating fat mass and nutrient homeostasis, releases a large number of bioactive mediators (adipokines) modulating hemostasis, blood pressure, lipid and glucose metabolism, inflammation, and atherosclerosis (see figure 1).

During positive caloric balance there are two factors important for the development of metabolic disease. First one is a type of the fat accumulation, i.e., due to increase in size (hypertrophy) or in number (hyperplasia) of fat cells. The next factor is a place of fat storage, i.e. subcutaneous (SC) or visceral (Vis) fat (Wajchenberg, 2000; Bays et al., 2008). In humans, white adipose tissue (WAT) produces over 50 'adipokines', including TNF-α which contributes to the low-grade inflammation found in obesity, leptin which has effects on food intake, and a host of other agents with a variety of effects (Lago et al., 2007). In parallel with these proinflammatory events, WAT also produces anti-inflammatory cytokines such as adiponectin (which, paradoxically, tends to be lower in obese individuals) and IL-10 and IL1R1 (IL-1Rα; production of which is proportional to body weight).

4.2 Leptin

Hyperleptinaemia is common in obesity and reflects increased adiposity and leptin resistance. Nevertheless, leptin resistance may not be complete as several actions of leptin, such as cardiovascular sympatho-activation, might be preserved in obese subjects known to be resistant to the metabolic effects of leptin (i.e. selective leptin resistance). Notably, the renal and sympathetic actions of leptin may play an important role in the pathogenesis of hypertension related to obesity and metabolic syndrome. Furthermore, the lipotoxic effect of leptin resistance may cause insulin resistance and β cell dysfunction, increasing the risk of T2D. Leptin has also been shown to possess proliferative, pro-inflammatory, pro-thrombotic, and pro-oxidative actions (Buettner et al., 2006).

4.3 Adiponectin

Adiponectin, referred to as adipocyte complement-related protein of 30 kDa (ACRP30), is a protein secreted from adipocytes (Correia & Rahmouni, 2006), that is abundantly present in plasma (Scherer et al., 1995; Berg et al., 2001). It is now well established that adiponectin has potent salutary actions on peripheral insulin sensitivity, and circulating adiponectin levels are reduced in obesity, insulin resistance and T2D (Scherer et al., 1995; Kern et al., 2003). Mice lacking adiponectin have reduced insulin sensitivity (Weyer et al., 2001; Kubota et al., 2002; Maeda et al., 2002); in contrast, adiponectin overexpression in ob/ob mice, confers dramatic metabolic improvements, e.g., in various mouse models, Holland et al. (2011) show that the insulin-sensitizing and antiapoptotic actions of adiponectin are partly related to its effects on sphingolipid metabolism, providing a new unifying mechanism for the pleiotropic beneficial actions of adiponectin. Adiponectin stimulates the cellular activity of ceramidase, which removes the fatty acyl chain from ceramides. This liberates sphingosine, which can subsequently be phosphorylated by sphingosine kinases to generate the antiapoptotic metabolite sphingosine-1-phosphate (S1P). Furthermore, liver-specific overexpression of the adiponectin receptors, AdipoR1 and AdipoR2, increased hepatic ceramidase activity and, concomitantly, reduced hepatic ceramide content. These *in vivo* models of varying adiponectin expression and AdipoR1 and R2 overexpression demonstrate a strong association between adiponectin levels, hepatic ceramide content and insulin sensitivity (Holland et al., 2011).

In the beta cell model of apoptosis, adiponectin protected against the development of hyperglycemia-a key feature of pancreatic insufficiency-by partially preserving beta cell mass and insulin content. Using mouse primary cardiomyocytes and a pancreatic beta cell line, Holland et al. (2011) showed that adiponectin prevents cell death induced by the saturated fatty acid palmitate and a short chain ceramide analog, C2-ceramide. Mechanistically, the insulin-sensitizing actions of adiponectin, which include enhanced glucose use and fatty acid oxidation (Yamauchi et al., 2003), inhibition of serine kinases that antagonize insulin signaling and enhanced mitochondrial biogenesis, are believed to occur via receptor-dependent activation of the 5′-AMP–activated protein kinase (AMPK). Intriguingly, it is known that adiponectin also exerts potent antiapoptotic effects and prevents myocardial apoptosis in response to ischemia-reperfusion injury (Shibata et al., 2005) and lipid-induced pancreatic beta cell apoptosis (Rakatzi et al., 2004).

Several studies have linked hypoadiponectinemia to diabetes (Kern et al., 2003), hypertension (Kim et al., 2007), atherosclerosis, and endothelial dysfunction (Chow et al., 2007). More recent studies have shown that the high-molecular weight (HMW) oligomer is inversely associated with the risk for diabetes independent of total adiponectin (Kadowaki et al., 2007), and the HMW oligomer is responsible for the association of adiponectin with traits of metabolic syndrome (Heidemann et al., 2008; Lara-Castro et al., 2006). On the other hand, adiponectin improves insulin sensitivity by increasing energy expenditure and fatty acid oxidation through activation of AMPK, and by increasing the expression of PPARα target genes such as CD36, acyl-coenzyme oxidase, and uncoupling protein-2 (Kadowaki et al., 2007). Alternatively, adiponectin may lead to an improved metabolic profile by the expansion of SC adipose tissue with decreased levels of macrophage infiltration (Nawrocki et al., 2006), similar to the actions of peroxisome proliferator-activated receptor (PPAR)-γ agonists; reduction of lipotoxicity and inflammation associated with obesity (Wang et al., 2007), and adiponectin has also had vasculoprotective effects mediated via an increase in endothelial nitric oxide production, or modulation of expression of adhesion molecules and scavenger receptors (Chow et al., 2007; Zhu et al., 2008).

In addition, work in experimental models has shown that adiponectin mediates beneficial actions in cardiovascular and metabolic-associated diseases (Sam & Walsh, 2010). For example, in mouse models, adiponectin modulates hypertrophic signals in the heart and exhibits direct anti-hypertrophic properties; in addition to improving vascular function and pathological remodeling (Antuna-Puente et al., 2008); the hypoadiponectinemia might be observed in subjects with hypertension and other cardiovascular diseases and could be a useful pharmacologic tool to improve membrane microviscosity in hypertension, via the NO dependent mechanisms (Tsuda, 2011).

It has been demonstrated that plasma adiponectin levels increased during weight reduction or blockade of the rennin angiotensin system indicating that adiponectin might be beneficial for preventing the development of atherosclerotic changes. The results of Kurata et al. indicate that blockade of Angiotensin II receptor ameliorates adipocytokine dysregulation and that such action is mediated, at least in part, by targeting oxidative stress in obese adipose tissue.

4.4 Resistin

Resistin is a 12-kDa peptide that was originally discovered as a result of examining differential gene expression of mouse adipose tissue after thiazolidinediones (TZD) treatment (Steppan et al., 2001). The thiazolidinediones a class of drugs that work through PPARγ agonism, are insulin sensitizers and have been shown to improve cardiac risk factors and decrease cardiovascular events; may potentially correct the inflammatory disarray, endothelial dysfunction, dyslipidemia, and plaque vulnerability associated with diabetic cardiovascular disease through their effects on insulin resistance and fat metabolism. Resistin was decreased by TZD treatment of mice and was increased in insulin-resistant mice. Furthermore, treatment with antiresistin antibody improved insulin sensitivity and glucose transport in mice and mouse adipocytes, respectively (Steppan et al., 2001). Additional studies in mice suggest that an important site of action of resistin is on hepatic glucose production (Rajala et al., 2003). Therefore, resistin is clearly an important adipokine that likely plays a role in the development of insulin resistance; however, it appears to be quantitatively less important in humans than other adipokines.

4.5 Visfatin

Visfatin is expressed in many cells and tissues, and was previously identified as a protein involved in B-cell maturation (pre-B colony enhancing factor) (Kitani et al., 2003; Samal et al., 1994). More recently, visfatin was described to be a highly expressed protein with insulin-like functions, and was predominantly found in visceral adipose tissue, from which the name visfatin was derived (Fukuhara et al., 2005). Injection of visfatin in mice lowered blood glucose, and mice with a mutation in visfatin, and nicotinamide adenine dinucleotide (NAD) biosynthetic activity ionotropy, which is essential for β-cell function (Revollo et al., 2007). In human studies, a positive correlation between visceral adipose tissue visfatin gene expression and BMI was noted, along with a negative correlation between BMI and SC fat visfatin (Berndt et al., 2005; Varma et al., 2007), suggesting that visfatin regulation in these different depots is different, and adipose depot ratios are highly dependent on the obesity of the subjects. Variable results were obtained regarding the relationship between visfatin and diabetes or insulin resistance (Varma et al., 2007; Chen et al., 2006; Hammarstedt et al., 2005; Haider et al., 2006). Therefore, there are a number of inconsistencies among the different studies of visfatin, and the role of this adipokine in obesity and insulin resistance is not clear.

4.6 Apelin

Apelin is another short peptide released from adipocytes upon stimulation by e.g. insulin and the endogenous ligand of the human orphan G-protein-coupled APJ receptor. In line with this, plasma apelin levels are increased in obesity associated with insulin resistance and hyperinsulinemia (Beltowski, 2006). In the cardiovascular system, apelin elicits endothelium-dependent, nitric oxide-mediated vasorelaxation and in rodents, apelin also increases cardiac contractility *in vivo* (Ashley et al., 2005; Atluri et al., 2007) and causes a rapid fall in both arterial blood pressure and systemic venous tone (Tatemoto et al., 2001; Lee, 2005) with corresponding reductions in left ventricular afterload and preload (Ashley et al., 2005; Tatemoto et al., 2001).

Apelin-APJ system, expressed in the central nervous system and in a variety of peripheral tissues, is involved in the regulation of the immune response, brain signaling, hemodynamic homeostasis, vasodilatation, inotropy, angiogenesis and glucose metabolism (Sorli et al., 2006; Zhang et al., 2009). In the cardiovascular system, high expression of APJ mRNA has been observed in the heart (Zhang et al., 2009). Apelin expression is restricted to endothelial cells and negligible in cardiomyocytes in normal myocardium, but detectable in failing hearts (Földes et al., 2003). Of all the active fragments identified to date, apelin-13 may represent the most potent biological ligand (Kawamata et al., 2001). Current studies suggest that apelin expression is at least maintained and possibly augmented in mild, compensated chronic heart failure but declines in severe disease (Japp & Newby, 2008). Exogenous apelin administration during myocardial injury can preserve cardiac function (Chandrasekaran, 2008). Some researchers suggested that apelin reduces infarct size and protects myocardial cells against ischemia-reperfusion (I/R) injury by activating the reperfusion injury salvage kinase (RISK) pathway. The RISK pathway incorporates phosphatidylinositol 3-OH kinase (PI3K)/Akt, p44/42 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated MAPK (ERK1/2) (Simpkin, 2007).

5. Inflammatory pathway activation and interactions with endothelial cells

5.1 Endothelial cells

The vascular endothelium, located at the interface of blood and tissue, is able to sense changes in hemodynamic forces and blood borne signals and react by synthesizing and releasing vasoactive substances. Vascular homeostasis is maintained by a balance between endothelium-derived relaxing and contracting factors. With disruption of this balance, mediated by inflammatory and traditional cardiovascular risk factors, the vasculature becomes susceptible to atheroma formation. Inflammatory mediators appear to play a fundamental role in the initiation, progression, and eventual rupture of atherosclerotic plaques.

5.1.1 Endothelial dysfunction

Endothelial dysfunction implies diminished production or availability of NO and/or an imbalance in the relative contribution of endothelium-derived relaxing and contracting factors, those included endothelin-1 (ET-1), angiotensin, and several oxidants. However, endothelial dysfunction, as assessed in terms of vasomotor dysfunction, can occur well before the structural manifestation of atherosclerosis and thus can serve as an independent predictor of future cardiovascular events (Behrendt & Ganz, 2002).

Hypercholesterolemia, traditional cardiovascular risk factor, promotes attachment of blood leukocytes to the endothelium. Oxidized low-density lipoprotein causes endothelial activation and changes its biological characteristics in part by reducing the intracellular concentration of NO (Cominacini, 2001).

On the other hand, angiotensin II can induce the production of ROS, increase the expression of the proinflammatory cytokines as IL-6 and monocyte chemoattractant protein-1 (MCP-1), and upregulate VCAM-1 on ECs. High levels of CRP can also promote endothelial dysfunction by quenching the production of NO and diminishing its bioactivity (Verma, 2002). These endothelial modifications promote inflammation within the vessel wall, setting the stage for the initiation and progression of an atherosclerotic lesion.

5.2 Adiponectin and inflammatory activation

Recent research has focused on the origin of the inflammatory markers in obesity and the extent to which adipose tissue has a direct effect. The production of adipokines by visceral adipose tissue is of particular interest since their local secretion by visceral fat depots may provide a novel mechanistic link between obesity and the associated vascular complications. Under conditions of inflammation associated with cardiovascular disease, as well as an increase in mobilization of fatty acids from adipose tissue, there is increased secretion of pro-atherogenic, pro-inflammatory adipocytokines and chemokines.

The cardiometabolic benefits of adiponectin may be driven largely through improvements in vascular homeostasis, especially through improving endothelial function. Some studies have demonstrated impaired endothelial function in adiponectin-deficient mice (Teoh et al., 2008) demonstrated that adiponectin plays an important role to limit endothelial activation

and inflammation in experimental sepsis. On the contrary, in adiponectin-deficient mice exhibit profound reduction in survival following cecal ligation and puncture.

5.3 Mediators of inflammation

The inflammatory processes are mediated by several factors secreted by adipocytes collectively called adipocytokines (adiponectin, leptin, ghrelin, visfatin and resistin) some of which seem to play an important role in obesity-associated insulin resistance and cardiovascular complications. Tissue levels of TNF-α, IL-6, leptin and visfatin were significantly higher in patients with CAD relative to control subjects. Significantly higher tissue levels of these four cytokines from abdominal fat depots were found compared to those from epicardial fat in CAD patients.

IL-6 is secreted by a wide variety of cells such as endothelial cells, adipocytes, β pancreatic cells, monocytes, and macrophages. This cytokine is essential in reducing the inflammatory process by promoting the synthesis of anti-inflammatory cytokines and by negatively regulating inflammatory targets. In humans, higher circulating IL-6 levels have been associated with obesity and visceral fat deposition, increased risk of impaired glucose tolerance, T2D and high blood pressure. IL-6 is a central mediator of the acute-phase response and a primary determinant of hepatic production of CRP. Visceral adipose tissue secretes about two to three times more IL-6 than subcutaneous tissue, secreting also other molecules that stimulate further IL-6 expression (Curti, 2011).

In obesity, the pro-inflammatory effects of cytokines through intracellular signaling pathways involve the NF-κB and JNK systems. Thus, it can be considered that obesity corresponds to a sub-clinical inflammatory condition that promotes the production of pro-inflammatory factors involved in the pathogenesis of insulin resistance (Bastard et al., 2002).

5.4 Vasculature as part of the immune system

Blood vessels are integral components of the immune system; they are important part in lymphocyte circulation and act as portals between tissue and blood compartments. Endothelial cells express toll-like receptors, (Kunjathoor, 2002) whose ligation induces expression of leukocyte adhesion molecules, inducible NO synthase 2, endothelin, IL-1, and other inflammatory molecules. These cells also express the scavenger receptors CD36 and LOX-1, and can internalize ligands such as modified LDL particles. ECs are located at the interface of blood and tissues, and play a pivotal role in the inflammatory response. Their activation causes leukocyte recruitment, increased permeability, edema, and other characteristic features of inflammation. Furthermore, ECs can activate adaptive immunity by presenting foreign antigens to specific T cells.

5.5 Mammalian Target Of Rapamycin (mTOR) signaling pathway

The mammalian target of rapamycin (mTOR) signaling pathway integrates both intracellular and extracellular signals and serves as a central regulator of cell metabolism, growth, proliferation and survival. Discoveries that have been made over the last decade show that the mTOR pathway is activated during various cellular processes (e.g. tumor

formation and angiogenesis, insulin resistance, adipogenesis and T-lymphocyte activation) and is deregulated in human diseases such as cancer and T2D (Laplante & Sabatini, 2009). *In vivo* stimulators of adipogenesis have not been clearly identified, but may include insulin, IGF-1, as well as certain fatty acids and/or their metabolites. Insulin/IGF-1 acts on cell surface receptors, activating key intracellular signaling proteins. One of these signaling pathways, mTOR that is binds to, and inhibited by, rapamycin, an immunosuppressant that blocks T cell proliferation. The mTOR protein is a 289-kDa serine-threonine kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and is conserved throughout evolution (Laplante & Sabatini, 2009). The role of mTORC1 in regulating lipid synthesis, which is required for cell growth and proliferation, is beginning to be appreciated. It has been demonstrated that mTORC1 positively regulates the activity of sterol regulatory element binding protein 1 (SREBP1) (Porstmann et al., 2008) and of PPAR-γ (Kim & Chen, 2004), two transcription factors that control the expression of genes encoding proteins involved in lipid and cholesterol homeostasis.

The binding of insulin to its cell-surface receptor promotes the tyrosine kinase activity of the insulin receptor, the recruitment of insulin receptor substrate 1 (IRS1), the production of phosphatidylinositol (3,4,5)-triphosphate [PtdIns(3,4,5)P_3] through the activation of PI3K, and the recruitment and activation of AKT at the plasma membrane. In many cell types, activation of mTORC1 strongly represses the PI3K-AKT axis upstream of PI3K. Activation of S6 kinase 1 (S6K1) by mTORC1 promotes the phosphorylation of insulin receptor substrate 1 (IRS-1) and reduces its stability (Harrington et al., 2005). This auto-regulatory pathway, characterized as the S6K1-dependent negative feedback loop, has been shown to have profound implications for both metabolic diseases and tumorigenesis (Manning, 2004) and pro-inflammatory cytokines, such as TNFα, activate IkB kinase-β (IKKβ), which physically interacts with and inactivates tuberous sclerosis complex 1 (TSC1), leading to mTORC1 activation (Lee et al., 2007). This positive relationship between inflammation and mTORC1 activation is thought to be important in tumor angiogenesis and in the development of insulin resistance.

5.6 Adiponectin as anti-inflammatory action

Adiponectin exerts potent anti-inflammatory effects, as documented in experimental studies where authors demonstrate that reduces TNF-α production in response to various stresses in plasma, adipose tissue, vascular wall, heart, and liver (Kojima et al., 2003; Ujiie et al., 2006). In addition, antagonizes several of the inflammatory effects of TNF-α (Ouchi et al., 2003); can facilitate the removal of early apoptotic cells by macrophages and modulate the processes of inflammation and autoimmunity. This activity was mediated by calreticulin expressed on the phagocytic cell surface and not by any of the previously identified adiponectin receptors. Because the accumulation of cell corpses can cause inflammation and immune system dysfunction, these authors suggest a mechanism by which hypoadiponectinemia can contribute to the development of diabetes, atherosclerosis, and other complex diseases in which chronic inflammation is a contributing factor (Takemura et al., 2007). Thus, while AdipoR1 and AdipoR2 may mediate the metabolic properties of adiponectin (Yamauchi et al., 2003), calreticulin controls aspects of adiponectin's antiinflammatory actions (Hug et al., 2004). *In vitro* studies demonstrate that adiponectin adheres to injured vascular endothelium

(Okamoto et al., 2000) and inhibits TNF-α-induced monocyte adhesion to endothelial cells. It also decreases the expression of endothelial cell adhesion molecules (Ouchi et al., 1999) and TNF-α-induced NFkB activation (Ouchi N et al., 2000).

Adiponectin has been shown to have a role in hepatic inflammation and steatosis. Hypo-adiponectinaemia is associated with nonalcoholic steatohepatitis (Targher et al., 2004) and adiponectin has been shown to have beneficial anti-inflammatory effects in liver, reducing steatosis, hepatomegaly and inflammation in mouse models of alcoholic and non-alcoholic fatty liver disease (Xu et al., 2003).

5.7 New mediators of inflammation and endothelial cell activation

5.7.1 Oxidized low-density lipoprotein receptor-1 and LOX-1

Oxidatively modified Ox-LDL and lectin-like oxidized LDL receptor-1 (LOX-1) are contributing factors of endothelial dysfunction, an early cellular event during atherogenesis. The primary receptor for Ox-LDL in endothelial cells is LOX-1. Under physiological conditions, LOX-1 may play a role in host defense (is expressed at low levels), whereas pathological states such as atherosclerosis, diabetes, dyslipedemia, hypertension dramatically and disease states that promote vascular injury, LOX-1 is highly expressed in blood vessels increase (Mattaliano et al., 2010), and may be involved in binding pro-atherogenic materials, such as ox-LDL, that activate the endothelium. With its ability to bind products that induce inflammation and endothelial activation, elevated LOX-1 expression was observed in both initial and advanced atherosclerotic lesions (Li et al., 2002). Induction of LOX-1 expression is mediated by angiotensin II and endothelin-1, both antagonists of NO (Chen et al., 2006). LOX-1 is a type II transmembrane glycoprotein that is known to recognize a wide array of structurally distinct ligands besides Ox-LDL. These include activated platelets, AGEs, apoptotic bodies, bacteria, and CRP. LOX-1 plays a critical role in the development of atherosclerosis. This may suggest that increased LOX-1 transcriptional promoter activity may equal increased LOX-1 gene expression and elevated risk of atherosclerosis. Accordingly, decreased LOX-1 promoter activation may reduce the incidence of atherosclerosis and related diseases (Chen et al., 2006).

5.7.2 Protease-Activated Receptors (PARs)

Protease-Activated Receptors (PARs) are a family of 7-transmembrane–domain, G-protein–coupled receptors that function to link tissue injury to appropriate cellular responses, such as inflammation and tissue repair, which may contribute to disease. Under the influence of the traditional cardiovascular risk factors, the endogenous defenses of the vascular endothelium begin to break down, resulting in endothelial dysfunction and injury (see figure 1). PAR activation is also linked to the secretion of IL–6, the cytokine that promotes CRP synthesis, which itself triggers many of the steps in the inflammatory process. Overall, PAR activation appears to promote the inflammatory response within the intimal tissue, enhancing the initiation and progression of atherosclerotic plaques. Rosiglitazone, a selective PPARγ agonist, exerts anti-inflammatory effects in both obese and T2D individuals by decreasing plasma concentrations of CRP, serum amyloid-A, and matrix metalloproteinase (Stienstra, 2007).

5.7.3 Lipocalin-2

A member of the lipocalin family, lipocalin-2, also known as neutrophil gelatinase–associated lipocalin, modulates inflammation and is another adipokine that is elevated in the adipose tissue of obese mouse models and in the plasma of obese and insulin-resistant humans. *In vitro* studies suggest that lipocalin-2 induces insulin resistance in adipocytes and hepatocytes. The plasma level of another member of the lipocalin family, lipocalin-type prostaglandin D synthase, serves as a biomarker of coronary atherosclerosis (Yan, 2007).

6. Oxidative Stress in conditions and comorbidities that aggregate with cardiovascular disease

Obesity, is associated with inflammation and ROS production, while advanced glycation end-products (AGEs), through their receptor (AGER or RAGE), play an important role on these processes. This is a multiligand receptor of the immunoglobulin superfamily that binds advanced glycation end-products. Thus, increased epicardial, pericardial (EAT), or subcutaneous adipose tissue (SAT) is associated with the presence and severity of coronary artery calcium. The AGE–RAGE engagement is widely related with CVD and ROS generation, mainly mediated by NADPH-oxidase. This enzyme consists in two membrane-bound subunits, the gp91-PHOX protein (NOX2) or some of its homologs (named NOX from 1 to 5) and p22-PHOX protein. Once activated, NADPH-oxidase produces superoxide anions from oxygen and NADPH or NADH. Enhanced ROS production is an important factor associated with some CVD such as CAD. Furthermore, Rodino-Janeiro et al. (2010, 2011) have previously observed that EAT may undergone higher oxidative stress than SAT in patients with CAD because of lower expression of some antioxidant enzymes, like catalase, and higher expression of RAGE in EAT than in SAT. Oxidation of phospholipids in LDL, which infiltrates into the injured vessel wall, results in the formation and accumulation of ox-LDL. These, is pro-atherogenic, produces several abnormal biological responses, such as attracting leukocytes to the intimal of the vessel, improving the ability of the leukocytes to ingest lipids and differentiate into foam cells, and stimulating the proliferation of leukocytes, endothelial cells, and smooth muscle cells, all of which are steps in the formation of atherosclerotic plaque. Furthermore, activated macrophages express scavenger receptors that internalize ox-LDL. However, unregulated uptake of ox-LDL leads to production of lipid-loaded foam cells (Hulsmans et al., 2011).

6.1 Oxidative stress and the beta-cell

Glucotoxicity, lipotoxicity, and glucolipotoxicity are secondary phenomena that are proposed to play a role in all forms of T2D. They are implicated in the pathogenesis of β–cell dysfunction (Poitout & Robertson, 2008). Hyperglycemia and hyperlipidemia follow the primary pathogenesis of diabetes and exert additional toxic effects on β-cells. The concept of toxicity derived because physiologically, it presents a continuous overstimulation of the β-cell by glucose could eventually lead to depletion of insulin stores, worsening of hyperglycemia, and finally deterioration of β-cell function. So, a prolonged *in vitro* exposure of isolated islets or insulin-secreting cells to elevated levels of fatty acids is associated with

inhibited glucose-induced insulin secretion, impaired insulin gene expression, and induction of cell death by apoptosis.

6.2 Oxidative stress and diabetic vascular complications

Cardiovascular risk factors promote the production of ROS, excessive generation of ROS and has expression of eNOS been implicated in a variety of pathological events such as diabetes, hypertension, atherosclerosis, ischemia-reperfusion injury, CVD and neurodegenerative disease (Halliwell & Gutteridge, 2007). Results from several studies showed that the increase in ROS levels precedes the hyperglycemia and insulin resistance, suggesting a causal role of ROS in the disease process. Atherosclerosis is considered as the underlying pathology of cardiovascular diseases such as peripheral vascular disease, stroke, and coronary heart disease. The pathology of atherosclerosis is complex and involves structural elements of the arterial wall, platelets, leukocytes, and inflammatory cells such as monocytes and macrophages (Libby et al., 2002; Weber et al., 2008). The endothelium is a dynamic interface between the arterial wall and the circulating cells. Therefore, endothelial dysfunction accounts for one of the primary causes of atherosclerosis. Since the endothelium is the major source of NO in the vasculature, loss of normal cellular function can result in altered NO synthesis. The endothelium provides a constitutive supply of NO from eNOS, and under certain conditions (e.g. inflammation) it can produce excessive NO from the inducible isoform of NOS (iNOS). Therefore, regulation of NOS is central in the development and progression of atherosclerosis.

In particular, increased glucose leads to increased mitochondrial formation of ROS. Superoxide is a ROS that produces peroxynitrite when reacting with NO. Peroxynitrite induces cellular damage through depletion of the co-factor of the eNOS, tetrahydrobiopterin (BH4). Also, it activates the denominated classic pathways of diabetic complications, including: a) the polyol pathway, b) the AGE pathway, c) the protein kinase C (PKC) pathway, and d) the hexosamine pathway. Several studies suggested that intermittent low and high glucose conditions are even more deleterious to endothelial cell function than a steady, constant increase of glucose. These conditions also induce endothelial cells to enter into a proinflammatory state, and this state is associated with the upregulation of various adhesion molecules and proinflammatory cytokines (Piconi et al., 2004).

iNOS is very relevant to diabetic pathophysiology. Recent reports reveal that decreased expression of eNOS accompanies increased expression of iNOS and nitrotyrosine during the progression of diabetes in rats (Nagareddy et al., 2005). This finding suggests that induction of iNOS in cardiovascular tissues is dependent on the duration of diabetes and contributes significantly to depressed responses to vasoactive agents. *In vivo* studies revealed that oxidative stress due to hyperglycemia, occurring before late complications, become clinically evident (Pitocco et al., 2009). This finding suggests that oxidative stress plays a crucial role in the pathogenesis of late diabetic complications. It has also been described in human studies that endothelial cells in diabetes fail to produce sufficient amount of NO and fail to relax in response to endothelium-dependent vasorelaxants e.g. acetylcholine, bradykinin, shear stress, etc (Avogaro et al., 2006).

Further clinical data have demonstrated that rapid glycemic swings are associated with an exacerbated degree of oxidant production in human diabetes (Monnier et al., 2006), and are

deleterious to the endothelial function of T2D patients (Ceriello et al., 2008). Overall, these data outline the importance of steady glucose control and the potential involvement of oxidative and nitrosative stress in the pathogenesis of complications due to poorly controlled diabetes. Diabetic subjects have reduced antioxidant capacity which could favor oxidative stress. A decline in important cellular antioxidant defense mechanisms, including the glutathione redox system and vitamin C-vitamin E cycle, significantly increases the susceptibility to oxidative stress. Thus, attempts have been made to reduce oxidative stress-dependent cellular changes in patients with diabetes by supplementation with naturally occurring antioxidants, especially vitamins E and C, lipoic acid levels are reduced in diabetic patients.

It has now been established that measurement of F2-isoprostanes is the most reliable approach to assess oxidative stress status *in vivo*, providing an important tool to explore the role of oxidative stress in the pathogenesis of human disease. In addition, products of the isoprostane (IsoP) pathway have been found to exert potent biological actions and therefore may be pathophysiologic mediators of disease. IsoPs, 8-iso-PGF2α and 8-iso-PGE2 possess potent biological effects in various systems and they also serve as mediators of oxidant stress through their vasoconstrictive and inflammatory properties (Kaviarasan et al., 2009). There exists a significant correlation between blood glucose and urinary IsoPs levels, suggesting that peroxidation is related to glycemic control. In vascular smooth muscle cells, F2-IsoPs formation was found to be induced *in vitro* by high glucose concentrations.

6.3 Cardiovascular disease

The diverse responses of the microvasculature to CVD risk factors include oxidative stress, enhanced leukocyte and platelet-endothelial cell adhesion, impaired endothelial barrier function, altered capillary proliferation, enhanced thrombosis, and vasomotor dysfunction (Granger et al., 2010).

As shown in figure 1, an imbalance between the production and detoxification of ROS in vascular endothelial cells can result in the oxidative modification of cell components, impair cell function and/or can enhance cell death via apoptosis or necrosis. The oxidative activation of enzymes (phospholipase A2) and transcription factors (nuclear factor kB, NFkB) that accompanies excess ROS production can also result in an enhanced biosynthesis of lipids (platelet activating factor, leukotrienes) and proteins (adhesion molecules, cytokines) that promote inflammation. Superoxide, by virtue of its ability to inactivate nitric oxide (an anti-inflammatory molecule), is another link between oxidative stress and the induction of a pro-inflammatory phenotype in the vasculature. This oxidative stress in the vessel wall is often accompanied by an increased production of superoxide anion by circulating immune cells, and there is evidence for a causal link between these two sources of ROS: circulating cells and vessel wall.

Different enzymatic sources have been implicated in the enhanced ROS production, including NADPH oxidase, xanthine oxidase, mitochondrial enzymes, and uncoupled nitric oxide synthase. It remains to clarify whether the pro-hypertensive effects of the superoxide anion relate to its ability to inactivate NO or to indirectly promote the production of endogenous vasoconstrictors, such as endothelin. NADPH-oxidase has received the most

attention as a potential source of ROS in hypertension (HTN), followed by xanthine oxidase. Both endothelial cell- and leukocyte-associated NADPH-oxidase have been implicated in HTN-induced superoxide production, and there is evidence linking both cellular sources of the enzyme to activation of the angiotensin II type 1 receptor (AT1r) and to cytokines (TNF-α) derived from circulating immune cells (Crimi et al., 2007; Harrison & Gongora, 2009).

While some adipokines as leptin and adiponectin have been shown to promote the expression of endothelial cell adhesion molecules (CAMs) and leukocyte-endothelial cell adhesion (LECA) are known to exert an inhibitory effect on these responses. The absence of LECA in the microcirculation of obese mice under basal conditions suggests either that the pro- and anti-adhesive adipokines are in balance or that the systemic plasma levels achieved by these mediators do not cause overt inflammation in tissues distant from their source (adipose tissue). The latter possibility is supported by evidence of an increased sensitivity (priming) of endothelial cells and leukocytes in obese animals to inflammatory stimuli.

However, within the microvasculature of adipose tissue, a robust inflammatory response is noted under basal conditions, as reflected by an increased expression of the endothelial cell adhesion molecules ICAM-1 and E- and P-select in, with an accompanying recruitment of rolling and firmly adherent leukocytes, and the formation of platelet-leukocyte aggregates. The reduced LECA may be linked to adiponectin deficiency since the adipokine is a potent inhibitor of LECA and its production/release is diminished during adipogenesis (Singer & Granger, 2007).

6.4 Oxidative stress in aortic valves

Superoxide levels also are increased in stenotic aortic valves from humans. Heistad et al., (2009) found, in stenotic valves removed during surgical replacement of the aortic valve, that superoxide is increased greatly near calcified regions of the valve. Others authors (Miller et al., 2008) also found, in valves obtained at surgery or autopsy, that oxidative stress is increased in stenotic aortic valves. Thus, in calcified stenotic aortic valves as well as in atherosclerotic lesions, oxidative stress is increased. But, there are important differences in mechanisms that account for oxidative stress in aortic valves and in atherosclerotic arteries. In calcific aortic stenosis, increased production of superoxide may be mediated by "uncoupling" of NOS, as NOS primarily produces superoxide instead of nitric oxide NAD(P)H expression and activity do not appear to be increased in aortic valves (Miller et al., 2008). In striking contrast, increased expression and activity of NADPH-oxidase appears to be a major mechanism for oxidative stress in atherosclerotic lesions. Oxidative stress, in addition to contributing to fibrosis, may activate matrix metalloproteinases (MMPs) in the aortic valve and arteries. In the valve, MMPs may play a permissive role in expansion of calcification of the valve, and degraded fragments of collagen and elastin also may increase pro-calcific signaling in valvular interstitial cells. Activation of MMPs in arteries probably is harmful in a different way, by contributing to plaque rupture.

6.5 Atrial fibrillation

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia in clinical practice and contributes to impaired quality of life, and increased morbidity and mortality. The

mechanisms underlying both the initiation and perpetuation of AF are not well established but are thought to involve inflammation and oxidative stress. Furthermore, a number of studies have shown that concentrations of inflammatory mediators or markers, such as IL-6 and high-sensitivity CRP (hs-CRP), are increased in patients with AF. One mechanism that may mediate the effects of inflammation in AF is oxidative stress. Elevated inflammatory biomarkers are strongly associated with AF. Inflammation has important prognostic implications in AF; large prospective studies have shown that elevated hs-CRP levels correlated with risk factors for stroke and overall prognosis. The positive correlation between elevated levels of TNF-α and N-terminal pro-brain natriuretic peptide (NTpBNP) and severity of AF suggests that these biomarkers could be prognostic markers for AF in clinical practice (Li et al., 2010).

6.6 Insulin resistance

A large number of studies have evidenced the pivotal role of oxidative stress in insulin resistance states such as metabolic syndrome, obesity, and T2D (Atabek et al., 2004; Block et al., 2002). Decreased antioxidant capacity, increased production of ROS with oxidation products of lipids, DNA, and proteins have been reported in plasma, urine, and various tissues, suggesting systemic and organ-specific oxidative stress. Recent evidence for systemic oxidative stress includes the detection of increased circulating and urinary levels of the lipid peroxidation product F2-isoprostane (8-epi-prostaglandin F2α) in both T1D and T2D patients (Davi et al., 2003). As described above, ROS and reactive nitrogen species (RNS) are able to directly modify the expression of adiponectin. Secreted almost exclusively from adipocytes, it is inversely correlated with fat mass in obesity and with its associated cardiovascular risk. It should be considered that, plasma and urinary lipid peroxidation markers indicative of systemic OS correlated with lower circulating adiponectin levels.

7. Conclusion

Figure 1 summarizes much of the content of this chapter, because it shows most of the cellular elements and signaling pathways in which highlights the participation of adipocytokines involved in the immune response and oxidative stress on the vascular endothelium. These alterations lead to development of atherosclerosis. And finally this endothelial damage, together with the increase in free radicals can cause multiorgan damage.

In conclusion, abnormal adipocytokine expression with consequent inflammation, oxidative stress itself may result from the inflammatory changes that occur in obesity. Therefore, a vicious cycle that provokes increased oxidative stress in obesity may exist. Reactive oxygen species that lead to increased oxidative stress can be generated in adipocytes and in other cell types such as leukocytes, all of which can be a source of increased oxidative stress in obese humans. Increased oxidative stress is independently associated with obesity measures including body mass index and waist-hip ratio. It is also associated with several CVD risk factors including smoking, blood glucose, and hyperlipidemia. Oxidative stress and increased adipocytokines may also promote endothelial dysfunction, atherogenesis, and coronary heart disease independent of traditional risk factors.

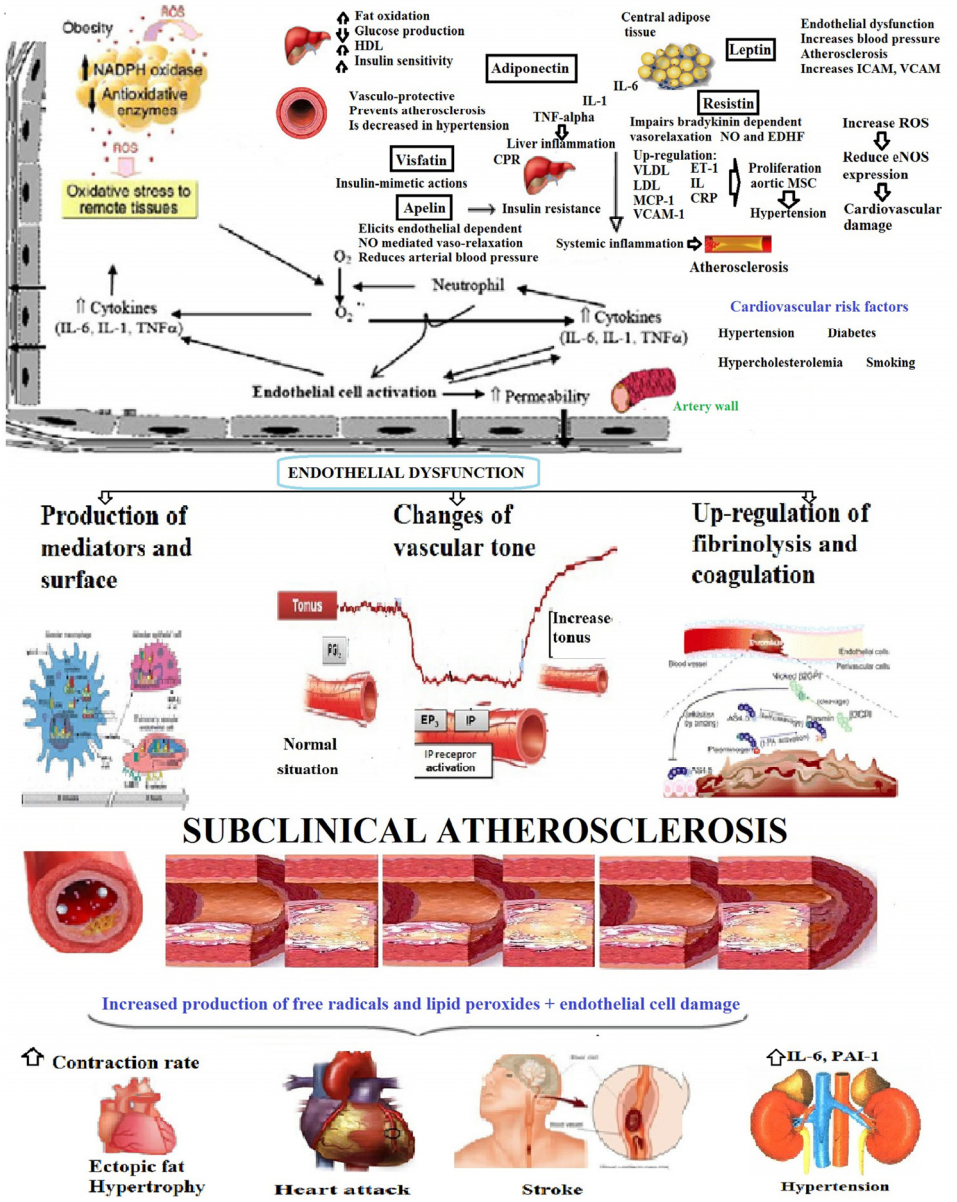

Fig. 1. Impaired cardiovascular functions and adipocytokines actions on oxidative stress. This figure shows the majority of cellular elements and signaling pathways in which highlights the participation of adipocytokines (framed) involved in the immune response and oxidative stress on the vascular endothelium. These alterations lead to development of atherosclerosis. And finally, this endothelial dysfunction can generate harmful free radicals and cause tissue and multiorgan damage.

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5

Oxidative Stress in the Carotid Body: Implications for the Cardioventilatory Alterations Induced by Obstructive Sleep Apnea

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1. Introduction

The obstructive sleep apnea (OSA) syndrome is recognized as an independent risk factor for systemic hypertension. The OSA syndrome is characterized by cyclic episodes of oxygen desaturation due to the partial or complete obstruction of the air flow during sleep. Among the disturbances produced by OSA, the chronic intermittent hypoxia is considered the main factor for developing hypertension. Oxidative stress, inflammation, and sympathetic hyperactivity have been proposed as pathogenic mechanisms involved in the hypertension. However, evidence for a single mechanism has been difficult to establish in OSA patients, because of concomitant comorbidities. Since OSA patients show augmented reflex sympathetic, cardiovascular and ventilatory responses to acute hypoxia, it has been proposed that an enhance carotid body responsiveness to hypoxia is involved in the pathological alterations induced by OSA. This proposal has received further support, since studies performed in animals have shown that intermittent hypoxia selectively enhances the carotid body chemosensory and ventilatory responses to acute hypoxia, producing long-term potentiation of the motor ventilatory and sympathetic discharges. The mechanisms underlying the enhanced carotid body chemosensory reactivity to hypoxia induced by intermittent hypoxia are not completely known. Nevertheless, the available evidence indicates that the repeated episodes of hypoxia-reoxygenation produce local oxidative stress in the carotid body due to the accumulation of reactive oxygen species. In this chapter, we will review and discuss the new evidence supporting the essential role played by the carotid body chemoreceptors, and the contribution of the oxidative stress, endothelin-1 and pro-inflammatory cytokines to the progression of the cardioventilatory alterations induced by chronic intermittent hypoxia.

2. The carotid body chemoreceptors

Most of the mammalian cells respond to hypoxia modifying the expression of genes and proteins, which induce a physiological response to recover the tissue oxygen levels (i.e.

angiogenesis). However, gene expression induction is not fast enough to counteract a rapid drop in systemic oxygen levels. Only the peripheral chemoreceptors located in the carotid and aortic bodies are capable to evoke fast systemic adjustments to overcome a hypoxic episode. The carotid body located in the bifurcation of the carotid arteries is the main arterial chemoreceptor in terms of its contribution to the reflex ventilatory responses to hypoxia (Gonzalez et al., 1994). In humans and mammals, the carotid body initiates the hyperventilatory response induced by hypoxia and activates the sympathetic nervous system. The carotid body is a complex chemoreceptor organ with a high blood flow formed by different types of cells. The glomus cells are considered the oxygen sensors in the carotid body. Glomus cells establish synaptic contacts with the nerve terminals of the primary sensory neurons, whose soma are located in the petrosal ganglion (Gonzalez et al., 1994, Iturriaga & Alcayaga, 2004, Iturriaga et al., 2007). The current model for oxygen chemoreception in the carotid body states that low oxygen induced the inhibition of a voltage-independent potassium TASK-like current, leading to the depolarization of the glomus cells, followed by the entry of Ca^{2+} through L-type Ca^{2+} channels and the subsequent release of one or more excitatory transmitters, which in turn increase the discharges of action potentials in the nerve endings of the chemosensory neurons (Iturriaga & Alcayaga 2004, Iturriaga et al., 2007). The glomus cells contain several molecules proposed as putative excitatory transmitters, such as dopamine, acetylcholine, adenosine nucleotides and peptides. Among these molecules present in glomus cells, acetylcholine and adenosine triphosphate fulfill most of the criteria to be considered as the excitatory transmitters between the glomus cells and petrosal nerve ending (Iturriaga et al., 2007). However, other molecules such as dopamine, histamine, nitric oxide and endothelin-1 acts as modulators of the chemosensory process, acting on the glomus cells or controlling the vasomotor tone of the blood vessel (Iturriaga et al., 2007). More recently, it has been proposed that pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6) and interleukin 1β (IL-1β) are excitatory modulators of the chemoreception process in the rat carotid body (Lam et al., 2008, Liu et al., 2009, Shu et al., 2007).

3. Cardiovascular alterations in patients with obstructive sleep apnea and animals exposed to intermittent hypoxia

The OSA syndrome, a highly prevalent sleep-breathing disorder is now recognized as an independent risk factor for systemic hypertension. Approximately 50% of the OSA patients develop systemic diurnal hypertension and 30% of the hypertensive patients have OSA. The OSA syndrome is also associated with stroke, pulmonary hypertension, coronary artery disease and atrial fibrillation (Garvey et al., 2009, Parati et al., 2007, Somers et al., 2008). The OSA syndrome affect up to 5% worldwide adult population, but according to the report of the American Heart Association in collaboration with the National Heart, Lung, and Blood Institute National Center on Sleep Disorders Research (Somers et al., 2008) "85% of patients with clinically significant and treatable OSA have never been diagnosed, and referral populations of OSA patients represent only the *tip of the iceberg* of OSA prevalence". OSA is characterized by recurrent episodes of partial or complete obstruction of the air flow during sleep produced by the collapse of the pharyngeal airway. The interruption of the air flow produces hypoxia and hypercapnia, negative intrathoraxic pressure, sleep fragmentation

and arousal. During the airway occlusion, hypoxia and hypercapnia stimulate the carotid body chemoreceptors increasing the respiratory muscle effort, the vascular sympathetic tone and the arterial blood pressure. Finally, the stimulation of the carotid body chemoreceptors and probably the pulmonary mechanoreceptors elicit arousal and restores the ventilation. Among the disturbances produced by OSA, the exposure to intermittent hypoxia is considered the main factor for developing hypertension. Oxidative stress, inflammation and sympathetic overflow have been proposed as potential pathogenic mechanisms involved in the onset of the hypertension and cardiovascular diseases (Arnardottir et al., 2009, Garvey at al., 2009, Lavie 2003, Somers et al., 2008). However, conclusions from studies performed in humans are conflictive, because of concomitant effects of comorbidities (obesity, cardiovascular diseases, diabetes, etc) associated with OSA (Gozal & Kheirandish-Gozal, 2008, Somer et al., 2008). Thus, animal models of chronic intermittent hypoxia (CIH), which simulate the hypoxic-reoxygenation cycles observed in OSA patients, reproduce several pathologic cardiovascular features of OSA (Fletcher et al., 1992, Pack 2009; Schulz et al., 2008). The hypoxic-reoxygenation episodes in OSA patients enhance the cardiorespiratory and sympathetic responses to acute hypoxia (Carlson et al., 1993; Narkiewicz et al., 1998a, 1998b, 1999); impair the autonomic regulation of the heart rate and the arterial blood pressure (Narkiewicz et al., 1999b, Shiomi et al., 1996) and exacerbate the renin-angiotensin system (Fletcher et al., 2002, Moller et al., 2003). Similarly, animals exposed to intermittent hypoxia show potentiated sympathetic discharges and vascular responses to hypoxia, and develop systemic hypertension (Dick et al., 2007; Fletcher et al., 1992, Greenberg et al., 1999, Zoccal et al., 2008). The autonomic hyperactivity is associated with a reduction of the efficiency of the baroreflex control of heart rate and alterations of heart rate variability in OSA patients (Narkiewicz et al., 1998b, Shiomi et al., 1996) and animals exposed to intermittent hypoxia (Lai et al., 2006; Lin et al., 2007; Rey et al., 2004, 2008). Thus, it is likely that the enhanced sympathetic activity along with the reduction of the baroreflex efficiency would impair the regulation of heart rate and the vasomotor tone of blood vessels eliciting hypertension. Besides that, it has been found that intermittent hypoxia produces parasympathetic withdrawal, attributed in part to neuronal loss in the vagal ambiguous nucleus (Lin et al., 2007, Yan et al., 2008).

4. Contribution of the carotid body to the cardiorespiratory alterations in obstructive sleep apnea and animal exposed to intermittent hypoxia

Patients with recently diagnosed OSA show enhanced ventilatory, sympathetic and vasopressor responses to acute hypoxia, attributed to a potentiated hypoxic chemoreflex (Cistulli & Sullivan, 1994). Narkiewicz et al., (1999) studied the ventilatory, tachycardic and hypertensive responses to acute hypoxia in untreated normotensive OSA patients, and found that the hypoxic stimulation evokes higher ventilatory, tachycardic, and blood pressor responses in OSA patients than control subjects, but the ventilatory and blood pressor responses induced by hypercapnia and by the cold pressor tested in OSA patients were not different from control subjects. Loredo et al., (2001) reported that OSA hypertensive patients present higher basal tidal volumes, suggesting an enhanced carotid body chemosensory drive. Leuenberger et al., (2007) measured changes in sympathetic discharges recorded from the peroneal nerve of normal humans in response to acute

hypoxic stimulation before and after the exposure to 30 episodes of apnea. The episodes of apnea do not only increased sympathetic discharges and produced mild increases in arterial blood pressure, but also enhanced the sympathetic neural response to acute hypoxia, indicating that short-term intermittent hypoxia produces a facilitation of the hypoxic chemoreflex in normal humans. Thus, the available evidence supports the proposal that the enhanced oxygen chemoreflex response in OSA patients is produced by the intermittent hypoxia. Similarly, rats and cats exposed to chronic intermittent hypoxia show enhanced hypoxic ventilatory responses to acute hypoxia (Iturriaga et al., 2009, Rey et al., 2004; Reeves et al., 2003) and long-term facilitation of respiratory motor responses (McGuire et al., 2003, Dick et al., 2007, Prahbakar et al., 2005). The long-term potentiated ventilatory responses to acute hypoxia observed in animals exposed to intermittent hypoxia has been attributed to a central facilitation of the serotonin-mediated motor ventilatory output (McGuire et al., 2003). Although, Narkiewicz et al., (1998a, 1998b) found that sympathetic, pressor and ventilatory responses to acute hypoxia were enhanced in OSA patients, and Fletcher et al., (1992) reported that the bilateral carotid body denervation prevents the hypertension in rats exposed to intermittent hypoxia, the idea that carotid body chemoreceptors are involved in the progression of the hypertension did not receive much attention. However, new evidence obtained in the last decade have shown that an abnormal potentiated carotid chemosensory reactivity to hypoxia is crucial to potentiate the sympathetic activity (Iturriaga et al., 2005, 2009, Feng et al., 2008, Garvey at al., 2009; Prabhakar et al., 2005, Rey et al., 2004; Smith & Pacchia, 2007).

5. Intermittent hypoxia enhanced the carotid body chemosensory responses to acute hypoxia

Recording of chemosensory discharges from the carotid sinus nerve have shown that chronic intermittent hypoxia produces long-term potentiation of the carotid body chemosensory responses to acute hypoxia. Indeed, exposure of cats and rats to intermittent hypoxia for 4 to 10 days increases the basal carotid body chemosensory discharges measured in normoxia and enhances the chemosensory responses to acute hypoxia (Peng et al., 2003, Rey et al., 2004, Del Rio et al., 2010). Peng et al., (2003) found that the baseline carotid discharge and the chemosensory responses to acute hypoxia were higher in rats exposed to short cyclic hypoxic episodes followed by normoxia, applied during 8 hrs for 10 days. Similarly, we found that cats exposed to intermittent hypoxia during 8 hrs for 4 days showed enhanced CB chemosensory and ventilatory responses to acute hypoxia (Rey et al., 2004). In rats, we found that intermittent hypoxia for 7 days potentiates the carotid chemosensory responses to acute hypoxia, effect that persisted until 21 days of intermittent hypoxia when animals developed hypertension (Del Rio et al., 2011). Figure 1 illustrates representative recordings of carotid chemosensory responses induced by short hypoxic challenges in a sham rat and in one carotid body from a rat exposed to 5% O_2, 12 times/hr during 8 hrs for 21 days. As is shown in fig. 1, chronic intermittent hypoxia increased the baseline carotid chemosensory discharges measured in normoxia and induced a potentiation of chemosensory responses to acute hypoxia. Since these alterations in the carotid chemosensory function occurred without significant elevation of the arterial blood pressure until 21 days of intermittent hypoxia, the hypertension was preceded by an early potentiation of the carotid body chemosensory and ventilatory responses to hypoxia.

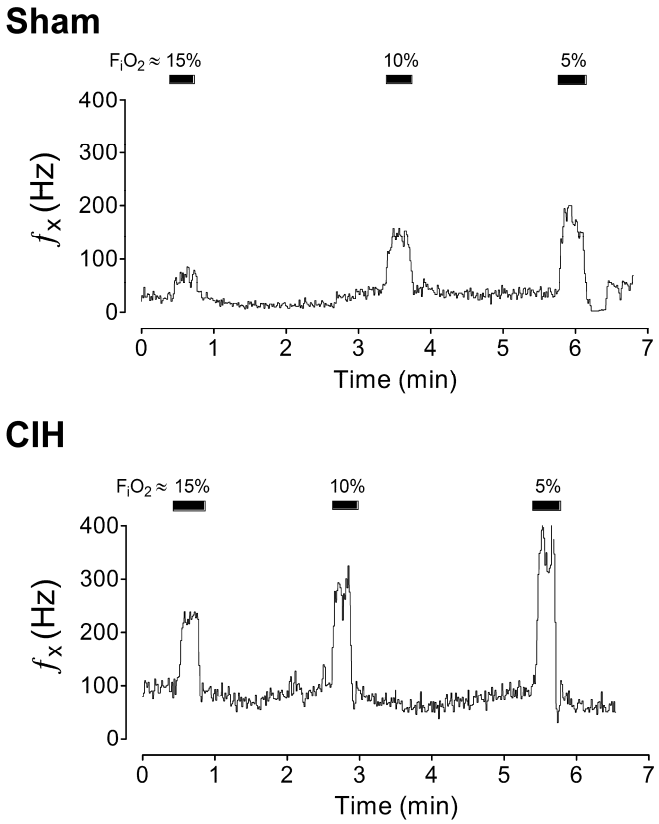

Fig. 1. Carotid body chemosensory potentiation induced by intermittent hypoxia in the rat. The carotid chemosensory discharges in response to various levels of inspired O_2 (FiO_2 ~15 to 5%) were measured from the carotid sinus nerve of a sham rat exposed to air to air cycles and a rat exposed to chronic intermittent hypoxia (CIH) for 21 days. f_x, frequency of carotid chemosensory discharges, expressed in Hz.

6. Mechanisms underlying the potentiation of carotid body chemosensory responses to hypoxia induced by chronic intermittent hypoxia

The enhance carotid chemosensory responses to hypoxia has been associated to increased levels of reactive oxygen species (Peng et al., 2003, Iturriaga et al., 2009, Del Rio et al., 2010) and endothelin-1 within the CB (Rey et al., 2006, Pawar et al., 2009), but it is possible that pro-inflammatory cytokines, which increased in the plasma of OSA patients (Lavie 2003, Jelic et al., 2008) may also contributes to the enhanced carotid body chemosensory responses to acute hypoxia (Iturriaga et al., 2009; Del Rio et al., 2011). Although some studies addressed the effects of intermittent hypoxia on transmitter production and release in the carotid body, very little is known on the functional significance of the role played by the neurotransmitters in the carotid body chemosensory potentiation induced by intermittent hypoxia (See for review Kumar, 2011).

6.1 Reactive oxygen and nitrogen species

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been proposed as mediators of the cardiovascular and cognitive morbidities in several diseases including the OSA syndrome (Christou et al., 2003; Gozal & Kheirandish-Gozal, 2008, Lavie 2003) and in pathological consequences of intermittent hypoxia in animal models (Chen et al., 2005, Del Rio et al., 2010, Peng et al., 2003, Peng et al., 2009). Studies performed in OSA patients and animals exposed to chronic intermittent hypoxia have shown that the cyclical episodes of hypoxia-reoxygenation produces systemic oxidative stress due to the accumulation of ROS and RNS, which are well known potential sources of cellular damage. Peng et al., (2003) found evidence that the superoxide radical participates in the potentiation of the rat carotid chemosensory responses to hypoxia induced by intermittent hypoxia. They found that pre-treatment of rats for 10 days before the exposure to intermittent hypoxia with the superoxide dismutase mimetic, manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP) prevents the potentiation of the carotid body chemosensory response to hypoxia. In addition, they found that intermittent hypoxia decreases the activity of the ROS sensitive enzyme aconitase in the whole carotid body, as well as the activity of the complex I of the mitochondrial electron transport chain, suggesting that the mitochondria is one of the sources of ROS (Peng & Prabhakar, 2003). More recently, Peng et al., (2009) tested the hypothesis that ROS generated by NADPH oxidase (NOX) mediate the intermittent hypoxia-induced carotid body potentiation. They found that acute hypoxia produced a larger increase in NOX activity in carotid body of rats exposed to intermittent hypoxia for 10 days than that of control carotid bodies. The carotid body chemosensory potentiation was prevented by NOX inhibitors and was not observed in NOX2 deficient mice. On the other hand, MacFarlane and Mitchell (2008) found that application of MnTMPyP into the intrathecal space of the cervical spinal cord abolished the phrenic long-term potentiation induced by acute intermittent hypoxia in rats, suggesting that ROS production is needed for enhancing the phrenic nerve ventilatory discharge. Consequently, ROS formation seems to be necessary for respiratory plasticity induced by intermittent hypoxia, at the level of the carotid body and respiratory motor output. Recently, we tested the hypothesis that oxidative stress contributes to the carotid chemosensory potentiation and the progression of the hypertension in rats exposed to intermittent hypoxia (Del Rio et al., 2010). We hypothesized that oral supplementation of the common antioxidant ascorbic acid (vitamin C) may prevent the carotid chemosensory potentiation and the cardioventilatory alterations including the hypertension induced by the intermittent hypoxic exposure. Accordingly, we studied the effects of ascorbic acid supplementation in the drinking water (1.25 g/l) on plasma lipid peroxidation, arterial blood pressure, and carotid chemosensory responses to acute hypoxia in rats exposed to short hypoxic episodes (5% O_2, 12 times/hr for 8 hrs) for 21 days (Del Rio et al., 2010). We found that exposure of the rats to intermittent hypoxia increased the plasma lipid peroxidation and the formation of 3-nitrotyrosine in the carotid body, the arterial blood pressure and enhanced the carotid chemosensory and ventilatory responses to hypoxia. Ascorbic acid treatment reduced the increased plasma lipid peroxidation and the formation of 3-nitrotyrosine in the carotid body, the potentiation of carotid body chemosensory responses (See Fig. 2), the ventilatory responses to acute hypoxia, as well as the hypertension.

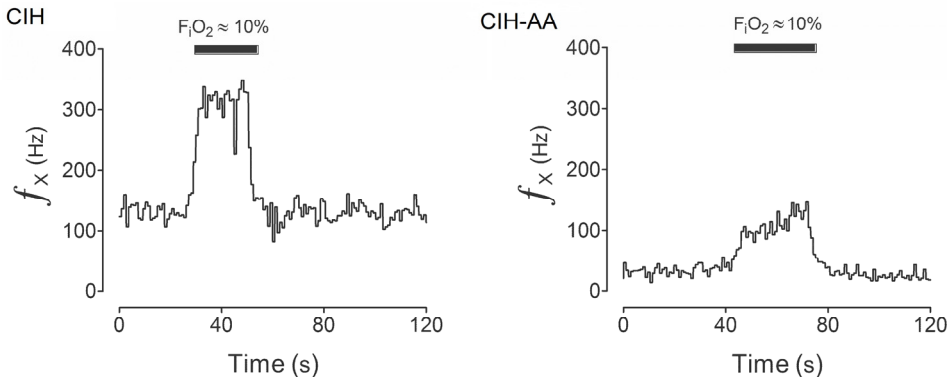

Fig. 2. Effect of ascorbic acid on the potentiated carotid body chemosensory responses to hypoxia induced by intermittent hypoxia. CIH, rat exposed to intermittent hypoxia. CIH-AA, rat exposed to intermittent hypoxia and treated with ascorbic acid. Note that ascorbic acid reduced both the baseline and the chemosensory response to 10% O_2. f_x, frequency of carotid chemosensory discharges, expressed in Hz.

Although the current information suggests that an increased local oxidative stress contributes to the carotid body chemosensory potentiation induced by intermittent hypoxia, the direct participation of ROS on the oxygen chemotransduction process is matter of debate, because no chemosensory excitatory effects of ROS have been observed (Gonzalez et al., 2007). A possible explanation is that an increased level of the superoxide radical in the carotid body may reacts with nitric oxide generating peroxynitrite, a powerful oxidizing agent that nitrates tyrosine residues forming 3-nitrotyrosine. We already found the excessive formation of 3-nitrotyrosine in glomus cells and blood vessels from carotid bodies harvested from rats exposed to intermittent hypoxia (Del Rio et al., 2010, 2011), as is shown in fig. 3. The increased formation of 3-nitrotyrosine indicates that the carotid body tissue is continuously exposed to oxidative stress during the intermittent hypoxic exposure. In addition, we found that a correlation between the marked increase of 3-nitrotyrosine immunoreactivity in the carotid body exposed to intermittent hypoxia and the enhanced carotid chemosensory responses to acute hypoxia (Del Rio et al., 2011), supporting and extending the idea that oxidative-nitrosative stress plays a critical role in the CB chemosensory potentiation (Iturriaga et al., 2009, Peng & Prabhakar, 2003). In OSA patients, Jelic et al., (2008) found that the expression of 3-nitrotyrosine in endothelial cells was greater than controls subjects, indicating that the oxidative stress contributes to the endothelial dysfunction caused by the intermittent hypoxia. In addition to the formation of nitrotyrosine residues, peroxynitrites may also modify iron sulfur clusters, zinc thiolates and other residues. Moreover, peroxynitrites may react with inorganic molecules such as CO_2 producing other free radicals that may modify DNA, lipids or proteins.

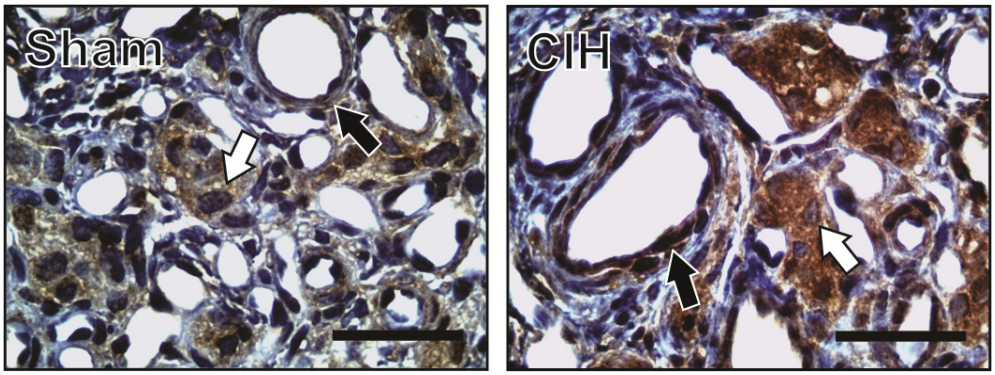

Fig. 3. Exposure to CIH increased 3-nitrotyrosine formation in the glomus cells (white arrows) and endothelial cells (black arrows) from rat carotid bodies. Scale bar, 20 μm.

6.2 Vasoactive molecules

An interesting molecule, which may mediate the carotid body chemosensory potentiation induced by intermittent hypoxia, is endothelin-1 (ET-1). It is known that the plasmatic ET-1 level increases in rats exposed to intermittent hypoxia (Kanagy et al., 2001) and OSA patients (Phillips et al., 1999). This potent vasoconstrictor peptide is expressed in the endothelium, blood vessels and glomus cells of the carotid body (Rey et al., 2007). The application of ET-1 produces chemosensory excitation in both *in situ* and *in vitro* carotid body perfused preparations, but not in the superfused preparation devoid of vascular effects (Rey & Iturriaga, 2004). We found that ET-1 was increased locally in the carotid body of cats exposed to 4 days to intermittent hypoxia by ~10-fold, while ET-1 plasma levels remains unchanged (Rey et al., 2006). The enhanced carotid body chemosensory responses to hypoxia were reduced by the ET receptor blocker bosentan in the intermittent hypoxic - treated cats, but have no effects on the carotid body chemosensory activity in control animals (Rey et al., 2006), indicating that a local increase of ET-1 contributes to enhance the carotid chemosensory responses. Pawar et al., (2009) tested the hypothesis that ET-1 induced by ROS plays a role in intermittent hypoxia induced chemosensory potentiation in the rat neonatal carotid body. They found that intermittent hypoxia enhanced the release of ET-1 and the expression of the ET-A receptor in response to intermittent hypoxia. Systemic administration of MnTMPyP, which prevent the elevation of ROS, reduced the increased basal release of ET-1, the overexpression of ET-A receptor mRNA and the enhanced carotid body chemosensory response to acute hypoxia. These results support the idea that a ROS-induced increase of ET-1 release is involved in the potentiation of carotid body chemosensory response elicited by intermittent hypoxia. Increased plasmatic levels of ROS and ET-1 have been also implicated in the hypertension induced by intermittent hypoxia. Troncoso-Brindeiro et al., (2007) reported that the concurrent treatments of rats exposed to intermittent hypoxia with the SOD mimetic, 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPOL), prevents the increased ROS plasmatic level and the hypertension. However, it is worth noting that intermittent hypoxia increases the expression of ET-1 in the rat CB during the first week of hypoxia, and later the ET-1 levels returned back to the control levels (Del

Rio et al., 2011), suggesting that ET-1 may contribute to the enhanced carotid body responsiveness to hypoxia in the early phase of the intermittent hypoxic exposures. In addition to the transient changes in ET-1 expression, we found a significant decrease in the eNOS expression in the rat carotid body at 7 days of intermittent hypoxia (Del Rio et al., 2011), suggesting that chronic intermittent hypoxia may decrease the nitric oxide (NO) levels within the carotid body. Since NO at low concentration is an inhibitory modulator of the carotid chemosensory activity (Iturriaga et al., 2000), a reduced NO level may contribute to enhance the carotid body chemosensitivity, as well as to amplify the vasoconstrictor effect of ET-1. This interpretation is supported by the finding that intermittent hypoxia decreases the expression of the neuronal NO synthase in the rat carotid body (Marcus et al., 2010), suggesting that the removal of the inhibitory NO effects may also contribute to enhance the carotid chemosensory responses to hypoxia. Our results also showed that carotid body iNOS immunorreactive levels increased after 21 days of intermittent hypoxic exposure. Since iNOS produce higher amounts of NO, it is plausible that the NO levels in the carotid body will increase during long-term intermittent hypoxic exposure. It is worth noting that NO has a dual effect on carotid chemosensory discharges. Indeed, Iturriaga et al., (2000) found that at low levels NO is predominantly an inhibitor of the chemosensory discharges, whereas at high concentration NO increases carotid body chemosensory discharges. Thus, it is plausible that high NO levels in the carotid body following long-term intermittent hypoxia may partially contribute to maintain the carotid body chemosensory potentiation.

6.3 Pro-inflammatory cytokines

Endothelial dysfunction has been related to the progression of hypertension in OSA patients and animals exposed to intermittent hypoxia due to the increased plasmatic levels of pro-inflammatory cytokines (Biltagi et al., 2008, Jelic et al., 2008, Jun et al., 2008, Tam et al., 2007, Williams & Scharf, 2007). It is likely that an increased production of ROS induced by the hypoxia-reoxygention cycles may evoke the expression of genes and the synthesis of pro-inflammatory cytokines, mediated by the activation of transcription factors such as the nuclear factor kappa B (NF-κB), the activator protein 1 and HIF-1α (Semenza & Prahbakar, 2007). In response to oxidative stress, HIF-1α induces the expression of several genes including ET-1 and iNOS, but ROS also produces the translocation of NF-κB to the nucleus, increasing the expression of several inflammatory genes such as IL-1β, IL-6, TNF-α, adhesion molecules, iNOS and ET-1 (Janseen-Heininger et al., 2000). Recently, we found that intermittent hypoxia increased the levels of TNF-α and IL-1β in the rat carotid body after 21 days of exposure (Del Rio et al, 2011). We found that glomus cells constitutively expresses TNF-α and IL-1β in the cell bodies and that chronic intermittent hypoxia up-regulates the expression of both TNF-α and IL-1β, without inducing carotid body tissue infiltration with macrophages or changes in TNF-α and IL-1β plasmatic levels (Del Rio et al., 2011). Our results showed that exposure to intermittent hypoxia enhances the rat carotid chemosensory responses to acute hypoxia, and progressively increase the immunorreactive TNF-α and IL-1β expression in the carotid body, suggesting a potential role for this cytokines in modulating the enhanced carotid body chemosensory activity after exposure to intermittent hypoxia.

7. Proposed targets of the effects of ROS in the carotid body

The available evidence indicates that oxidative stress mediated the potentiation of the carotid body chemosensory responses to acute hypoxia, induced by the exposure to intermittent hypoxia. However, the nature of the molecular mechanism by which ROS induced chemosensory potentiation is not known. Based on the presented evidences, we hypothesized that chronic intermittent hypoxia may increase the expression of pro-inflammatory cytokines and other chemosensory modulators, such as ET-1 and NO, which may potentially contribute to enhance the carotid body chemosensory responses to hypoxia. Figure 4 summarized the possible targets of the effects of ROS on oxygen chemoreception in the carotid body. It is plausible that excessive amounts of free radicals may modify the O_2-sensitive K^+ channels, increasing the intracellular Ca^{2+} levels, which in turn evokes the release of excitatory transmitters, but a direct participation of ROS on the O_2 chemotransduction process in the carotid body is not clear (Gonzalez et al., 2007). ROS or other molecules, produced downstream of the ROS signal, which act upon the mitochondria, membrane channels or the gene expression machinery may modify the oxygen sensing in the carotid body. Further studies are required to determine which protein or enzyme complexes involved in the carotid body chemosensory process are affected by ROS or ROS-dependent molecules induced by intermittent hypoxia.

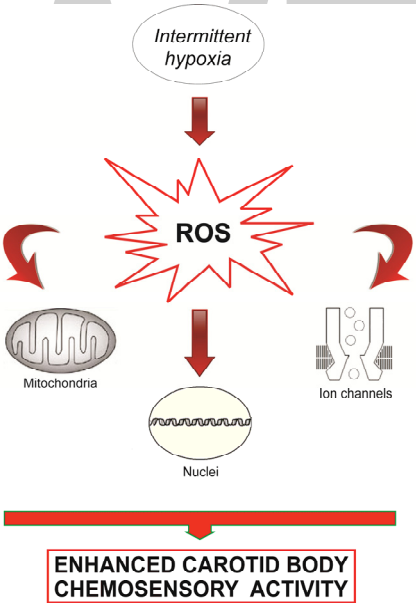

Fig. 4. Proposed targets of the effects of ROS on the potentiation of the carotid body induced by intermittent hypoxia.

8. Integrative model

Figure 5 shown a diagram of the proposed mechanisms involved in the potentiation of the carotid body chemosensory responses to hypoxia induced by intermittent hypoxia, which

finally contributes to the development of the hypertension. We postulate that cyclic episodes of hypoxia-reoxygenation enhance the carotid body chemosensitivity to hypoxia, which in turn contributes to elicit a persistent facilitation of the sympathetic neural output. The enhanced sympathetic activity along with a reduction of the baroreflex efficiency should impair the regulation of the heart rate variability and the vasomotor tone of blood vessels, resulting in an elevation of arterial blood pressure. On the other hand, systemic oxidative stress and the inflammation *per se* may contribute to the endothelial dysfunction, leading to the hypertension.

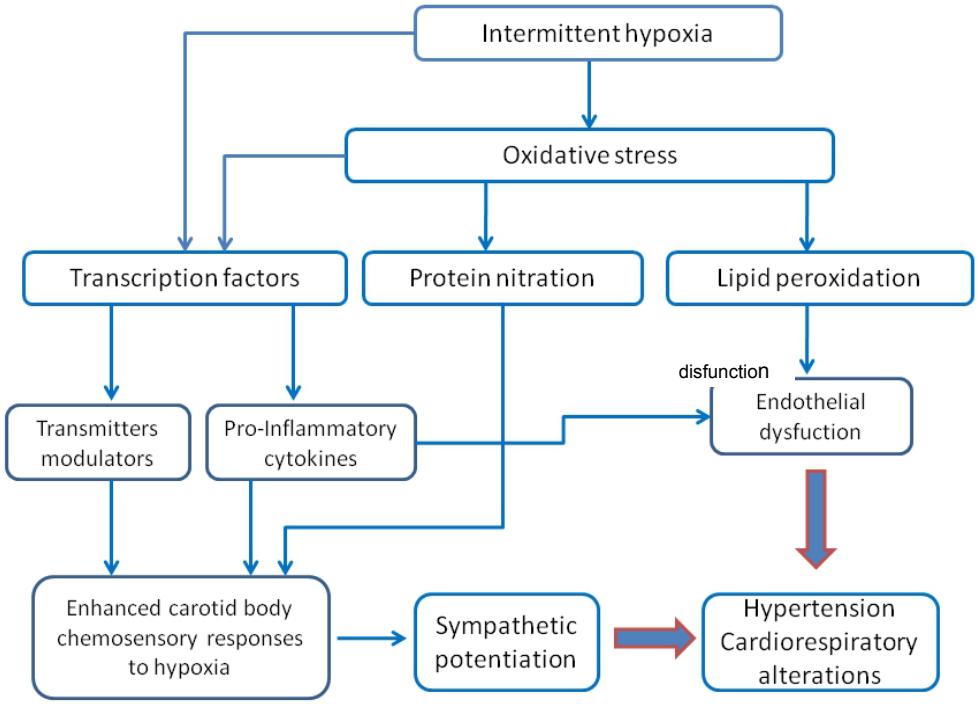

Fig. 5. Proposed mechanisms involved in the hypertension induced by the potentiation of the carotid body chemosensory responses to hypoxia induced by intermittent hypoxia, and the systemic oxidative stress.

9. Conclusion

Autonomic dysfunction has been associated to exposure to chronic intermittent hypoxia in animal models, and is thought to be involved in the increased risk of hypertension and cardiovascular mortality in OSA patients. The cyclic hypoxic episodes in OSA patients potentiate cardiovascular and sympathetic responses induced by hypoxic stimulation of peripheral chemoreceptors, and impair the regulation of arterial blood pressure and the renin–angiotensin system. Intermittent hypoxia enhances the ventilatory and cardiovascular responses to acute hypoxia, suggesting a major role of the carotid body in the pathological

cardiorespiratory consequences of intermittent hypoxia. New evidences indicate that intermittent hypoxia induced oxidative stress in the carotid body, which contributes to potentiate the carotid body chemosensory responses to acute hypoxia. Understanding how the oxidative stress interacts with the carotid body chemoreceptor system will provide new insights into the pathophysiological cardiovascular consequences of OSA. Thus, upcoming new knowledge in the field will serve to propose new therapies to moderate the severity of the cardiovascular alterations induced by OSA. We believe that is possible to ameliorate or prevent the hypertension using antioxidants, which will reduced the systemic oxidative stress as well as the carotid body potentiated chemosensitivity to hypoxia.

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6

Oxidative Damage in Cardiac Tissue from Normotensive and Spontaneously Hypertensive Rats: Effect of Ageing

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1. Introduction

The spontaneously hypertensive rat (SHR) is a laboratory model of naturally developing hypertension and heart failure that appears to be similar in many aspects to essential hypertension in humans (Trippodo & Frohlich, 1981). Systolic blood pressure in SHR rapidly increases during 5 to 10 weeks of age and develops cardiac hypertrophy between 9 and 12 weeks of age (Shimamoto et al., 1982). Increasing evidence from different experimental models supports the concept that oxidative stress contributes to the pathogenesis of myocardial hypertrophy and in the process of myocardial remodeling leading to heart failure (Yücel et al., 1998; Lasségue & Griendling, 2004).

The oxidative stress is the result of an increase of reactive oxygen species (ROS) and/or inadequate antioxidant defense mechanisms. It has been shown that an increase in the activity and expression of myocardial NAD(P)H oxidase (NOX) is the main source of ROS in cardiac hypertrophy (Bendall et al., 2002; Griendling et al., 2000; Xiao et al et al., 2002). However, existing data about the antioxidant status in hypertension are inconsistent. Some studies have shown that the activities of one or more antioxidant enzymes are lower (Ito et al, 1995; Newaz & Nawal, 1999), higher (Czonka et al., 2000) or without changes (Gómez-Amores et al., 2006; Girard et al, 2005) compared with normotensive controls. Although the underlying causes of these discrepancies are unknown, it may be possibly due to the use of different hypertension models, animals at different hypertensive stages and/or different experimental preparations.

On the other hand, ROS are thought to be a key mechanism in the aging process (Beckman & Ames, 1998; Colavitti & Finkel, 2004; Harman, 1988) and there are arguments that NOX-

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derived ROS may lead to cellular senescence (Ago et al., 2010a; Ago et al., 2010b; Imanishi et al., 2005). Thus, lipid peroxidation and oxidative modification of proteins by ROS like peroxynitrite-the product of combination of superoxide ($O_2^{-\cdot}$) and nitric oxide (NO)- are implicated in the pathogenesis of hypertrophy (Nadruz et al., 2004) and in cardiac normal aging (Beal, 2002).

The aim of this study was to assess the oxidative stress in hearts from young and old SHR compared to age-matched Wistar rats.

2. Methods

Experiments were conducted with 40 days and 4-, 11- and 19-month-old male SHR and age-matched Wistar rats. All animals were identically housed under controlled lighting (12 hs) and temperature (20 °C) conditions with free access to standard rat chow and tap water. The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised in 1996). Systolic blood pressure (SBP) was recorded by the tail-cuff method (Camilión de Hurtado et al., 2002). Left ventricular hypertrophy (LVH) was evaluated by the ratio between heart weight (HW) and tibia length (TL) as previously described (Yin et al., 1982). Wistar strain was used as normotensive control rat. For the biochemical determinations SHR and Wistar rats of 4- and 19 months-old were used. The animals were decapitated and hearts were quickly removed and perfused with ice-cold saline solution (0.9% NaCl) to remove the blood. Left ventricle (LV) samples were taken to assay NOX activity, superoxide production and protein nitration. The rest of the heart was homogenized in 5 volume of 25 mM PO_4KH_2 - 140 mM ClK at pH = 7.4 containing protease inhibitors cocktail (Complete Mini Roche) with a Polytron homogenizer. An aliquot of heart homogenate was used to assess lipid peroxidation. The remaining homogenate was centrifuged at 12000 x g for 5 min at 4º C and the supernatant stored at -70 ºC until superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities were assayed. Protein concentration was evaluated by Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

2.1 Assessment of lipid peroxidation

Lipid peroxidation was determined by measuring the level of thiobarbituric acid reactive substances (TBARS), expressed as nmol/mg protein. Heart homogenates were centrifuged at 2000 x g for 10 min. Supernatants (0.5 ml) were mixed with 1.5 ml trichloroacetic acid (30 % w/v), 1 ml thiobarbituric acid (0.7% w/v) and 0.5 ml water followed by boiling during 15 min. After cooling, absorbance was determined spectrophotometrically at 535 nm, using a ε value of 1.56×10^5 M^{-1} cm^{-1} (Buege & Aust, 1978).

2.2 Assessment of protein nitration

The interaction of peroxynitrite leads to nitrotyrosine formation actually considered as an indirect marker of oxidative /nitrosative stress (Halliwell, 1997). Thus, we assessed nitrotyrosine level by Western blot analysis. A sample of left ventricle was homogenized in lysis buffer (300 mM sucrose; 1 mM DTT; 4 mM EGTA, protease inhibitors cocktail: 1 tablet/15 ml of buffer; 20 mM Tris-HCl, pH 7.4). After a brief centrifugation proteins were

denatured and equal amounts of protein subjected to PAGE and electrotransferred to PVDF membranes. Membranes were incubated with an anti-nitrotyrosine polyclonal antibody (Cayman Chemical). A peroxidase-conjugated, anti-rabbit IgG (Santa Cruz Biotechnology) was used as secondary antibody, and finally bands were visualized with ECL-Plus chemiluminescence detection system (Amersham). Autoradiograms were analyzed by densitometric analysis (Scion Image).

2.3 Determination of NAD(P)H oxidase (NOX) activity

Left ventricular slices (LVS, 1 x 5 mm, 3 – 3.5 mg dry weight) were incubated for 5 min at 37 °C in Krebs-Hepes buffer (in mmol/l: 99 ClNa, 4.69 ClK, 1.87 Cl_2Ca, 1.2 SO_4Mg, 1.03 K_2PO_4, 25 CO_3HNa, 20 Hepes, 11.1 glucose) bubbled with 95% O_2 - 5% CO_2 to maintain pH 7.4 and then transferred to glass scintillation vials containing the same buffer with 5 μM lucigenin. Chemiluminiscence was assessed at 37°C over 15 minutes in a Scintillation counter (Packard 1900 TR) at 1-minute intervals. Vials containing all components without tissue were previously counted and the values were substracted from the chemiluminiscence signals obtained in the presence of LVS. NOX activity was measured in the presence of 100 mM NAD(P)H and expressed as cpm/mg dry weight of LVS (Souza et al., 2002).

2.4 Measurement of superoxide ($O_2^{-\cdot}$) production

Superoxide production was measured in LVS with lucigenin-enhanced chemiluminiscence in Krebs-Hepes buffer with 5 μM lucigenin (Khan et al., 2004). The chemiluminiscence in arbitrary units (AU) was recorded with a luminometer (Chameleon, Hidex) during 30 seconds each with 4.5 min interval during 30 minutes. $O_2^{-\cdot}$ production was expressed as AU per mg dry weight per minute. To determine the involvement of NOX in $O_2^{-\cdot}$ production, the slices were pretreated during 30 min with 300 μM apocynin.

2.5 SOD, CAT and GPx activities assays

SOD activity was determined by inhibition of formazan production (produced by nitroblue tetrazolium (NBT) reduction by superoxide anion) at pH 10.2 and 25º C. The reaction mixture consists in: 100 μM xanthine, 100 μM EDTA, 25 μM NBT, 50 mM CO_3Na_2, pH 10.2. The reaction was started by the addition of xanthine oxidase, reading the absorbance at 560 nm each 30 sec for 5 min (Beauchamp & Fridovich, 1971). One unit of SOD assay was defined as the amount of enzymatic protein required to inhibit 50 % of NBT reduction.

CAT activity was determined by the procedure of Aebi (1984). Decrease in absorbance at 240 nm by the addition of 30 mM H_2O_2 was monitored each 15 sec and for 30 sec. One unit of CAT assay was defined as the amount of the enzyme that decomposed 1 μmol of H_2O_2.

The GPx activity was measured according to Lawrence and Burk method (1976). The assay reaction comprised 50 mM K_2HPO_4 buffer, 1 mM EDTA, 1 mM NaN_3, 1 mM reduced glutathione, 0.2 mM NADPH, 0.25 mM H_2O_2 and 1 U/ml glutathione reductase. Gpx activity was assayed by following NADPH oxidation at 340 nm, measuring the absorbance each 15 sec for 5 min. The activity was calculated using a molar extinction coefficient for NADPH of 6.22 10^3 M^{-1} x cm^{-1} at 340 nm. One unit of the enzyme was represented the decrease of 1 μmol of NADPH/min under assay conditions.

2.6 Statistical analysis

Data are presented as mean ± SE. Differences between Wistar and SHR, young and old groups were analyzed using two-way analysis of variance (ANOVA) with the Newman-Keul's post-hoc test used for multiple comparisons among groups, considering $P < 0.05$ as statistically significant.

3. Results

Comparing to age-matched W rats, SBP of SHR was higher at all ages examined. The analysis of the time course of SBP showed that as early as at 40-day-old the SHR exhibited higher SBP values compared to age-matched W rats. At 4-month-old SBP increased more in comparison to the youngest rats and it remained elevated throughout the last stage studied. LVH significantly increased in SHR at 4, 11 and 19-month-old compared to age-matched W rats. Higher values were obtained at 11 and 19-month-old SHR when compared to younger SHR. An increase in LVH was also observed in W rats with aging (11 and 19-month-old) compared to younger rats (Table 1).

	SHR		Wistar	
	SBP (mmHg)	**LVH**	**SBP (mmHg)**	**LVH**
40 days-old	154 ± 5 *	2.04 ± 0.11	115 ± 5	1.56 ± 0.15
4 months-old	187 ± 2 *#	2.72 ± 0.17 *	116 ± 3	2.05 ± 0.12
11 months-old	178 ± 1.5 *#	3.18 ± 0.23 *#	116 ± 3	2.56 ± 0.09 §
19 months-old	191 ± 5 *#	3.40 ± 0.26 *#	107 ± 6	2.46 ± 0.04 §

Table 1. Values of systolic blood pressure (SBP) and left ventricular hypertrophy (LVH) of SHR and Wistar rats of 40 days and 4, 11 and 19 months-old. * $P < 0.05$ in SHR vs. Wistar; # $P < 0.05$ in SHR vs. 40-day-old; § $P < 0.05$ in Wistar vs.to 40-day-old.

Fig. 1 shows TBARS content in hearts from 4-, and 19-month-old SHR and Wistar rats. In hearts from SHR there was a significantly higher TBARS level of approximately 87% at 19-month-old compared to age-matched Wistar rats. No differences in TBARS with aging were observed in Wistar rats.

Nitrotyrosine levels from hearts of 4 and 19-month-old Wistar and SHR are depicted in Fig. 2. Immunoblotting assays showed a statistically significant increase of approximately 40 % in nitrotyrosine levels at 19-month-old SHR compared to age-matched Wistar rats. The oldest SHR and Wistar rats exhibited an increase of 200 and 120 %, respectively, in nitrotyrosine levels compared to their respective younger group.

Although there were no significant differences in NOX between SHR and Wistar hearts from young animals, an increase in aged rats (approximately 30% for Wistar and 60% for SHR) was obtained showing SHR the highest values (Fig. 3).

Similar $O_2^{-\cdot}$ production was obtained in hearts from Wistar rats and SHR at 4 months of age, whereas in older animals SHR showed a significantly higher $O_2^{-\cdot}$ production (approximately 170%) in comparison with age matched Wistar rats (approximately 70%) (Fig. 4). Anyway, aged rats produced a higher $O_2^{-\cdot}$ amount that younger. The addition of the selective NOX inhibitor apocynin decreased $O_2^{-\cdot}$ production in hearts of aged SHR and Wistar rats. In 4-month-old SHR and Wistar rats $O_2^{-\cdot}$ production was lower in the presence of apocynin, but

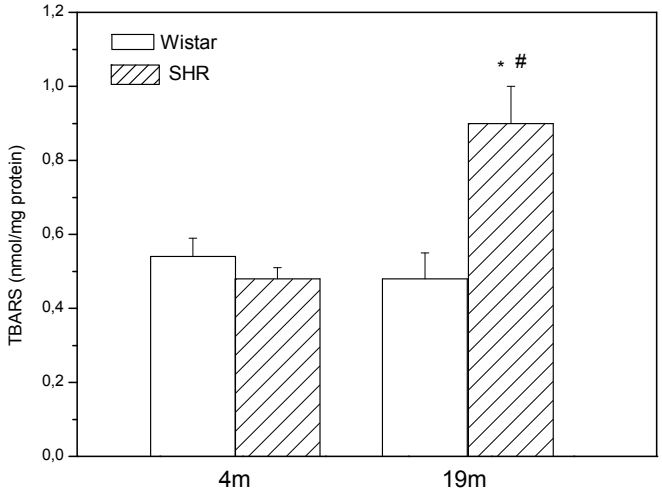

Fig. 1. TBARS content in nmol/mg protein, expressed in nmol/mg protein in hearts from SHR and Wistar rats at 4, and 19 months-old . * P < 0.05 in SHR vs Wistar ; # P < 0.05 vs 4 months-old SHR.

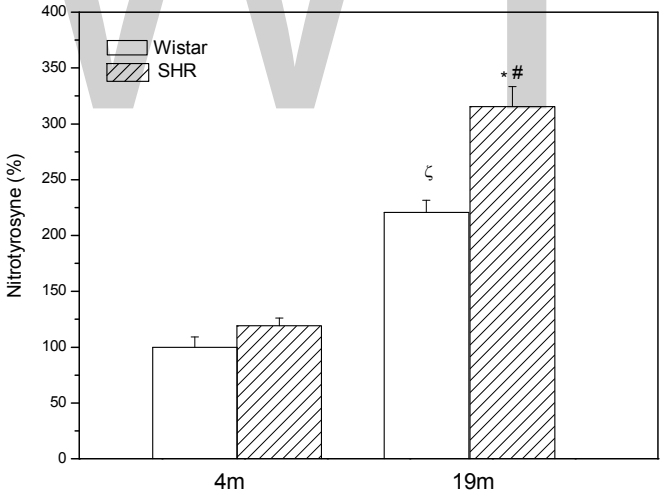

Fig. 2. Nitrotyrosine content, expressed as percentage with respect to 4-month-old Wistar rats in hearts from SHR and Wistar rats at 4 and 19 months-old. * P < 0.05 in SHR vs Wistar ; # P < 0.05 vs 4 months-old SHR; ς P < 0.05 vs 4-month-old Wistar rats.

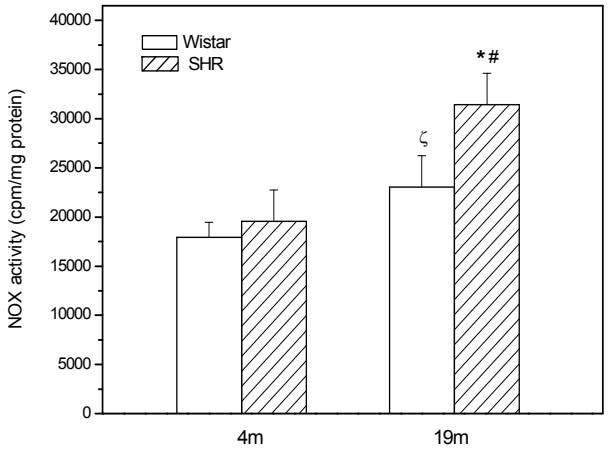

Fig. 3. NOX (NAD(P)H oxidase) activity, expressed as cpm/mg protein in hearts from SHR and Wistar rats at 4 and 19-month-old. * P < 0.05 in SHR vs Wistar; # P < 0.05 in 19- vs 4-month-old SHR; ζ P < 0.05 in 19- vs 4-month-old Wistar.

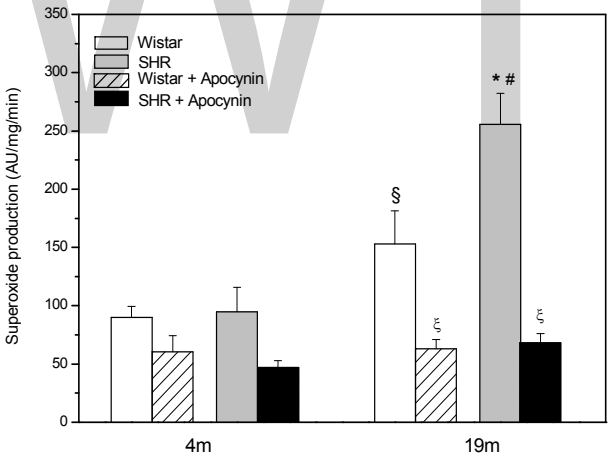

Fig. 4. Superoxide production, expressed as arbitrary units AU/mg/min, in hearts from SHR and Wistar rats at 4 and 19 months of age in the absence and presence of apocynin. *P < 0.05 in SHR vs. Wistar rats, # P < 0.05 in 19- vs. 4-month-old SHR, § P< 0.05 in 19- vs. 4-month-old Wistar rats, ξ P < 0.05 in 19-month-old SHR and Wistar rats in the presence vs. absence of apocynin.

the difference was not statistically significant. This may have been because the lucigenin method was unable to detect very small differences in $O_2^{-\cdot}$ levels that were only slightly above the background levels (Dikalov et al., 2007).

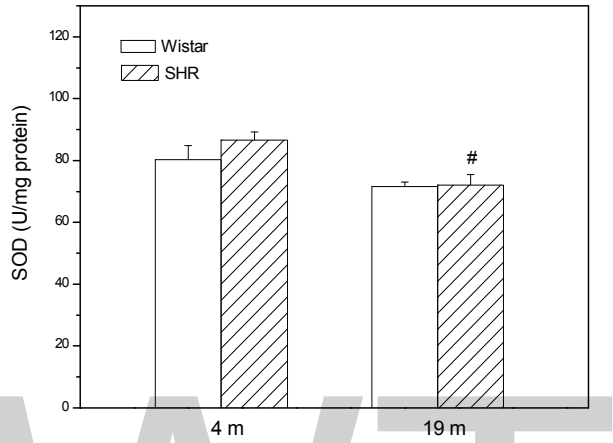

Fig. 5. Superoxide dismutase (SOD) activity, expressed as U/mg protein, in SHR and Wistar hearts of 4 and 19-month-old. # P < 0.05 in 19- vs 4-month-old SHR.

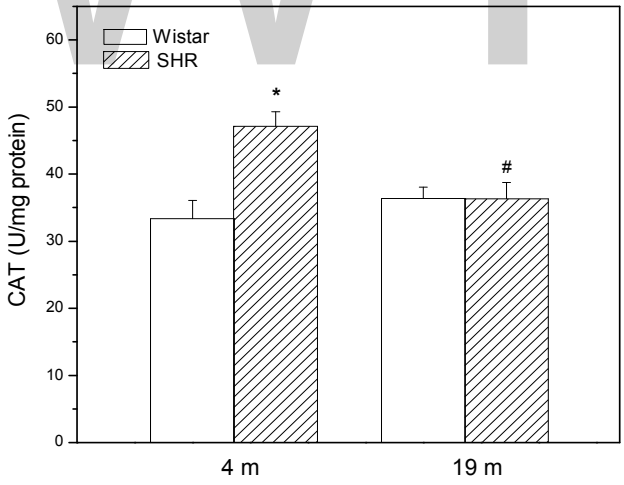

Fig. 6. Catalase (CAT) activity, expressed as U/mg protein, in SHR and Wistar hearts of 4 and 19-month-old. * P < 0.05 in SHR vs Wistar ; # P < 0.05 in 19- vs 4-month-old SHR.

The activities of antioxidant enzymes are shown in Fig. 5, 6 and 7. SOD activity significantly decreased in older hearts from SHR (approximately 17 %) while not significant differences were detected in Wistar rats with aging (Fig. 5).

Hearts from 4-month-old SHR exhibited a higher catalase activity (approximately 40%) in comparison to hearts from age-matched Wistar rats and it decreased in 19-month-old SHR. In Wistar rats CAT activity did not change with aging (Fig. 6).

Compared to younger animals, a significant decrease of GPx activity was detected in hearts from 19-month-old SHR and Wistar rats. No differences were detected between SHR and age-matched Wistar rats (Fig. 7).

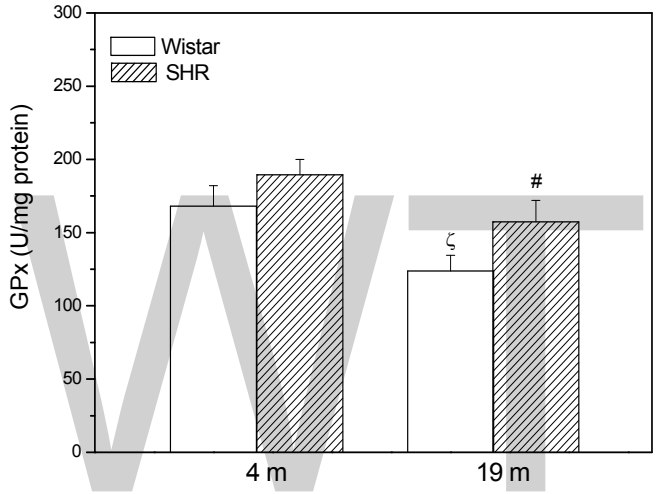

Fig. 7. Glutathione peroxidase (GPx) activity, expressed as U/mg protein, in SHR and Wistar hearts of 4 and 19-month-old. # $P < 0.05$ in 19- vs 4-month-old SHR; ς $P < 0.05$ in 19- vs 4-month-old Wistar.

4. Discussion

The present study shows an increase of oxidative stress associated to ageing in both rat strains, showing SHR the highest values. Oxidative stress is a major contributor to the aging process (Fukagawa, 1999) and appears to be a common feature of hypertensive disorders from diverse origins (Ito et al., 1995; Dobrian et al., 2003; Vaziri & Sica, 2004; Swei et al., 1997). The damage caused by oxidative stress during aging becomes more evident when analyzing the effect of ROS on organic macromolecules, like proteins and lipids. Lipid peroxidation is a major contributor to the age-related loss of membrane fluidity, especially related to increase in the levels of two aldehydic lipid peroxidation products, malonyldialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE). Therefore, it is not surprising that lipid peroxidation is increased in the aged heart as demonstrated by higher levels of

MDA (Cocco et al., 2005) or HNE (Judge et al., 2005). However, in the present study, in accordance with previously reported data (Muscari et al., 1990; Navarro-Arévalo et al., 1999; Cand & verdetti, 1989), we did not find any increase of TBARS in hearts from normotensive rats with aging. These results can be explained considering that the normal hearts have a reduced amount of substrate for the lipoperoxidation (Cand & Verdetti, 1989) or /and the end products of lipoperoxidation are readily metabolized (Muscari et al., 1990) or possess efficient antioxidant defence system . However, we detected an increase in TBARS content with aging in hearts from SHR, compared to age-matched Wistar rats. Moreover, 19-month-old SHR exhibited the highest hypertrophy index and level of lipid peroxidation suggesting that an increase of oxidative damage can be the consequence or the reason for the persistent elevated systolic blood pressure and/or increased cardiac hypertrophy in addition to aging.

Nitric oxide (NO) plays pivotal roles in the maintenance of blood pressure and vascular tone (Loscalzo & Welch, 1995). Superoxide avidly reacts with NO and in the process produces highly reactive and cytotoxic products, like peroxynitrite (ONOO-). Peroxynitrite, in turn, reacts with and modifies various molecules, namely lipids, DNA, and proteins. For instance, peroxynitrite reacts with the tyrosine and cysteine residues in protein molecules to produce nitrotyrosine and nitrocysteine, leading to inactivation of important antioxidant enzymes, like SOD (Mac Millan-Crow & Cruthirds, 2001; Alvarez et al., 2004). In addition to these and other harmful biochemical reactions, the oxidation of NO by ROS inevitably results in functional NO deficiency, which can contribute to pathogenesis and maintenance of hypertension and its long-term consequences. In agreement with previous findings in the vasculature of hypertensive animals (Mc Intyre et al., 1999; Zalba et al., 2001), we detected a higher $O_2^{-\cdot}$ production in cardiac tissue of aged SHR compared to age-matched normotensive Wistar rats. The fact that blood pressure of SHR decreased with antioxidant therapy implies that oxidative stress is involved in the genesis and/or maintenance of hypertension (Vaziri et al., 2000). Recent investigations using hypertensive models other than SHR have shown that an increase of cellular tolerance to oxidative stress is one of the mechanisms responsible for the efficacy of anti hypertensive treatments such as calcium antagonists (Umemoto et al., 2004; Hirooka et al., 2006), angiotensin II type 1 receptor antagonists, or angiotensin-converting enzyme inhibitors (Takai et al., 2005; Tanaka et al., 2005). In our study, hearts from 4-month-old SHR and Wistar rats showed a similar nitrotyrosine content. In addition to lipid peroxidation data, this result is another demonstration that the higher LVH observed in young SHR relative to age-matched Wistar rats was not accompanied by higher nitrosative damage. Aged Wistar rats exhibited an increase in nitrotyrosilation compared with young animals. This increase was lower in Wistar in comparison to SHR, indicating that the addition of hypertrophy to aging process leads to a high degree of nitration due to an increased imbalance in myocardial production of either NO or $O_2^{-\cdot}$. Although we did not measure the expression or activity of NOS, it has been reported that aged hearts exhibited increased myocardial NOS-cGMP signaling associated with an up-regulation of NOS (Zieman et al., 2001; Llorens et al., 2005). Therefore, higher levels of nitrotyrosine in aged SHR hearts would be attributed to an increase of peroxynitrite derived from an excessive production of both reactive species, NO and $O_2^{-\cdot}$. Another possibility for explaining the higher oxidative and nitrosative stress of aged SHR compared to Wistar rats is a decrease in NO availability due to an increase in $O_2^{-\cdot}$ production.

Mitochondria occupy a central position in the metabolism of ROS, supporting the so-called "free radical theory of aging" (Beckman & Ames, 1998; Hardman, 1956; Hardman, 1988). Other cardiovascular sources of ROS include the enzymes xanthine oxidoreductase (Berry & Hare, 2004), NOX (multisubunit membrane complexes) (Griedling et al., 2000) and eNOS uncoupling (Kuzkaya et al., 2003; Landmesser et al., 2003). This eNOS transformation takes place when its essential cofactor (6R)-5,6,7,8-tetrahydro-L-biopterin (BH4) is oxidized by ONOO- then a functional NOS is converted into a dysfunctional $O_2^{-\cdot}$ generating enzyme that contributes to oxidative stress. Abnormal activation and expression of myocardial NOX have been suggested to be the mains sources of ROS in the hypertrophic and failing myocardium (Bendall et al., 2002; Li et al., 2002). A recent paper of Miyagawa et al. (2007) shows that the production of $O_2^{-\cdot}$ by NOX in femoral arteries of SHR in comparison to WKY is enhanced, resulting in the inactivation of NO and impairment of endothelial modulations of vascular contractions. In our study, whereas young SHR showed a similar NOX activity as age-matched Wistar, an increase in the activity of this enzyme was detected in aged SHR, suggesting that NOX-dependent ROS production would be mediating both the hypertrophic response and aging. Apocynin is a well characterized inhibitor of NOX (Meyer & Schmitt, 2000). It acts by impeding the assembly of the p47-phox and p67-phox subunits within the membrane NOX complex (Meyer & Schmitt, 2000; Hamilton et al., 2001). Some of the effects of apocynin treatment are protection of the endothelium from the initiating events of atherosclerosis (Hamilton et al., 2001), a reduction of p22-phox mRNA expression and cardiac hypertrophy in aldosterone-infused rats (Park et al., 2004), and a prevention of hyperglycemia-induced intracellular ROS elevation and myocyte dysfunction (Privratsky et al., 2003). Aponycin has also been shown to reduce oxidative stress in stroke-prone spontaneously hypertensive rats, leading to the suppression of cardiac hypertrophy, inflammation and fibrosis (Yamamoto et al., 2006). Under our experimental conditions, apocynin blunted the $O_2^{-\cdot}$ production in hearts from aged SHR and Wistar rats. Although a significant increase in NOX activity was only evident in aged SHR hearts, we suggest that NOX–dependent ROS production would mediate both the hypertrophic response and aging.

In the myocardium, as in other tissues, antioxidant enzymes protect cells by maintaining ROS at low levels, thus preventing oxidative damage to biological molecules. SOD rapidly converts $O_2^{-\cdot}$ to H_2O_2, which is further degraded by CAT and GPx. The levels of the antioxidant enzymes are sensitive to the oxidative stress and increased or decreased levels have been reported in different pathologies in which an enhancement of ROS is cause or consequence of the disease (Navarro-Arévalo et al., 1999; Ulker et al., 2003). Our data show that SOD activity in hearts from young SHR was slightly but not significantly higher than Wistar rats. The lack of significant difference between SOD activities of hearts from both rat strains is in accordance with previous findings (Gómez-Amores et al., 2006; Wilson & Johnson, 2000; Robin et al., 2004). GPx activity was slightly but no significantly higher in hearts from young SHR compared to age-matched Wistar rats whereas CAT activity showed a significant increase. An opposite result has been recently demonstrated in thoracic aorta of SHR in which a CAT activity decreased and a concomitant increase of H_2O_2 were detected (Ulker et al., 2003). Although we did not have experimental evidence, the increase in CAT activity without GPx one changes detected in young SHR would indicate that CAT is acting as compensatory mechanism. This action could lead to a diminution of H_2O_2 amount in our preparations and could explain the similar TBARS and nitrotyrosine content obtained in young hearts from both rat strains. Aged Wistar rats did not exhibit any change in SOD and GPx activities. However, a significant diminution of antioxidant enzymes was evident in aged compared to younger SHR.

These data are in concordance with those reported by Ito et al. (1995) and opposed to recent observations of Csonka et al. (2000). In addition, both rat strains of 19 months old showed similar antioxidant enzyme activity. Therefore, this fact could not explain the differences of oxidative damage detected between aged SHR and W rats. These differences could be attributed to a significantly higher NOX activity in aged than young SHR in accordance with the increased $O_2^{-\cdot}$ production with aging, indicating that the compensatory mechanism detected in young rats will be abnormal in cardiac tissue from aged SHR. In this regard, it is worth noting a previous report that an increase of SOD pharmacology potency by lecithinization is able to protect endothelial cells against alterations induced by ROS (Igarashi et al., 1992). Another explanation to the differences observed would be related to angiotensin II content, which appears involved in the genesis of oxidative stress in another tissue than heart in the SHR model (De Godoy & Rattan, 2006). This hypothesis was supported by the recent experiments performed in vascular tissue of stroke-prone SHR (Takai et al., 2005; Tanaka et al., 2005) in which the inhibition of angiotensin receptor or angiotensin-converting enzyme system produced a reduction of ROS production. Our results are also consistent with investigations showing that cardioprotective treatments are mediated by a restoration or up-regulation of antioxidant enzyme (Umemoto et al., 2004; Tanaka et al., 2005). Accumulating evidence has suggested that ROS are capable to activate directly intracellular cascades involved in the regulation of hypertrophic growth (Takano et al., 2003). It has been reported that Rho family proteins, specially Rac1, play critical roles in mechanical stress-induced hypertrophy responses and are involved in ROS-mediated activation of MAP kinases (such as p38, ERK1/2) and activation of nuclear factor-κB. Moreover, Rac 1 is essential for assembly of plasma membrane NOX (Griendling et al., 2000). Thus, in our experimental conditions, sustained hemodynamic load in SHR would modulate the action of extracellular stimuli (such as angiotensin II, norepinephrine, tumor necrosis factor-α, epidermal growth factor) on Rac1 activation leading to NOX activation. The increase in $O_2^{-\cdot}$ production by NOX would, in presence of a deficient endogenous antioxidant system, activate redox-sensitive kinase cascades and transcription factors. These actions would produce an induction of immediate early genes, reexpression of fetal genes, increased mRNA content and protein synthesis thus leading to the increase in myocyte cross-sectional area and fibrosis observed in aged SHR heart.

5. Conclusion

This study shows that an increase in $O_2^{-\cdot}$ production in NOX dependent way and consequently higher oxidative damage appears associated to the aging process and to the increase in cardiac hypertrophy detected in hearts of SHR compared to age-matched Wistar rats. Thus, oxidative stress would be the cause and/or consequence of hypertrophy development in the SHR model.

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7

Oxidative Stress and Mitochondrial Dysfunction in Cardiovascular Diseases

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1. Introduction

Reactive oxygen species (ROS) include a wide variety of molecules and free radicals derived from molecular oxygen. O_2 being highly electrophilic can be reduced by one electron at a time producing relatively stable intermediates, such as superoxide anion ($O_2\bullet^-$), precursor of most ROS and a relevant mediator in many biological reactions. Dismutation of $O_2\bullet^-$ produces hydrogen peroxide (H_2O_2), which, in turn, may be fully reduced to water or partially reduced to hydroxyl radical (•OH), one of the strongest oxidants in nature. The formation of •OH is catalyzed by reduced transition metals. On the other hand, superoxide anion is able to reduce transition metals and intensify in this way hydroxyl generation. In addition, $O_2\bullet^-$ may react with other radicals, in particular, with nitric oxide (•NO) in a reaction controlled by the rate of diffusion of both radicals. The product, peroxynitrite, is very powerful oxidant. The oxidants derived from •NO have been recently called reactive nitrogen species (RNS). Oxidative stress is an expression used to describe various deleterious processes resulting from an imbalance between the formation and elimination of ROS and/or RNS by antioxidant defenses. While small fluctuations in the steady-state concentration of these oxidants play an important role in intracellular signaling, uncontrolled increase in their concentration produces free radical-mediated chain reactions, which indiscriminately target proteins, lipids, polysaccharides, and DNA. Mitochondria are a major source of ROS, converting as much as 0.2-2% of molecular oxygen to superoxide as a by-product of the electron transfer activity (Wittenberg & Wittenberg, 1989; Alvárez et al., 2003). Other enzymatic sources of ROS include NADPH oxidases, located on the cell membrane of polymorphonuclear cells, macrophages, endothelial and other cells, and cytochrome P450-dependent oxygenases, along with the proteolytic conversion of xanthine dehydrogenase to xanthine oxidase, which produces both $O_2\bullet^-$ and H_2O_2.

Mitochondria's critical role in cardiomyocyte survival and death has become an exciting finding in the field of cardiac biology. Indeed, it is accepted that mitochondrial dysfunction plays a crucial role in the pathogenesis of multiple cardiac diseases, mainly due to the imbalance of the fine interplay between aerobic metabolism, calcium homeostasis, and ROS production. Reactive oxygen species generated in the mitochondria, unless adequately

neutralized, cause mitochondrial oxidative stress and, through reactions with polyunsaturated fatty acids, form lipid hydroperoxides and unsaturated aldehydes that propagate among cellular compartments and react with proteins and nucleic acids. In the myocardium, the oxidative stress cascade impairs several functions, like mitochondrial biogenesis, fatty acid metabolism, ionic homeostasis, and antioxidant defense mechanisms, leading to diminished cardiac energetic efficiency, altered bioenergetics, apoptosis and degradation. Besides the obvious relevance of mitochondria in energy production, new processes like mitochondrial fusion and fission are reported to be linked to ROS generation and now are included in the cast of key players in cardiac disease. In this chapter, we explore the mechanisms of mitochondrial dysfunction driven by ROS generation associated with the pathophysiology of cardiovascular diseases.

2. Mitochondrial reactive oxygen species generation

The standard reduction potential for the conversion of molecular oxygen to $O_2\bullet^-$ is -0.160 V (Wood, 1987). Given the highly reducing intramitochondrial environment, various respiratory components, including complexes I and III of the mitochondrial respiratory chain (Paradies et al., 2001; Tompkins et al., 2006), flavoproteins (Prosser et al., 2011), iron-sulfur clusters (Napoli et al., 2006), and ubisemiquinone (Wen & Garg, 2008) are thermodynamically capable to donate one electron to oxygen. Moreover, most steps in the respiratory chain involve single-electron reactions, further favoring the monovalent reduction of oxygen. On the other hand, the mitochondrion possesses various antioxidant defenses designed to eliminate both $O_2\bullet^-$ and H_2O_2. As a result, the steady state concentrations of $O_2\bullet^-$ and H_2O_2 have been estimated to be around 1×10^{-10} M and 5×10^{-9} M, respectively (Cadenas & Davies, 2000). Mitochondrial sources of $O_2\bullet^-$ include several respiratory complexes and individual enzymes. Superoxide formation occurs on the outer mitochondrial membrane, in the matrix, and on both sides of the inner mitochondrial membrane (Table 1, Figure 1). While superoxide anion generated in the matrix is mainly eliminated in that compartment, part of the $O_2\bullet^-$ produced in the intermembrane space may be carried to the cytoplasm via voltage-dependent anion channels (Han et al., 2003). The relative contribution of every site to the overall $O_2\bullet^-$ production varies from organ to organ and also depends on whether mitochondria are actively respiring (State 3) or if the respiratory chain is highly reduced (State 4) (Barja, 1999). Complex III appears to be responsible for most of the $O_2\bullet^-$ produced in heart mitochondria (Turrens & Boveris, 1980; Turrens et al., 1982), although Complex I is thought to be the primary source of ROS in a variety of pathological scenarios ranging from ageing to Parkinson's disease (Betarbet et al., 2002; Sherer et al., 2003a). The rate of $O_2\bullet^-$ formation by the respiratory chain is controlled primarily by mass action law, increasing both when electron flow slows down (the concentration of electron donors, R• is higher) and when the concentration of oxygen increases[1] (Turrens et al., 1982).

$$d[O_2]/dt = k\,[O_2]\,[R\bullet] \quad (1)$$

The electron flow through the respiratory chain establishes an H^+ gradient across the inner mitochondrial membrane, used to drive ATP synthesis through the ATP synthase complex (Complex V). In the absence of ADP, the movement of H^+ through ATP synthase is ceased and the H^+ gradient builds up causing slowdown of electron flow and reduction of the

respiratory chain (State 4). As a result, the physiological steady state concentration of $O_2^{\bullet-}$ formation increases. The formation of $O_2^{\bullet-}$ may be further increased in the presence of certain inhibitors (for example rotenone, which inhibits Complex I, or antimycin an inhibitor of Complex III), which cause those carriers upstream from the site of inhibition to become fully reduced. In Complex I, the primary source of $O_2^{\bullet-}$ appears to be one of the iron-sulfur clusters, whereas in Complex III, most of $O_2^{\bullet-}$ results from ubisemiquinone auto-oxidation, both on the outer and inner sides of the inner mitochondrial membrane (Table 1).

Component	Localization	References
Complex I (NADH dehydrogenase)	Inner membrane/ inner side	(Turrens & Boveris, 1980; Turrens *et al.* 1982; Genova *et al.* 2001; Kushnareva *et al.* 2002)
Complex II (succinate dehydrogenase)	Inner membrane/ inner side	(Zhang *et al.* 1998; Lenaz, 2001)
Complex III (ubiquinol–cytochrome *c* reductase)	Inner membrane/ inner side	(Boveris *et al.* 1976; Cadenas *et al.* 1977; Turrens *et al.* 1985)
Complex III (ubiquinol–cytochrome *c* reductase)	Inner membrane/ outer side	(Han *et al.* 2001; Starkov & Fiskum, 2001)
External NADH dehydrogenase (yeast)	Inner membrane/ outer side	(Fang & Beattie, 2003)
Glycerolphosphate dehydrogenase	Inner membrane/ outer side	(Drahota *et al.* 2002)
Dehydroorotate dehydrogenase	Matrix	(Forman & Kennedy, 1976)
Mono amino oxidase	Outer membrane/ inner side	(Hauptmann *et al.* 1996; Cadenas & Davies, 2000)

Table 1. Compartmental localization of the main mitochondrial sources of superoxide anion. Modified from Turrens, 2003.

Although $O_2^{\bullet-}$ production increases as the respiratory chain becomes more reduced, not all mitochondrial inhibitors have this effect. Most of the production of $O_2^{\bullet-}$ by Complex III is actually inhibited if electron flow between the Rieske Fe–S protein and oxygen is blocked, for example by myxothiazol, cyanide or cytochrome *c* depletion (Turrens et al., 1985). This inhibitory effect indicates that $O_2^{\bullet-}$ must be produced as a result of the autoxidation of semiquinone (Q•), an intermediate produced in Complex III during the Q-cycle (Trumpower, 1990; Figure 2). Coenzyme Q is fully reduced and converted to ubiquinol (QH_2) in the inner side of the mitochondrial membrane and then migrates to the outer side of the inner membrane carrying two protons that become part of the pool needed to sustain ADP phosphorylation. Once on the outer side of the membrane, one electron is transferred to cytochrome *c*1 (via the Rieske Fe–S protein), resulting in the formation of Q•. In a second cycle, a new QH_2 transfers one of its electrons to the iron-sulphur protein (ISP) and then to cytochrome c1, whereas the second electron reduces cytochrome b_{566} and then cytochrome b_{540} (Turrens et al., 2003). This second electron reduces the Q• produced in the first cycle, yielding QH_2. Despite the high efficiency of redox reactions in the Q cycle, some electrons leak and react with oxygen producing $O_2^{\bullet-}$ (Figure 1).

Another modulator of mitochondrial ROS production is the membrane potential ($\Delta\psi_m$), thus it has been reported that both, uncouplers and uncoupling proteins (UCPs), minimize ROS production by enhancing proton leak and providing a negative feedback loop for

mitochondrial ROS production. A direct impact of ROS on the glutathionylation status of UCPs has been invoked to explain the activation of such proteins (Mailloux & Harper, 2011).

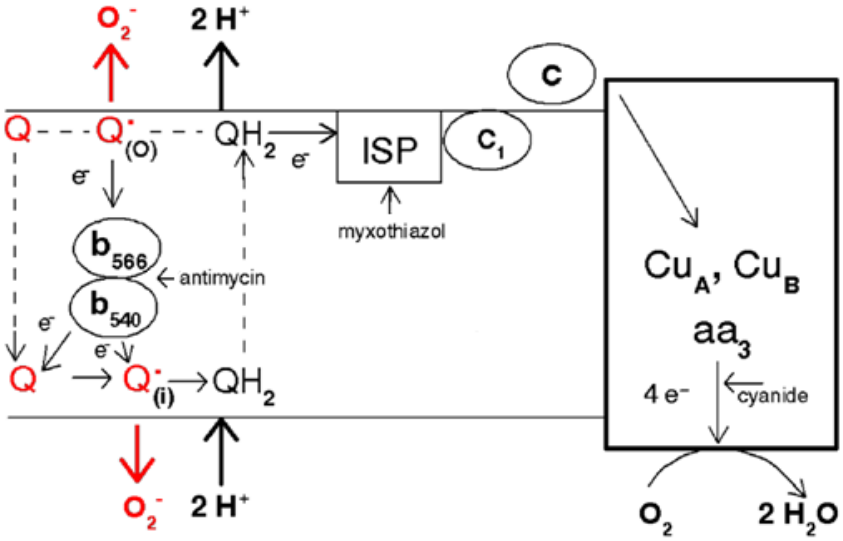

Fig. 1. Electron flow in the Q-cycle. Ubiquinone (Q) is reduced by electrons transferred from NADH or succinate producing ubiquinol (QH_2). The reduced form undergoes a two-cycle reoxidation in which the semiquinone (Q•) is a stable intermediate that transfers electrons to oxygen via the Rieske iron-sulfur protein (ISP), cytochrome *c*1 (c1) and cytochrome *c* (c). The terminal oxidase catalyzes the oxidation of reduced cytochrome c and the concomitant four-electron reduction of one O_2 molecule producing water. Superoxide may be produced on both sides of the inner membrane via the autoxidation of Q, but the contribution of each pool has not yet been determined.

Special mention is worth about how Ca^{2+} modulates mitochondrial ROS generation. The primary role of this cation in mitochondria is the stimulation of oxidative phosphorylation by allosteric activation of pyruvate dehydrogenase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, as well as other proteins of the phosphorylating machinery (Brooks et al., 2004). Thus, the stimulation of oxidative phosphorylation would enhance ROS production, as mitochondria are being forced to work faster and to consume more O_2. Experimental observations are diverse, overall it appears that physiological $[Ca^{2+}]_m$ has no direct effect on respiratory chain function or oxidation/reduction process, however pathological mitochondrial Ca^{2+} overload can lead to ROS increase. Possible mechanisms include Ca^{2+} stimulated increase of metabolic rate; Ca^{2+} stimulated nitric oxide production, which inhibits complex IV; Ca^{2+} induced cytochrome c dissociation that would inhibit the distal respiratory chain; Ca^{2+} induced cardiolipin peroxidation and Ca^{2+} induced mitochondrial permeability transition pore opening (Peng & Jou, 2010).

On the other hand, simultaneous formation of $O_2^{\bullet -}$ and nitric oxide can produce peroxynitrite, a very strong oxidant and nitrating agent. Nitric oxide is a vasodilator

resulting from the convertion of arginine to citrulline, in a reaction catalyzed by a family of NADPH-dependent enzymes called nitric oxide synthases. It has been reported that the mitochondrial matrix contains a unique form of nitric oxide synthase (Alvarez et al., 2003). Although its physiological role is still unclear, the formation of nitric oxide in mitochondria may have important pathological consequences, as it binds to the heme group of cytochrome oxidase, inhibiting respiration (Poderoso et al., 1996). ROS are also produced within mitochondria at sites other than the inner mitochondrial membrane (Dowrakowski et al., 2008), by proteins such as monoamine oxidase (MAO) and p66Shc (reviewed in Di Lisa et al., 2009).

3. Antioxidant systems in mitochondria

Mammalian mitochondria possess a multi-leveled ROS defense network of enzymes and non-enzymatic antioxidants. Thus, constantly generated ROS, essential for normal cellular physiology and signaling process, are maintained at specific levels by intrinsic antioxidant defenses, avoiding oxidative stress. In healthy mitochondria, ROS contention is driven by manganese-dependent superoxide dismutase (MnSOD), gluthatione peroxidase (GPx), thioredoxin ($TrxSH_2$) and thioredoxin reductase (TrxR), peroxiredoxin (Prx), and glutaredoxin (Grx), as well as water- and lipid-soluble antioxidants, i.e. vitamins C, α-tocopherol (α-toc), reduced glutathione (GSH), and melatonin. It has been proposed that under certain circumstances, the mitochondrial respiratory chain can also contribute to mitochondrial antioxidant defense.

3.1 Non-enzymatic antioxidants

The tripeptide GSH is the main non-protein molecule, containing reactive thiol (-SH) groups, with scavenging properties, that provides an abundant source of reducing equivalents (Stowe & Camara, 2009). GSH reacts with hydroxyl radical (•OH), hypochlorous acid (HOCl), peroxyl radical (RO_2•), carbon-centered radicals, and peroxynitrite anion ($ONOO^-$) producing thiyl radical (GS•), which potentially generates O_2•$^-$ among other ROS (Ježek & Hlavatá, 2005). Despite its exclusive synthesis in the cytosol, GSH is distributed in intracellular organelles, including the endoplasmic reticulum (RE), nucleus, and mitochondrion (Marí et al., 2009; Figure 2). GSH synthesis involves a two step reaction that requires ATP. Glutamate and cysteine are converted to γ-glutamyl-cysteine in a rate-limiting reaction driven by γ-glutamylcysteine synthetase. Then, γ-glutamylcysteine and glycine produce GSH by the action of the enzyme GSH synthetase (Figure 2). The first reaction is inhibited by GSH, a mechanism that regulates cellular GSH concentration (Marí et al., 2009). In the cytosol, GSH concentration is around 11 mM (Griffith & Meister, 1979; Mårtensson et al., 1990) and is transported into the mitochondrial matrix through a non-described high affinity carrier and a low affinity carrier, that could be the mitochondrial oxoglutarate carrier (Coll et al., 2003) or the dicarboxylate carrier (Lash et al., 2002). In mitochondria, GSH levels may fluctuate from 5 to 11 mM (Valko et al., 2007). Mitochondrial GSH plays a critical role in cell survival, as toxic cell death often correlates better with depletion of the mitochondrial GSH pool than with overall intracellular GSH depletion (Orrenius et al., 2007).

The importance of UCPs in the control of mitochondrial ROS generation remains unclear: it is known that UCPs are inner membrane carriers that transfer protons across the mitochondrial inner membrane (MIM), by-passing ATPase (Stuart et al., 2001). A putative

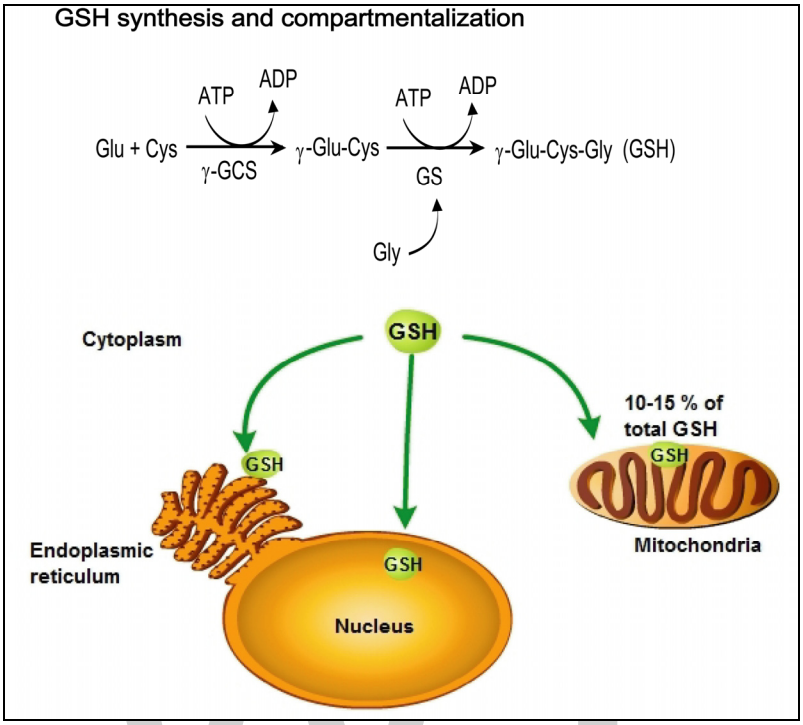

Fig. 2. GSH synthesis and compartmentalization. GSH is synthesized by γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GS) in the cytoplasm and then redistributed to mitochondria, nucleus, and endoplasmic reticulum.

role in redox homeostasis control has been associated with their capacity to reduce mitochondrial ROS production by modulating $\Delta\psi_m$ (Haines et al., 2010). In fact, UCPs reduce the damaging effects of ROS during cardiac ischemia/reperfusion injury (Barreiro et al., 2009). UCP1 isoform is involved in thermogenesis and expressed specifically in brown adipose tissue mitochondria, in which it confers a regulated proton leak across the inner membrane, whereas the physiologic functions of the other isoforms remain unclear. UCP2 and UCP3 are present in the heart and provide cardioprotection. In rat neonatal cardiomyocytes, UCP overexpression confers tolerance against oxidative stress by a mechanism related with calcium uptake (Sack, 2006b).

α-Tocopherol and melatonin scavenge lipid peroxyl radicals much faster than these radicals can react with adjacent fatty acid side-chains, so they are probably the most important inhibitors of the free-radical chain reaction of lipid peroxidation in animals (Maroz et al., 2009; Gavazza & Catalá, 2009). Aside from its various physiological functions, melatonin could acts as a scavenger of the particularly toxic •OH and carbonate radical, which is important because of their presumed role in mitochondrial damage (Srinivasan et al., 2009, Hardeland & Coto-Montes, 2010). Besides, melatonin could act as an indirect antioxidant promoting *de novo* synthesis of GSH, stimulating the activity of γ-glutamylcysteine synthetase and also through its effects on GPx, Grx, SOD, and CAT gene expression

(Rodríguez et al., 2004), helping in GSH recycling and maintaining a high GSH/GSSG ratio. Melatonin is synthesized and released to the circulation by the pineal gland, and its amphiphilic properties lead to its free access to all compartments in the cell, being concentrated especially in the nucleus and mitochondria (Escames et al., 2010). A direct role of melatonin in regulation of Complex I and IV activity and in other mitochondrial functions has been suggested. This effect, not shared by other antioxidants, would reflect redox interactions with the electron transfer chain complexes, stimulating electron flow, limiting electron leakage, and ROS generation (Escames et al., 2010). Interestingly, it has also been reported that melatonin protects mitochondria from oxidative damage by preventing cardiolipin oxidation (Paradies et al., 2010).

3.2 Enzymatic antioxidants

MnSOD converts superoxide anion ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2) in the matrix side of the inner mitochondrial membrane (Liochev & Fridovich, 2010), while some $O_2^{\bullet -}$ released into the intermembrane space is partially dismutated by copper-zinc containing superoxide dismutase (CuZnSOD) (Figure 3). Disruption of the MnSOD gene in mice has been

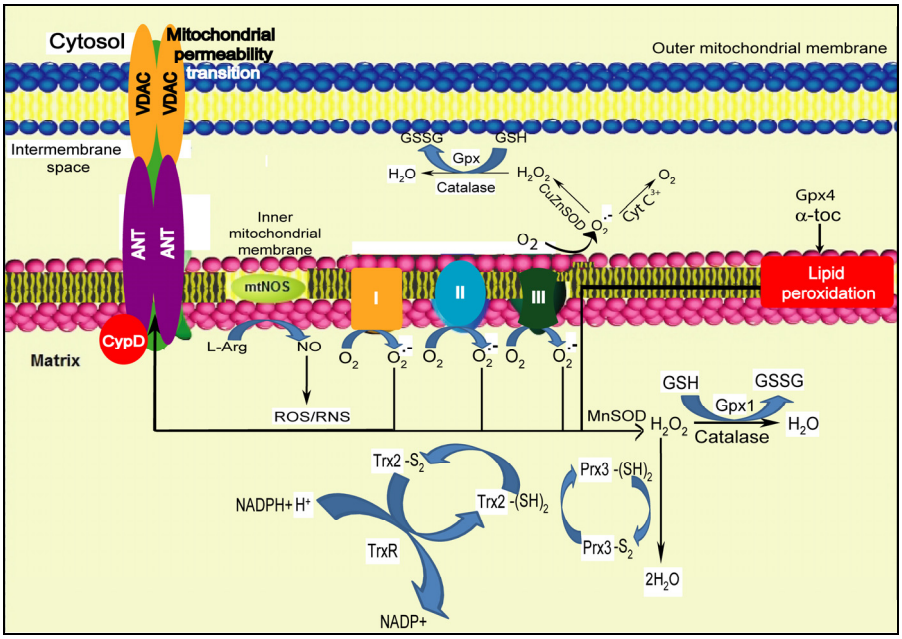

Fig. 3. Mitochondrial antioxidant network. Mitochondria are normally protected from oxidative damage by a network of mitochondrial antioxidant systems. See text for further details. α-toc: α-tocoferol; Cyt *c*3+ (reduced cytochrome *c*) ; CuZnSOD: copper-zinc superoxide dismutase; Gpx: glutathione peroxidase; GSH: reduced glutathione, GSSG: oxidized glutathione; L-Arg: L-arginine; mtNOS: mitochondrial nitric oxide synthase, NO: nitric oxide; Prx3-$(SH)_2$: peroxiredoxine 3 reduced, Prx3-S_2: peroxiredoxine 3 oxidized; ROS: reactive oxygen species, RNS: reactive nitrogen species; Trx2-$(SH)_2$: tioredoxine 2 reduced, Trx2-S_2: tioredoxine oxidized.

associated with early postnatal lethality (Li et al., 1995), while MnSOD overexpression was shown to protect mitochondrial function and block apoptosis (Holley et al., 2010). The activity of MnSOD should be coordinated with H_2O_2-removing enzymes. Thus, H_2O_2 produced by MnSOD could be metabolized by Gpx, Prx, or by catalase that has been found in extremely small amounts in the mitochondrial matrix.

Besides of the importance of GSH as a direct antioxidant, it participates in multiple GSH-linked enzymatic defense systems. Among others, GSH acts as electron donor in the reduction of H_2O_2 and different hydroperoxides by GPx1 and GPx4 (Camara et al., 2011). Five different isoforms of GPx have been identified GPx1, GPx2, GPx3, GPx 4, and GPx 6. GPx1 is the major isoform and is localized predominantly in the cytosol, but a small proportion is also present within the mitochondrial matrix (Orrenius et al., 2007). GPx4 is a unique intracellular antioxidant enzyme that directly reduces peroxidized lipids produced in cell membranes (Nomura et al., 2000). Because GPx4 is membrane-associated, with a fraction localized in the intermembrane space of the mitochondria, possibly at the contact sites of the two membranes, and due to its small size and large hydrophobic surface it can interact with, and detoxify, membrane lipid hydroperoxides much more efficiently than the alternative pathway, phospholipase A_2-GPx1 (PLA2) (Antunes et al., 1995). Hence, GPx4 is considered to be the primary enzymatic defense system against oxidative damage to cellular membranes. Accordingly, GPx4-null mice are embryonically lethal, while the heterozygotes are more sensitive to oxidants than wild type mice (Ran et al., 2003).

Other mitochondrial GSH-linked enzymes are glutaredoxins (Grx), which catalyze glutathione-dependent dithiol reaction, reducing protein disulfides, and monothiol reactions reducing mixed disulfides between proteins and GSH. An interesting member of this family is glutaredoxin 2 (Grx2), which was cloned and found to be present as both mitochondrial and nuclear isoforms (Lundberg et al., 2001). Modeling suggests that the GSH binding site and the hydrophobic surface of Grx2 are similar to those of Grx1 (Lundberg et al., 2001), although Grx2 lacks one of the conserved non-active site cysteine residues of Grx1 (Lundberg et al., 2001), hence it is more resistant to oxidants and oxidized glutathione (GSSG) action. Furthermore, Grx2 can be reactivated directly by thioredoxin reductase (TrxR) as well as by GSH (Johanson et al., 2004). GSSG is reduced by glutathione reductase (GR) with NADPH as a cofactor. In turn, mitochondrial NADPH can be regenerated by matrix dehydrogenases and by reaction of hydride ion transfer, which is proton motive force-dependent, utilizing intramitochondrial NADPH to reduce $NADP^+$. Besides NADPH *per se* can serve directly as a non-enzymatic antioxidant, according to some authors (Kirsch & De Groot, 2001). Another potential source of disulfide reductase activity in mitochondria is the thioredoxin system, which includes thioredoxin 2 (Trx2) and thioredoxin reductase (TrxR2). Trx2 catalyzes the reduction of protein disulfides at much higher rates than Grx (Arner & Holmgren, 2000). This enzyme is important for life, given that disruption of the Trx2 gene in the homozygous mouse causes massive apoptosis and, finally, results in embryonic lethality (Nonn et al., 2003). Specific glutathione S-transferase (GST) isoforms: GSTα1-1, GSTα4-4, and GSTμ1-1, which neutralize reactive molecules such as 4-hydroxy-2-noneal (4-HNE), incorporating GSH to the radical molecule, have been found in mitochondria (Raza et al., 2002).

The intermembrane space of mitochondria contains ~0.7 mM cytochrome *c* (Hackenbrock et al., 1986) capable of superoxide removal. Cytochrome *c* can be alternatively reduced by the

respiratory chain or superoxide. A diminution in its concentration may inhibit the distal respiratory chain and increase ROS production. Indeed, it has been reported that upon cytochrome *c* release during apoptosis initiation, mitochondrial ROS production increases (Cai & Jones, 1998).

As mentioned before, ubiquinone (Q) acts as a pro-oxidant in its semiquinone form, however when fully reduced it acts as an antioxidant. Ubiquinol (UQH_2) contains phenolic hydrogen atoms that can be donated to a carbon- or oxygen centered radical, converting it to a non-radical molecule (Ježek & Hlavatá, 2005). Lipid peroxidation prevention by UQH_2 has been reported (James et al., 2004). Preferential succinate oxidation can provide a tool against excessive accumulation of ROS by increasing the proportion of fully reduced ubiquinone. The antioxidant activity of UQH_2 is independent from the effect of α-tocopherol (Ernster et al., 1992), which acts as a chain-breaking antioxidant, inhibiting the propagation of lipid peroxidation (Maroz et al., 2009). Even more, UQH_2 can efficiently sustain the effect of α-tocopherol by regenerating it from the tocopheroxyl radical, which otherwise must rely on water-soluble agents such as ascorbate (vitamin C) (Ernster & Forsmark-Andree, 1993).

4. Oxidative stress and cardiovascular diseases

The pathological role of increased mitochondrial ROS in heart disease has been established from studies in gene-modified mice with altered mitochondrial antioxidant levels. Deletion of mitochondrial thioredoxin reductase 2 is embryonically lethal in mice because of impaired hematopoiesis and impaired cardiac function (Conrad et al., 2004). Fatal dilated cardiomyopathy in mice is developed after complete deletion of mitochondrial Mn superoxide dismutase (Li et al., 1995). In contrast, attenuated left ventricular remodeling after myocardial infarct was observed in transgenic mice overexpressing mitochondrial peroxiredoxin III (Matsushima et al., 2006) or glutathione peroxidase (Shiomi et al., 2004). Also, mice with a mitochondrial-targeted overexpression of catalase have a prolonged life span and improved cardiac function (Schriner et al., 2005).

A critical role of intracellular Ca^{2+} overload and oxidative stress in the genesis of myocyte dysfunction is well established in cardiovascular diseases like atherosclerosis, hypertension, ischemia/reperfusion damage, cardiac hypertrophy, and heart failure (HF). In general, Ca^{2+} overload can be induced by direct effect of ROS on Ca^{2+} handling proteins or indirectly, by inducing membrane lipid peroxidation (Santos et al., 2011). Recent evidence suggests that redox modification of ryanodine receptor (RyR2) may contribute to abnormal Ca^{2+} handling in disease states. RyR2 dysfunction with an increase in diastolic Ca^{2+} leak from the sarcoplasmic reticulum (SR) may reduce calcium transients and contribute to a reduced contractile force in the failing heart, as well as an increased likelihood of arrhythmia (González et al., 2010). In humans, increased cytosolic calcium ($[Ca^{2+}]_c$) has been related with augmented oxidative stress in atherosclerosis. A growing body of evidence indicates that the production of ROS is tightly linked with Angiotensin II-induced action. In this respect, a causative link between superoxide production and hypertension has been established in experiments in which SOD reduced blood pressure by 50 mm Hg in vascular smooth muscle cells (VSCM) from Angiotensin II-infused rats (Laursen et al., 1997). Much less attention has been paid to other reactions catalyzed by ROS. However, it is known that ATPase activity and inhibition of ATP-independent Ca^{2+} binding are severely depressed in

sarcolemmal membranes exposed to hydrogen peroxide and Fe^{2+} (Kukreja et al., 1992, Kaneko et al., 1989). In addition, augmented levels of iron pool in atherosclerotic lesions suggest that iron-catalyzed formation of free radicals may take place in the development of this pathology (Yuan & Li, 2003). High-fat diets stimulate stress response (heat shock protein 70) and signal transduction genes (Ras, MAPK1), inhibiting SOD and GPx gene expression. These effects could be prevented by scavengers of peroxides and antioxidant supplementation of the high-fat diet and caloric restriction (Rosier & Saes, 2006).

5. Mitochondrial dysfunction

Mitochondrial myopathies were described in the early 1960s, when systematic ultrastructural and histochemical studies revealed excessive proliferation of abnormally looking mitochondria in muscle of patients with weakness or exercise intolerance (Shy & Gonatas, 1964). Mitochondrial dysfunction, reflected in the structure, function and number of mitochondria within the cardiomyocyte, leads to diminished energy production, loss of myocyte contractility, altered electrical properties, and eventual cardiomyocyte cell death (Capetanaki, 2002). In addition, cardiotoxic stimuli often lead to excessive production of ROS and to Ca^{2+} overload in the mitochondrial matrix (Ragoni & Condolini, 2009). Evidences for a pathological role of mitochondrial ROS comes from studies in animal models of myocardial infarction, in which increased mitochondrial ROS production was observed, accompanied by decreases in mtDNA copy numbers, in mitochondrial-encoded gene transcripts, and in related enzymatic activities (complexes I, III, and IV), and from studies of genetically modified animals. Overexpression of Prx-3 (a mitochondrial antioxidant protein) improved post-myocardial infarction left ventricular function by restoring mitochondrial activity and DNA copy numbers (Matsushima et al., 2006). Other examples are studies in mice with complete deletion of mitochondrial MnSOD, which developed severe fatal dilated cardiomyopathy (Li et al., 1995). Decreased vascular SOD activities have also been associated with increased susceptibility to ischemia/reperfusion mediated damage; whereas overexpression of mitochondrial antioxidants increased cardiac tolerance to ischemia (Madamanchi, et al., 2005). Recently, the causal role of mitochondrial ROS in Angiotensin II-induced cardiomyopathy was shown by the observation that mice that overexpress catalase targeted to mitochondria, but not mice that overexpress wild-type peroxisomal catalase, are resistant to cardiac hypertrophy, fibrosis and mitochondrial damage induced by angiotensin II (Dai et al., 2011). Monoamine oxidase (MAO) has been shown to play a prominent role in myocardial injury caused by post-ischemic reperfusion (Bianchi et al., 2005a) and to contribute to the maladaptive evolution from myocardial hypertrophy to heart failure (Kaludercic et al., 2010). MAO-mediated ROS production has been related with serotonin-induced myocyte hypertrophy *in vitro* (Bianchi et al., 2005b) and in mitogenic signaling induction by a process that may involve the activation of the metalloproteinase MMP-2, in smooth muscle cells (Coatrieux et al., 2007).

Mitochondria isolated from hearts of rabbits exposed to hypercholesterolemic diet showed significantly reduced respiration rates (state 3 and state 4) (Kojik et al., 2011), whereas increased cholesterol is related with diminution of the mitochondrial membrane potential and mitochondrial pore opening (Chávez et al., 1998; Martínez Abundis et al., 2007) and activation of apoptosis (Martínez-Abundis et al., 2009).

5.1 Metabolic adaptation

Complex mechanisms have evolved to maintain the balance between myocardial O_2 supply and O_2 consumption under pathological stresses such as hypoxia, ischemia, pressure, and volume overload. These mechanisms induce changes in cardiomyocyte structure and/or function through coordinated changes in gene and protein expression and/or the activities of various proteins. Redox mechanisms are involved in the signaling pathways underlying many of these mechanisms, both via the direct effects of O_2 levels in the cardiomyocyte and through the effects of ROS (Santos et al., 2011). Metabolically, the adult mammalian heart normally uses lipids as the major fuel, and mitochondria supply over 90% of the total ATP through β-oxidation of plasma fatty acids (Opie & Sack, 2002). During hypoxia, under ischemia or settings of increased cardiac workload, there is a substantial increase in glycolytic ATP generation, which may be cardioprotective during ischemia/reperfusion by ensuring an adequate ATP supply for membrane and sarcoplasmic reticulum ion pumps (Opie & Sack, 2002; Correa et al., 2008a). Recent studies suggest that a metabolic shift to glycolysis is related with the redox status in the heart. NADPH has been recognized as a critical modulator of the antioxidant defense through the regeneration of reduced pools of glutathione, while G6PDH activity was shown to be of major importance for the maintenance of redox status, Ca^{2+} homeostasis, and contractile function in cardiomyocytes subjected to oxidative stress (Jain et al., 2003) .

5.2 Mitochondrial biogenesis

Mitochondrial biogenesis can be defined as a chain of events that promote growth and division of preexisting organelles. Mitochondrial biogenesis includes the synthesis, import and incorporation of proteins and lipids, as well as the replication of mitochondrial DNA (mtDNA). Replication and transcription of mtDNA are controlled by mtTFA (mitochondrial transcription factor A), and two specific transcription factors TFB1M and TFB2M (mitochondrial transcription factor B1/B2), an RNA polymerase (POLRMT), and a mitochondrial transcription termination factor (mTERF). The coordination between the expression of mitochondrial and nuclear genes is directed by nuclear respiratory factor (NRF-1 and/or NRF-2), the peroxisome proliferator-activated receptors (PPARs), estrogen receptor (ERR), and co-activators of peroxisome proliferator-activated receptor gamma (PGC-1α) (Scarpulla, 2008). PGC-1α is also involved in regulation of fatty acid oxidation (FAO) and in co-activation of ERRα (Figure 4).

Mitochondrial biogenesis decreases in aging, obesity, insulin resistance, dyslipidemia, and hypertension, co-morbidities associated with cardiovascular diseases. Impaired activation of the renin-angiotensin-aldosterone system (RAAS) has been associated with such pathologies (Cooper et al., 2007). In fact, elevated levels of angiotensin II (Ang II) and aldosterone promote alterations in insulin metabolism, endothelial dysfunction, and loss of myocardial function (Kim et al., 2008; Sowers et al., 2009). RAAS increases the activity of NADPH oxidase and stimulates ROS generation resulting in mitochondrial damage, decreased ATP production, diminished NO availability, and attenuated mitochondrial biogenesis. Clinical and experimental observations report the loss of expression of PGC-1α, and mtTFA NRFs in hypertension (Whaley-Connell et al., 2009). In addition, down-regulation of the mitochondrial biogenesis co-activator PGC-1α and its downstream nuclear

factors have been associated with myocardial contractile dysfunction, intracellular Ca^{2+} mishandling, ROS accumulation, mitochondrial damage, and loss of mitochondrial density and mtDNA content in high-fat diet–induced obesity (Dong et al., 2007).

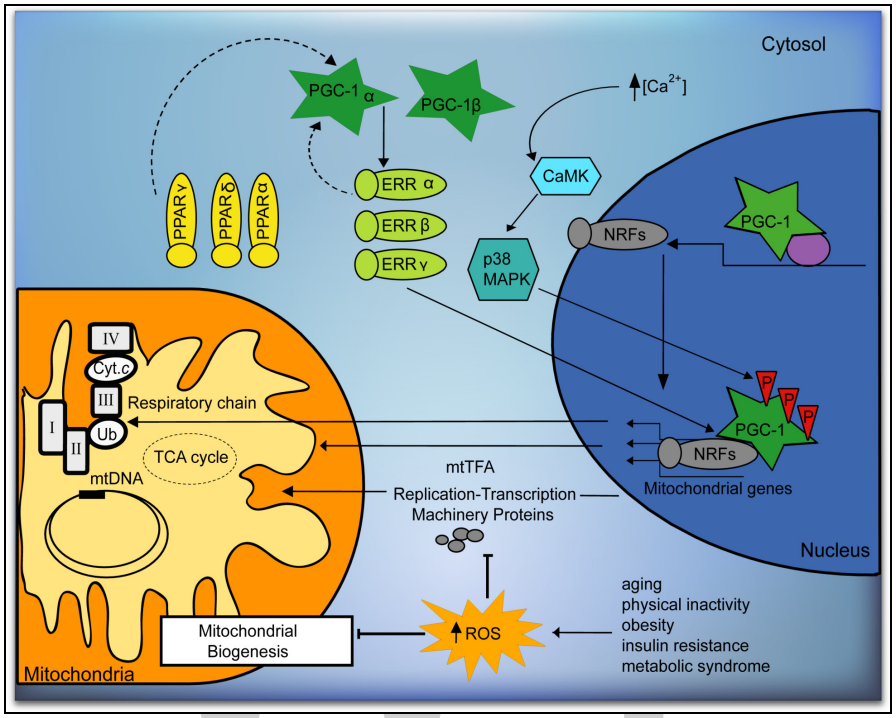

Fig. 4. The transcriptional network that controls mitochondrial biogenesis. See text for further details.

5.3 Mitochondrial ion channels

5.3.1 Permeability transition pore

General membrane damage secondary to ROS-mediated lipid peroxidation is one mechanism by which changes in mitochondrial permeability can occur; however, severe oxidative stress accompanied by calcium overload in the mitochondrial matrix favors the formation of the pathological and non-specific mitochondrial permeability transition pore (mPTP). Opening of the mPTP induces depolarization of the mitochondrial inner membrane (MIM) leading to ATP depletion and further ROS production. The increase in MIM permeability enhances colloidal osmotic pressure in the mitochondrial matrix ultimately leading to matrix swelling and rupture of the mitochondrial outer membrane (MOM). Rupture of the MOM results in release of pro-apoptotic proteins from the mitochondrial intermembrane space to the cytoplasm initiating both caspase-dependent and caspase-independent apoptosis. Permeabilization of the MOM may also occur due to formation of non-selective channels induced by translocation of pro-apoptotic Bcl-2 family proteins to

mitochondria. Due to its central role in cell death triggering, the mPTP represents a potential therapeutic target in some cardiovascular diseases. Studies performed over 20 years have demonstrated that acute cardiac ischemia followed by reperfusion damage is associated with mPTP opening (Arteaga et al., 1992; Griffiths & Halestrap, 1993; Chipuk et al., 2006; Lucken-Ardjomande et al., 2008; Halestrap and Pasdois, 2009). Furthermore, pharmacological and conditional inhibition of mPTP formation significantly improved cardiac function reducing ischemic injury and myocardial infarction size in animal models (Argaud et a., 2005; Hausenloy & Yellon, 2003; Correa et al., 2008b) and in patients (Shanmuganathan et al., 2005; Piot et al., 2008). mPTP opening also causes cell death in isolated endothelial and vascular smooth muscle cells. Indeed, atherosclerosis is exacerbated when mitochondrial antioxidant defenses are hampered and a decrease in mitochondrial ROS formation reduces atherogenesis.

Regulation of the mPTP by a variety of signaling molecules and cellular metabolites and ions is a complex process, and mPTP formation ultimately depends on the balance between factors favoring and inhibiting pore opening (Figure 5). The precise metabolic role of mPTP formation

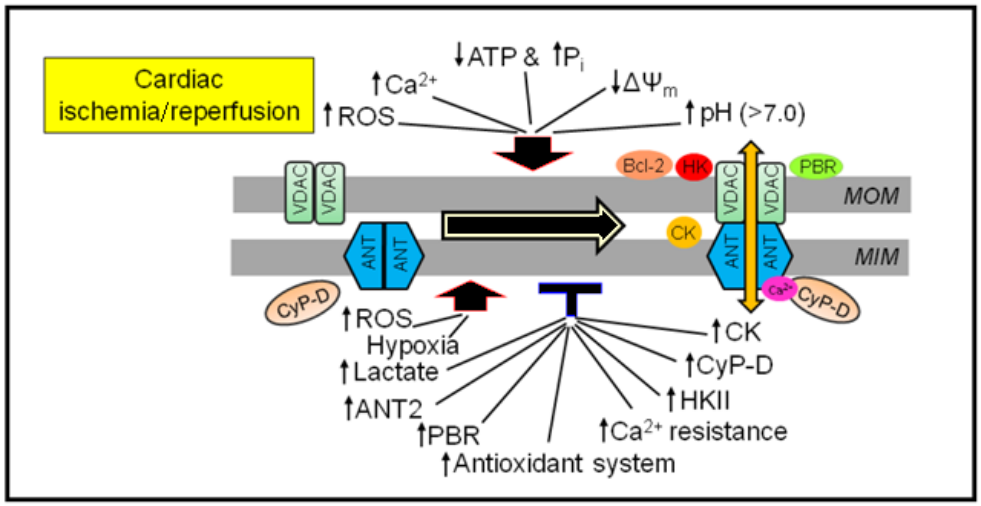

Fig. 5. Metabolic inducers and inhibitors of mPTP opening in cardiac ischemia/reperfusion and tumorigenesis. ANT, adenine nucleotide translocase; CK, creatine kinase; CyP-D, cyclophilin D; HK, hexokinase; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane; Pi, inorganic phosphate; PBR, peripheral benzodiazepine receptor; SOD, superoxide dismutase; VDAC, voltage-dependent anion channel.

under physiological conditions is still being debated; reverse pore opening with a low conductance may be attributed to regulation of mitochondrial Ca^{2+}. Low conductive mPT-induced matrix swelling and increased mitochondrial Ca^{2+} can regulate ATP synthesis through the tricarboxylic acid cycle, electron transport chain, and oxidative phosphorylation. Induction of mitochondrial permeability transition (mPT) in response to pathological stresses can also be regulated with the signaling protein kinases: PKA, PKCε

(protein kinase Cε) and GSK-3β (glycogen synthase kinase-3), which interact with the voltage-dependent anion channel (VDAC) (Bera et al., 1995; Baines et al, 2003; Javadov et al., 2009).

5.3.2 Structure of the mPTP complex

Although the crucial role of mPTPs in pathological conditions has been intensively studied in the heart, brain, and liver (Chipuk et al., 2006; Bernardi et al., 2006; Robertson et al., 2009), the actual molecular composition of the mPTP complex remains unclear. Until recently, three proteins had been accepted as key structural components of this megachannel: adenine nucleotide translocase (ANT), cyclophilin D (CyP-D), and the voltage-dependent anion channel (VDAC) located in the MIM, in the matrix and in the MOM, respectively. However, recent studies from different groups have questioned the molecular identity of the mPTP. A new model of the mPTP consisting of a phosphate carrier and ANT has been proposed where Ca^{2+} sensitivity of the pore is regulated by CyP-D binding to the phosphate carrier (Leung et al., 2008). Many studies have provided strong evidence that CyP-D plays a major regulatory role in mPTP formation (Baines et al., 2005; Nakagawa et al., 2005). Mitochondria isolated from CyP-D knockout mice were desensitized to the onset of the mPT, and required much higher concentrations of Ca^{2+} to induce pore opening compared to wild type animals, which is consistent with the role of CyP-D to regulate Ca^{2+}-mPTP interactions (Nakagawa et al., 2005). Recent studies on transgenic mice questioned the role of ANT and VDAC as essential components of the mPTP suggesting a regulatory, rather than structural role in pore formation.

5.3.3 mPTP formation in cardiac Ischemia/Reperfusion (I/R)

mPTP opening has been examined extensively in cardiac pathological conditions, mostly in I/R (Chipuk et al., 2006; Correa et al., 2007; Halestrap & Pasdois, 2009). Acute I/R does not affect the expression of mPTP compounds due to its short duration, although it induces conformational changes in essential mPTP proteins, modifying their interactions with the pore effectors in the cytoplasm and mitochondrial matrix. Although many factors that induce pore opening are present during ischemia, including ATP depletion, Ca^{2+} overload, increased phosphate and ROS levels, it has been demonstrated that pore opening occurs during reperfusion rather than during ischemia (Griffiths and Halestrap, 1995). This is explained, in part, by the acidic conditions resulting from lactate and other acidic intermediates accumulation in the mitochondrial matrix. We and others demonstrated that delayed pHi recovery during reperfusion exerts beneficial effects on post-ischemic cardiac function, associated with improved mitochondrial function and inhibition of mPTP opening (Javadov et al., 2008; Correa et al., 2008a). In this regard, inhibition of the Na^+/H^+ exchanger 1(NHE-1) may be a promising therapeutic strategy against I/R damage (Linz & Busch, 2003; Karmazyn et al., 2001). mPTP opening causes mitochondrial uncoupling, thereby ATP is hydrolyzed rather than synthesized in the post-ischemic heart leading to myocardial death (Correa et al., 2005). mPTP opening also increases in Ca^{2+}-induced cardiomyopathy (Nakayama et al., 2007), in diabetic cardiomyopathy (Oliveira et al., 2003), in heart failure following myocardial infarction (Javadov et al., 2005), and in intracoronary microembolization (Sharov et al., 2007).

5.3.4 Mitochondrial K_{ATP} channels

Mitochondrial ATP-sensitive potassium channels (mK^+_{ATP}) were described about 20 years ago in mitoplasts obtained from rat liver mitochondria. First advances in mK^+_{ATP} research demonstrated that different drugs stimulate the opening of mK^+_{ATP} channels decreasing mitochondrial potential ($\Delta\psi_m$) and that mK^+_{ATP} channels are involved in mechanisms regulating cell volume (Garlid, 1988). Latter, it was observed that bradykinin (Yang et al., 2004), opioids (Jang et al., 2008), and adenosine (Kin H, 2005), activate signaling cascades that induce the opening of mK^+_{ATP} and provide cardioprotection against ischemia/reperfusion injury. Recently a causative link between the opening of mK^+_{ATP} and cardioprotection conferred by post-conditioning has been proposed. Garlid et al. (2008) suggested that post-conditioning induces protection via an early redox-sensitive mechanism, followed by persistent mK^+_{ATP} activation. A complex signaling system involving mitochondrial PKCε 1 and 2 may prevent the formation of the mPTP. The putative signaling cascade includes the activation of Gi protein-coupled receptors and cGMP-dependent protein kinase (PKG). At mitochondrial level, this kinase binds to a hypothetic receptor, called "R1" in the mitochondrial outer membrane. This receptor may phosphorylate PKCε 1 (Jaburek et al., 2006), which, in turn, phosphorylates the mK^+_{ATP} channel favoring its opening. Once activated, the entry of K^+ may produce alkalinization of the mitochondrial matrix and promote ROS production by Complex I. ROS could activate the mitochondrial matrix PKCε 2, and prevent the formation of mPTP, reducing cell death and infarction size (Costa et al., Garlid et al., 2009) (Figure 6).

Other proposals have been invoked to explain cardioprotection. One hypothesis is that K^+ flow into the matrix may depolarize the inner membrane and reduce the driving force that sustains Ca^{2+} overload (Holmuhamedov et al., 1991; Murata et al., 2001). However, although Ca^{2+} reduction in the mitochondrial matrix may reduce mPTP opening in the post-ischemic heart, it is difficult to explain how a small depolarizing effect generated by activation of mK^+_{ATP} channels could avoid Ca^{2+}-overload. Another assumption is that discrete mitochondrial swelling associated with K^+ flow would change the architecture and respiratory control of mitochondria, creating a state of mitochondrial "super-efficiency" (Garlid, 2000). Whatever the mechanism involved in mK^+_{ATP} opening, its association with myocardial protection is clear.

6. Programmed cell death and autophagy

ROS/RNS can cause cell death by non-physiological (necrotic) or regulated pathways (apoptotic) in many cardiovascular diseases such as atherosclerosis, ischemic heart disease, heart failure, stroke, hypertension, and diabetes. The mechanisms by which ROS/RNS cause or regulate apoptosis typically are caspase-dependent and include the activation of membrane receptors, Bcl-2 family proteins, and mitochondrial dysfunction. Autophagy, a caspase-independent mechanism of cell death that protects cells against oxidative damage and is involved in the degradation and recycling of oxidized proteins and damaged organelles in cells, yields amino acids for *de novo* protein synthesis or energy provision (Nishida et al., 2009). While programmed cell death participation in cardiovascular diseases is well established, insights into caspase-independent mechanisms of cell death have emerged recently.

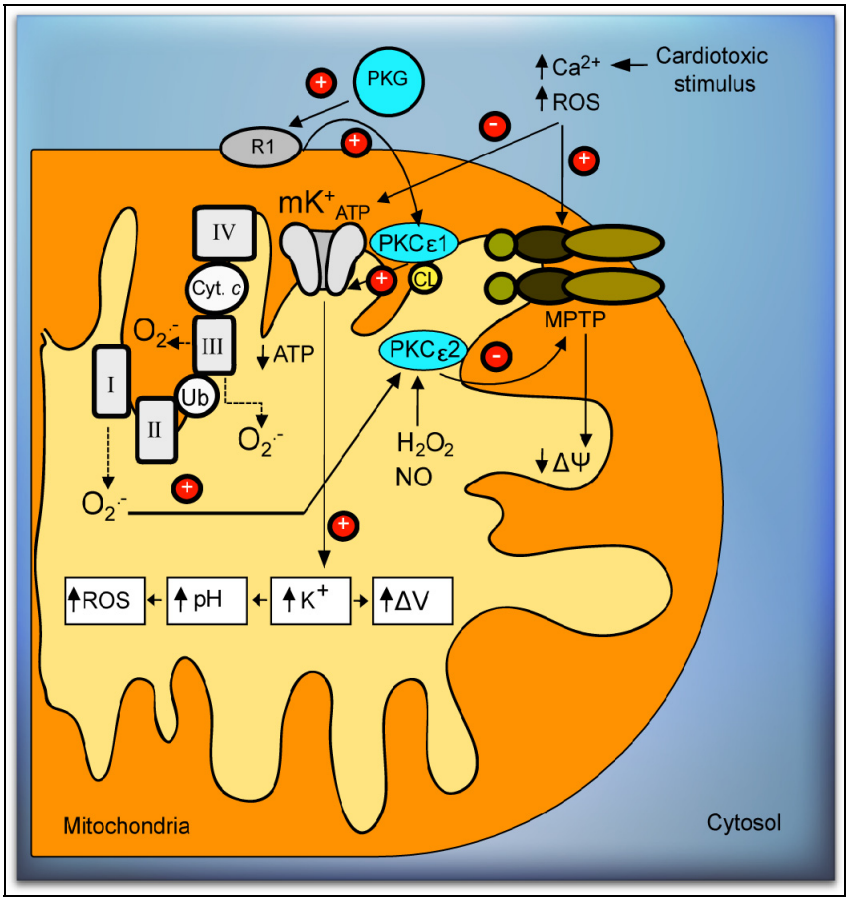

Fig. 6. Intramitochondrial signaling pathways. The pathways leading to mK^{+}_{ATP} opening, ROS production, and MPT inhibition are shown.

6.1 Apoptotic mitochondrial pathway

Mitochondria contain diverse pro-apoptotic factors within their intermembrane space, such as cytochrome *c*, apoptosis-inducer factor, and Smac / Diablo, which are released and propagate the death cascade. Two different pores have been described as pathways for cytochrome *c* release (reviewed in Kinally & Antonsson, 2007). The first one is the mitochondrial apoptosis-induced channel (MAC) formed by Bax and VDAC proteins (Shimizu et al., 2000). There are also reports indicating that only Bax-forming channels could account for cytochrome *c* release (Kuwana et al., 2002). In this sense, the contribution of Bax channels to cytochrome *c* release after reperfusion has been explored by Bombrun et al., 2003. A second possible pathway described for cytochrome *c* release is the mPTP. These proteins could be assembled into a continued unspecific channel, promoting mitochondrial swelling, MOM rupture, and pro-apoptotic proteins release (Figure 7).

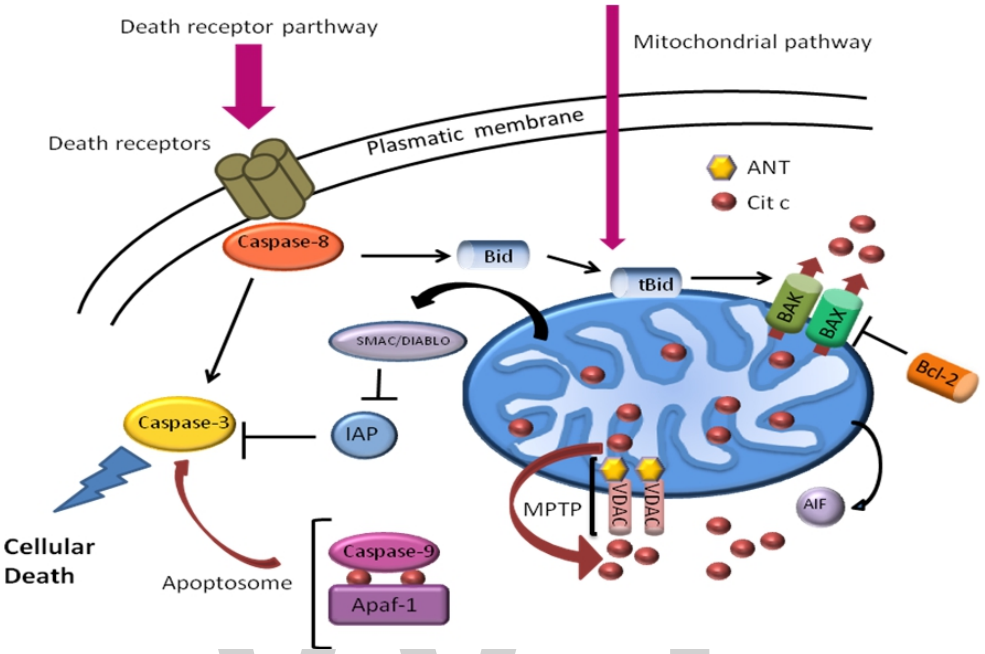

Fig. 7. Simplified model of cellular apoptotic pathways. Specific ligands bind to death receptors, activating initiator and executioner caspases. In the mitochondrial pathway, several stimuli are processed favoring membrane permeabilization and the release of pro-apoptogenic factors as cytochrome *c*, AIF, and Smac/DIABLO to the cytosol, through membrane pore forming proteins, such as BAK/BAX or as a consequence of the mPTP opening. Once in the cytosol, cytochrome c and APAF-1 bind to caspase-9, activating caspase-3. The extrinsic pathway could activate the mitochondrial pathway, through the Bcl-2 family member (Bid) that promotes BAX and BAK oligomerization.

Overexpression of antioxidant proteins in several models sustains the relevance of oxidative stress in cardiomyocyte apoptosis. Catalase, glutathione peroxidase 1, metalothionein, mitochondrial glutaredoxin-2, and peroxiredoxin 2 over- expression reduce apoptosis and improve contractile dysfunction after ischemia/reperfusion injury (Shiomi et al., 2004; Nagy et al., 2008; Zhao et al., 2009). As indicated, oxidative stress may activate the intrinsic apoptotic pathway in cardiomyocytes through multiple mechanisms, such as the induction of mPTP opening, DNA damage-induced, translocation of Bax and Bad to the mitochondria, and caspase activation. However, an alternative mode of oxidative stress-induced activation of the intrinsic apoptotic pathway may also involve induction of the ER stress response, leading to caspase-12 activation and/or Ca^{2+}-dependent opening of the mPTP (Foo et al., 2005). Catecholamines, angiotensin II, prostaglandin $F_{2\alpha}$, or endothelin-1, which interact

with G-protein coupled receptors and induce cardiomyocyte hypertrophy, may also induce apoptosis. A well characterized mechanism is a $G_{\alpha q}$-mediated PKC-dependent transcriptional upregulation of the Bcl-2 family member Nix, which activates the mitochondrial death pathway (Yussman et al., 2002)

6.2 Autophagy

Autophagy is a physiological process that is necessary for cell survival and maintains stable levels of nutrients to sustain cellular homeostasis. It is also involved in various pathophysiological processes, and shows increased activity in response to extracellular and intracellular stimulation such as nutrient starvation and hypoxia. During autophagy, cytoplasmic constituents are sequestered into the autophagosome, a closed double membrane vacuole that eventually fuses with a lysosome. In the new structure, named autolysosome, the contents are degraded and recycled for protein synthesis (Cuervo, 2004). ROS act as signaling molecules in the early events of autophagy induction. If the pro-survival attempt fails, ROS cause cell death which, depending on the experimental context, involves either the autophagic or the apoptotic pathway. Mitochondria are both the major source of intracellular ROS and, at the same time, targets of ROS. An enhanced oxidative stress may activate a signaling cascade involving the PKCβ-dependent phosphorylation of p66 protein and its translocation to the mitochondrial matrix. Damaged mitochondria are degraded by a specialized form of autophagy, called mitophagy, in which mitochondrial calcium plays an active role. Normally calcium homeostasis is tightly regulated and occurs at ER-mitochondria contacts where microdomains of high calcium concentration are present. This event causes a variety of responses depending on the amount of Ca^{2+} increase, from stimulation of metabolism and ATP production to ROS production, mPTP opening, and apoptosis (Figure 8). In this respect, recent data have proposed a role of p66Shc in mediating the response of mitochondria to ROS-induced apoptosis or autophagy (Mammucari & Rizzuto, 2010).

Autophagy increases in response to acute myocardial ischemia (AMI), chronic myocardial ischemia, heart failure, and cardiomyopathy degeneration (Cuervo, 2004). The effect of autophagy upregulation is under debate, as some studies have demonstrated that it leads to myocyte death after ischemia/reperfusion (Valentim et al., 2006), but others indicate that autophagy has a cardioprotective effect during myocardial ischemia. Three mechanisms are invoked to explain protection: 1) generation of free amino acids and fatty acids that contributes to maintain the mitochondrial energy supply, improving cell survival (Matsui et al., 2007), 2) elimination of disordered structural proteins, harmful to cardiac myocytes, and 3) removal of damaged mitochondria. In human myocardial depression associated to endotoxemia, Hickson-Bick et al. (2008) described that mitochondrial biogenesis observed in cardiomyocytes may reflect an effort to replace the mitochondria eliminated by autophagy. In HL-1 cells subjected to oxidative stress, the induction of autophagy by rapamycin suppressed ROS production and protected cells against death (Yuan et al., 2009). These results are consistent with the notion that autophagy is a protective mechanism in this setting and limits the production of harmful ROS, either by removing damaged mitochondria or by supporting *de novo* glutathione biosynthesis through the delivery of amino acids.

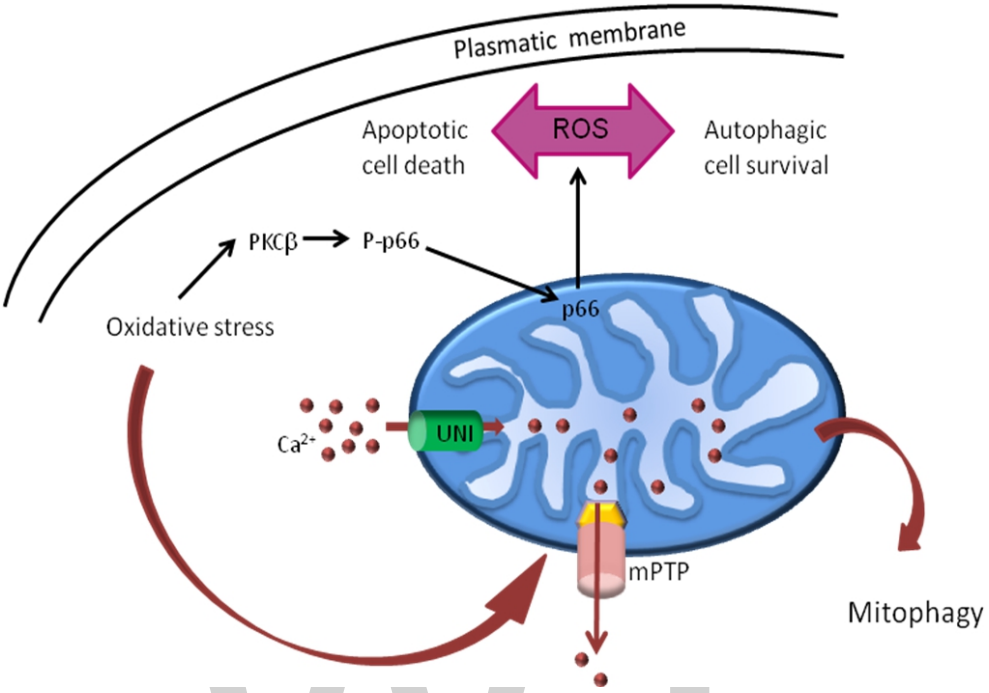

Fig. 8. Signaling pathways, regulating mitochondrial function. ROS production is induced by oxidative stress, which activates a signaling cascade involving the PKCβ-dependent phosphorylation of p66 its translocation to the mitochondrial matrix. Mitochondria are also targets of ROS damage. Damaged mitochondria are removed by mitophagy, a specialized form of autophagy, which is regulated by different pathways.

7. Mitochondrial fission and fusion

Mitochondria are highly dynamic organelles that undergo constantly fusion and fission as part of their normal function (Detmer & Chan, 2007). These two opposite processes are accurately coordinated and necessary for proper morphology and function and are thought to play critical roles during development, cell division, and apoptosis (Cerveny et al., 2007; Chan, 2006). Disruption of mitochondrial fission and fusion has been linked to the development and progression of some diseases.

Mitochondrial fusion facilitates the exchange of materials between mitochondria for the maintenance of functional mitochondria, whereas mitochondrial fission contributes to the elimination of damaged mitochondrial fragments through mitophagy and contributes to the proper distribution of mitochondria in response to the local demand for ATP. Multiple proteins have been identified to mediate mitochondrial fission and fusion processes (Chan, 2006). These two opposing processes are regulated by the mitochondrial fusion proteins, mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2, Fzo1 in yeast) (Eura et al., 2003), and optic atrophy protein 1 (Opa1, Mgm1 in yeast) (Cipolat et al., 2004), and the mitochondrial fission proteins

dynamin-related protein 1 (Drp1, Dnm1 in yeast) (Frank et al., 2001; Smirnova et al., 2001; Ingerman et al., 2005), and human mitochondrial fission protein 1 (hFis1) (Yoon et al., 2003). The balance between mitochondrial fusion and fission within a cell can be disrupted by a myriad of factors, including oxidative stress (Frank et al., 2001) and simulated ischemia (Brady et al., 2006), and has also been linked to aging (Kowald & Kirkwood, 2011) (Figure 9).

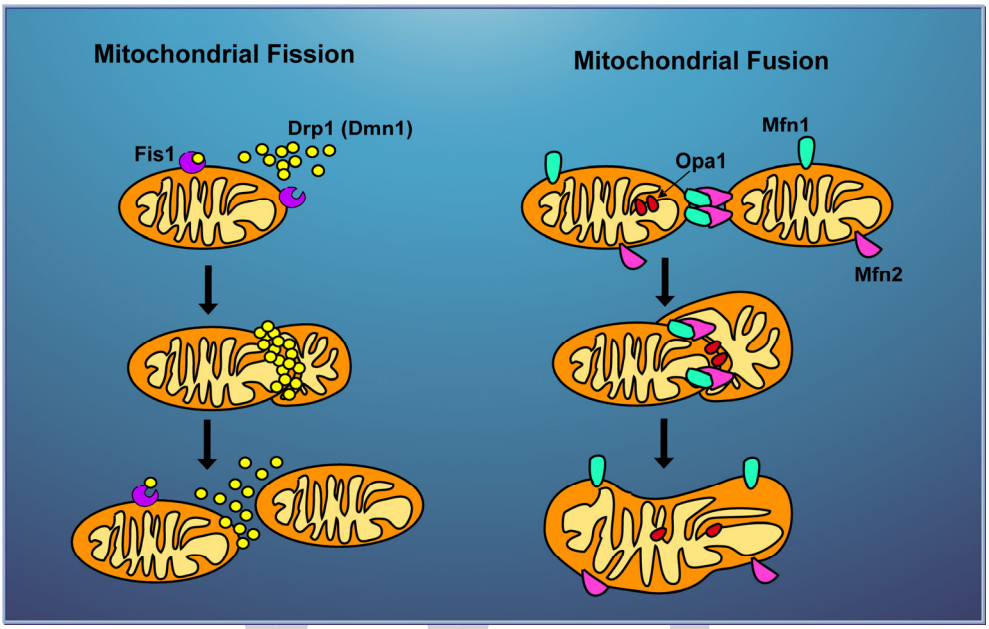

Fig. 9. Mitochondrial fission and fusion. Mitochondrial fission involves the action of Drp1, which can self-assemble into polymeric spirals and is recruited into the mitochondrial membrane by hFis1 and Mdv1/Caf4. Drp1 polymers wrap around the mitochondrion and constrict the membrane until fission occurs. Mitochondrial fusion involves the interaction of Mfn1 and Mfn2 proteins located in the outer mitochondrial membrane of two mitochondria until outer membranes fuse, consequently, inner mitochondrial membrane fusion occurs through interaction of Opa1 proteins. See text for further details.

7.1 Mitochondrial fusion

Data on fission and fusion proteins' role in heart diseases are scarce. Recently it has been reported that cardiac myocyte mitochondria, lacking the fusion protein Mfn2, are pleiomorphic and show enlarged morphopology. Consistent with an underlying mild mitochondrial dysfunction, Mfn2-deficient mice display modest cardiac hypertrophy accompanied by slight functional deterioration (Papanicolau et al., 2011). Expression of OPA1 is decreased in both human and rat failing hearts, which show small and fragmented mitochondria indicative of decreased fusion. OPA1 mRNA levels did not differ between failing and normal hearts, suggesting post-transcriptional control, possibly through degradation by proteases activated by ATP (Baricault et al., 2007).

7.2 Mitochondrial fission

It has been suggested that defects in mitochondrial fusion and fission processes are responsible for abnormal mitochondrial morphologies observed in many cardiac diseases. In cultured neonatal ventricular myocytes, inhibition of mitochondrial fission, by over-expressing a dominant-negative mutant form of Drp1, prevents ROS production, mitochondrial permeability transition pore opening, and subsequent cell death after ceramide treatment (Parra et al., 2008). An increase in the level of cytosolic Ca^{2+} induced by thapsigargin (Tg) causes cardiac mitochondrial fission associated to ROS generation in a Drp1-dependent pathway (Hom et al., 2010). Because calcium overload is a common feature in heart failure (HF), this may increase mitochondrial fission and dysfunction, thus further contributing to the decrease in the metabolic demand of the heart and increasing its injury.

8. Conclusion

Mitochondrial redox signaling is paramount to the maintenance of cardiomyocyte homeostasis. Therefore, oxidative deregulation of mitochondrial key players, like $mK^{+}_{ATP,}$ mPTP, ionic transporters, metabolism enzymes, and apoptotic machinery, has enormous impact on cardiovascular function. Intense research is devoted to obtain a better understanding of the complex regulatory mechanisms ruling these systems and to enable the development of more specific therapeutic strategies for heart diseases. In addition, fascinating links are beginning to be discovered between mitochondrial function and cardiac physiology and diseases in the context of diverse signaling mechanisms. Besides, proteins with previously known function, like those driving mitochondrial fusion and fission, are now reported to have emergent functions in intracellular calcium homeostasis, apoptosis, and vascular smooth muscle cell proliferation, all, key issues in cardiac disease. These processes broaden the traditional role in energy production undertaken by mitochondria and provide new directions for research in cardiovascular diseases.

9. Acknowledgment

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8

Role of Oxidized Lipids in Atherosclerosis

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1. Introduction

The role of oxidized lipids in cardiovascular diseases (CVD) has been investigated over the last three decades extensively. A number of studies have been carried out on the mechanisms, and pathways leading to the arterial atherosclerosis. These studies originated from the oxidation hypothesis of the atherosclerosis which was originally proposed more than 25 years ago (Steinberg et al., 1989), and since then experiments were performed by many investigators to further examine and explore the contribution of oxidation and oxidized lipids to cardiovascular diseases. Oxidized fatty acids in the ester and free forms, their decomposition products, cholesterol and its oxidized products, proteins with oxidized amino acid residues and cross-links, and polypeptides with varying extents of covalent modification with lipid oxidation products, and many others substances derived from oxidation have been the subject of detailed studies by many investigators. These products originated *in vivo* from oxidized lipoproteins and lipid membranes were linked to initiation and propagation of atherosclerosis (Zhang & Salomon, 2005; Mitra et al., 2011; Hulsmans et al., 2010). The effect of dietary oxidized fat as a contributor to the oxidative stress was also investigated by several groups including our group (Catapano et al., 2000; Drüeke et al., 2001; Garelnabi et al., 2008; Mitra et al., 2011). While there is a consensus in understanding of initial oxidative steps in the generation of early fatty streak lesions as well as the role of products of peroxidized lipid decomposition such as aldehydes in atherosclerosis, the role of further oxidation into neutral carboxylic acids is still obscure. In this chapter we will review the background of the oxidation theory of lipoproteins and the current state of the knowledge. We will review and summarizes data leading to the current understanding of the role of oxidized lipids in atherosclerosis and some pathways involved in this process. We will also discuss recent studies that elucidate factors leading to oxidative stress including chemical, physical and biological factors. In addition, we will explain the current knowledge of the use of antioxidants; and explain their benefits if any to inhibit oxidation of LDL. This part will discuss in brief some selected clinical data.

1.1 Atherosclerosis

Atherosclerosis is the principal contributor to the pathogenesis of myocardial and cerebral infarction, gangrene, and loss of function in the extremities. The process, which under normal circumstances is a protective response to insults against the endothelium and smooth muscle cells of arterial walls, consists of the formation of fibrofatty and fibrous lesions, and is preceded and accompanied by inflammation. The advanced lesions of atherosclerosis become pathologic, and may cause occlusion of the affected artery, result from an excessive inflammatory-fibroproliferative response to numerous different forms of insult (Ross, 1986).

The earliest recognizable lesion of atherosclerosis is the so-called 'fatty streak', an aggregation of lipid-rich macrophages and T lymphocytes within the innermost layer of the arterial wall, the intima. The ubiquity of the atherosclerotic process is attested by the finding of fatty streaks in the coronary arteries of half of the autopsy specimens from children aged 10 to 14 years (WHO, 1985). Animal observations have shown that fatty streaks precede the development of intermediate lesions, which are composed of layers of macrophages and smooth muscle cells and, in turn, develop into the more advanced, complex, occlusive lesions called fibrous plaques (Fig 1). The fibrous plaques increase in size and, by projecting into the arterial lumen, may impede the flow of blood. They are covered by a dense cap of connective tissue with embedded smooth muscle cells that usually overly a core of lipid and necrotic debris (Garelnabi, 2010).

Most of the sudden deaths from myocardial infarcts are due to ruptures or fissures, particularly in the margins of the fibrous cap where there are more macrophages, resulting in hemorrhage into the plaque, thrombosis, and occlusion of the artery (Ross, 1993). As the process continues, migrating cells reach further beneath the arterial surface, where the monocytes become macrophages, accumulate lipid, become foam cells, and together with the accompanying lymphocytes, become the fatty streak. These often form at sites of pre-existing collections of intimal smooth muscle. Thereafter, continued cell influx and proliferation lead to the more advanced lesions, distinguished by their fibrous character, and ultimately to the fibrous plaque (Ross, 1993).

Studies on animals with artificially induced hypercholesterolemia have confirmed that three processes are involved in the formation of atherosclerotic lesions : (1) The proliferation of smooth muscle cells, macrophages, and possibly lymphocytes; (2) the formation of a connective tissue matrix by smooth muscle cells comprised of elastic fiber proteins, collagen, and proteoglycans; and (3) the accumulation of lipid and mostly free esterified cholesterol in the surrounding matrix and the associate cells (Daley et al., 1994).

There are numerous signals, biochemical in nature, which underlie smooth muscle proliferation. Platelet derived growth factor (PDGF), the first postulated growth factor in atherogenesis is produced by many of the cells involved in the process (i.e., platelets, macrophages, endothelial cells and smooth muscle cells). Activated macrophages can also synthesize fibroblast growth factor (FGF), endothelial derived growth factor (EDGF), and transforming growth factor beta (β-TGF). The combination of these growth factors has been shown to be extremely potent in stimulating the migration and proliferation of fibroblasts and smooth muscle cells, as well as the formation of connective tissue element.

When platelets interact with or adhere to sub-endothelial connective tissue, they are stimulated to release their granule contents. Endothelial cells normally prevent platelet adherence because of the non-thrombogenic character of their surface and their capacity to form antithrombotic substances (e.g., prostacyclin and heparin). When endothelium is injured, platelets are promoted to adhere to its surface and thus, the release of platelet constituents, although it is not clear that platelet adherence to modified endothelium is a common event (Ross, 1986). Several investigators have demonstrated that if platelets are absent from the site of endothelial injury, or if are prevented from the injury sites pharmacologically as in experimental models, then the intimal proliferative lesions that usually accompany such injury will not occur (Friedman et al., 1977; Haker et al., 1983). Oxidized low density lipoproteins (OxLDLs) have been shown to play a key role in the pathogenesis of atherosclerosis, since they are present in atherosclerotic lesions. Indeed, oxidized LDLs inhibit endothelium-dependent relaxation of the rabbit aorta in response to acetylcholine, as well as of porcine coronary artery in response to serotonin and platelets (Tanner et al., 1990).

2. Oxidation of LDL

The major constituents of plaques are lipid-laden foam cells are formed and their remains. Foam cells form when macrophages or other cells uptake an excessive amount of LDL, and die. An oxidative hypothesis of atherosclerosis was proposed in 1989 and suggested modification of LDL as a primary reason of foam cell formation and development of atherosclerosis (Steinberget al, 1989; Parthasarathy et al., 2010). A massive amount of confirming data was collected since then. It is well accepted now that oxidative processes and oxidized lipids play pivotal role in initiation and progression of the disease.

LDL is a microparticle consisting of one ApoB protein molecule and a mixture of triacylglycerol, cholesterol and its esters, phospholidpids, and vitamin E. Oxidation of LDL is a gradual process starting with oxidation of vitamin E and polyunsaturated fatty acids. Peroxides, the primary oxidation products, undergo further transformations with generation of aldehydes among other products. Aldehydes modify amino acid residues of ApoB, primarily lysine, resulting in malondialdehyde modified ApoB (MDA-ApoB) and 4-hydroxy-2-nonenal modified ApoB (4-HNE-ApoB). Biological effect of oxidized LDL varies greatly depending on the grade of oxidation. There are several terms for oxidized LDL that indicate the level of oxidation, such as MM-LDL (minimally modified LDL), fully oxidized LDL, and MDA-LDL (malondialdehyde-modified LDL). It is difficult to determine the level of oxidation in many cases. The term OxLDL (oxidized LDL) is used for any oxidized LDL regardless of the extent of oxidation.

Development of atherosclerotic lesion starts with accumulation of OxLDL in intima, the innermost part of vessel, consisting of single layer of endothelial cells that rest on basement membrane. Intimal basement membrane separates endothelial cells and smooth muscle cells in arterial blood vessels. It consists of extracellular matrix, mostly collagen and proteoglycans, with sparse immune cells and smooth muscle cells (SMC) in it.

There is detectable level of OxLDL in circulating blood, and OxLDL is observed in vascular wall. Immunoglobulin M (IgM) is essential for noninflamatory clearance of OxLDL by macrophages. IgM co-localizes with CD68-positive macrophages in lesions. Double

knockout Ldlr-/- and soluble IgM-/- mice develop lesions seven time bigger than Ldlr-/- control. C1qa is a complement participating in IgM-mediated clearance. There is a pronounced increase in the size of aortic root lesion in double knockout Ldlr-/-, C1qa-/- mouse as compared to Ldlr-/- mouse/- (Lewis et al., 2009).

Immunization of atherosclerosis-prone Ldlr-/- mice with MDA-LDL or native LDL before feeding with cholesterol-rich atherogenic diet resulted in smaller lesion areas without significant reduction of plasma cholesterol (Freigang et al., 1998). Both type of immunization generated antibodies that recognize a wide pattern of modified and oxidized LDL likely because of some oxidation of LDL during immunization. Binding of OxLDL with antibodies demonstrated antiatherogenic effect, whether it limits the influx of OxLDL into artery wall or helps to clear retained OxLDL. Similar results were obtained in rabbit (Ameli et al., 1996).

While immunization with MDA-LDL prior or at initial stages of atherosclerosis suppresses growth of lesions in mouse and rabbit, there is a controversy in whether higher titer of antibodies to OxLDL in blood correlates with higher or lower grade of atherosclerosis (Palinski et al., 1995; Tsimikas et al., 2007, reviewed in Shoenfeld et al., 2004).

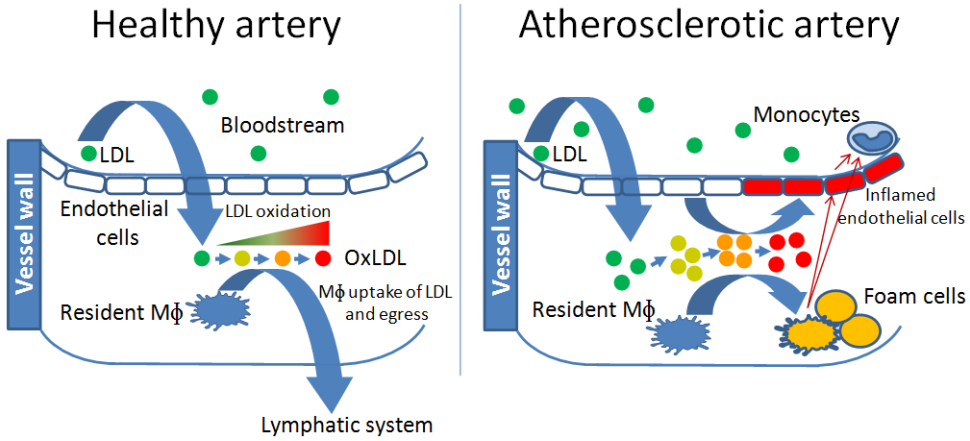

Fig. 1. **OxLDL effects and fate in healthy and atherosclerotic artery wall.** LDL (green circles) enter vessel wall and become gradually oxidized (depicted by changing circle color from green to red). In healthy artery tissue lymphocytes, primarily macrophages (Mϕ), uptake OxLDL, and egress the vessel to lymphatic system. The removal of OxLDL is impaired in atherosclerotic artery. Macrophages get overloaded with OxLDL and die generating foam cells. Overloaded macrophages release inflammatory signals that affect endothelial cells and patrolling leukocytes on the vessel surface (depicted with red arrows). Endothelial cells respond to accumulating OxLDL by inflammation as well.

Currently, the general consensus is that oxidation of LDL occurs mostly within vascular wall. Both native LDL and OxLDL are able to pass through endothelial layer passively through interendothelial junctions, or by endothelial transcytosis, an active transport process (von Eckardstein & Rohrer, 2009). LDL and OxLDL are retained in intima through interaction of the LDL protein ApoB-100 and proteoglycans. LDL undergoes oxidation in

intima and becomes absorbed by macrophages through scavenger receptors. There are many scavenger receptors that vary in the substrate specificity, expression in different tissues, and biological roles. Some of them play essential role in atherosclerosis (Table 1). Excessive loading of macrophages by OxLDL convert them to dysfunctional "foam" cells. OxLDL itself or products of spontaneous or enzyme-assisted decomposition act as pro-inflammatory, chemotactic, growth-promoting factors (Fig 1).

2.1 Induction of oxidative stress by OxLDL

OxLDL are cytotoxic for all spectra of atherosclerosis-related cells: T-cells (Alcouffe et al., 1999), macrophages, endothelial cells, smooth muscle cells. OxLDL cytotoxicity in human fibroblasts is mediated through OxLDL-derived lipid peroxides and hydroperoxides, but not superoxide (Coffey et al., 1995).

High load of OxLDL induces two separate lethal processes in macrophages. The first process is activation of caspases-3 in Fas-independent manner. Other caspases, caspase-6, caspase-8, caspase-9, are likely involved as well. It ultimately leads to apoptosis with characteristic DNA fragmentation. The second process is OxLDL-induced plasma membrane lysis (necrosis) mediated by reactive oxygen species (ROS). Both processes occur concurrently, however lysis of plasma membrane is likely the actual reason for macrophages death.

Caspase activation might contribute to macrophage death, however some experiments demonstrate that the extent of the activation is not enough for OxLDL cytotoxicity, since a higher level of caspase-3 activity through activation of Fas is not lethal for macrophages. At the same time inhibitors of caspase-3 do not suppress macrophage lysis by OxLDL, while peroxyl radical scavengers Trolox, and N,N'-diphenyl-1,4-phenylene diamine (DPPD) inhibit cytotoxicity of OxLDL. Generation of peroxyl radical as primary reactive oxygen species (ROS) in OxLDL-activated macrophages was confirmed with several specific ROS-sensitive fluorescent dyes. So, OxLDL cytotoxicity is mediated by peroxyl radicals, but not superoxide. ROS-mediated lysis and caspase activation are independent processes since inhibitors of caspase-3 do not suppress macrophage lysis by OxLDL, and Trolox does not inhibit caspase activation when it inhibits OxLDL-induced macrophage lysis (Asmis & Begley, 2003).

In response to OxLDL, macrophages start to generate intracellularly an increased amount of ROS. Excessive load with OxLDL and ROS generation leads to necrosis of foam cells. There are several NADPH oxidases expressed in macrophages. Nox2 (Gp91phox), a heme-containing subunit of NADPH oxidase, is the major source of ROS during phagocytosis. Nox2 likely does not contribute to atherosclerosis, since Nox2 knockout mouse does not slow development of lesions (Kirk et al., 2000).

Nox4 is another NADPH oxidase. Protein expression of Nox4 and its binding partner p22phox in macrophages is increased by OxLDL but not by native LDL through MEK1/2 pathway. Inhibition of MEK1/2 or siRNA knockdown of Nox4 suppresses ROS production and macrophage death assessed by membrane integrity (Lee et al., 2010).

2.2 NF-κB response to OxLDL and atherosclerosis

NF-κB is a family of transcription factors and their precursors sharing Rel homology domain. They function as homo or heterodimers, such as RelA/p50. In resting cells, NF-κB

dimer is associated with IκB, an inhibitory subunit of NF-κB. There are several members in IκB family. The canonical pathway of NF-κB activation is IκB phosphorylation by activated IκB kinase complex consisting of IKKα and IKKβ subunits and regulatory protein NEMO. Phosphorylated IκB becomes ubiquitinated and undergoes degradation. Degradation of inhibitory subunit releases NF-κB dimer, which translocates from cytoplasm to nucleus and initiates transcription of target genes. Various signals activate IKK complex including tumor necrosis factor (TNF) and interleukin-1 (IL-1). In an alternative pathway, activated NF-κB inducing kinase (NIK) phosphorylates precursor protein p100 that results in ubiquitination and proteasomal processing of a precursor protein p100 into mature p52 subunit. The subunit binds with RelB, and RelB/p52 dimer is an active transcription factor. B-cell–activating factor and other stimuli can activate NIK and thus initiate the alternative pathway. Factors such as lipopolysaccharide (LPS), CD40 ligand can activate both pathways, canonical and alternative. However, there is no data yet on regulation of NF-κB via alternative pathway in smooth muscle cells, macrophages, and endothelial cells (de Winther et al., 2005). OxLDL initiates inflammatory response in endothelial cells and leukocytes. Inflamed cells induce factors that attract leukocytes. Activation of NF-κB is one of the pathways that are involved in atherosclerosis. Activation of this pathway is observed in lesions in endothelial cells, macrophages and SMC (Brand et al., 1996).

OxLDL exerts dual effect on NF-κB activation in monocytes and macrophages. It activates NF-κB in short term, and suppresses it in long term (Brand et al., 1997; Eligini et al., 2002). Activation of NF-κB by OxLDL in atherosclerotic endothelial cells is more stable. An essential mechanism of NF-κB activation is mediated through scavenger receptor LOX-1 (lectin-like oxidized low-density lipoprotein receptor 1). Binding of OxLDL to LOX-1 induces superoxide and hydrogen peroxide generation, and NF-κB activation trough activation of p38 MAP kinase, PI3K, ERK1/2 pathway (Cominacini et al., 2000; Tanigawa et al., 2006). Knockdown of LOX-1 gene suppresses endothelial cell injury measured as LDH release, abates expression of MCP-1 and decreases monocyte adhesion to endothelial cells (Li & Mehta, 2000). Knockout of LOX-1 in Ldlr-/- mouse suppresses activation of p38 MAPK, decreases NF-κB p65 protein level, and inhibits development of atherosclerosis (Mehta et al., 2007).

The importance of NF-κB in endothelial cells in progression of atherosclerosis is demonstrated in ApoE-/- mouse. NF-κB pathway was disrupted by ablation of NEMO/IKKγ or expression of dominant-negative IκBα in endothelial cells. In both cases the lesions developed slower than in control ApoE-/- mouse (Gareus et al., 2008).

Inflammation is central process in development of atherosclerosis. Presentation of P-, E-, L-selectins by endothelial cells initiates vascular recruitment of circulating monocytes through selectin ligands that are expressed on surface of leukocytes, such as PSGL-1 (Yang et al., 1999; Sperandio et al., 2003). Inhibition of leukocyte recruitment slows development of atherosclerosis. Indeed, P-selectin knockout mice have smaller lesions than control animals (Dong et al., 2000). NF-κB regulates expression of P-selectin and other inflammation-related genes including E-selectin, ICAM-1, VCAM-1, and MCP-1 (Cominacini et al., 1997).

MCP-1 is another cytokine essential for development of atherosclerosis: Ldlr-/- Mcp1-/- mouse has smaller lesions compare to Ldlr-/- (Gu et al., 1998). VCAM-1 on endothelial cells

participates in tight adhesion of monocytes. VCAM-1 knockout is lethal for mouse; however a study of a transgenic mouse with suppressed expression of VCAM-1(D4D) demonstrated reduced lesion development (Cybulsky et al., 2001).

While NF-κB pathway responds to OxLDL, activation of NF-κB stimulates expression of Lox-1 and OxLDL uptake. A study of transgenic ApoE-/-, SIRT1+/- mouse with decreased SIRT1 function revealed that NF-κB inhibition decreases expression of Lox-1 and Ox-LDL uptake. SIRT1, a NAD-dependent class III deacetylases, is known to inhibit NF-κB activity by deacetylating RelA/p65. Indeed transgenic ApoE-/-, SIRT1+/- mouse has decreased SIRT1 activity an increased level of Lox-1 in aorta, and develops atherosclerosis faster compared to ApoE-/-, SIRT1+/+ mouse. Experiments with bone marrow transplantation revealed that pro-atherogenic effect of decreased SIRT1 function is mostly associated with leukocytes. ApoE-/-, SIRT1+/- peritoneal thioglycolate-elicited macrophages uptake showed increased uptake of OxLDL (Stein et al., 2010).

3. Lipid peroxidation: NO Implication

It is believed that lipid peroxidation is involved in the oxidative modification of low density lipoprotein (LDL) and the formation of the potent oxidant peroxynitrite (ONOO–) (Roger et al., 1994). Despite intensive research into this key step, the identity of the radical is still a mystery, especially for the in vivo situation. It may result from preformed or lipoxygenase-derived lipid hydroperoxides or hydrogen peroxide, which decompose in the presence of metal ions to lipid alkoxyl radicals and lipid peroxyl radicals and to hydroxyl radical, respectively. Once formed, the carbon-centred PUFA radical reacts very quickly with molecular oxygen yielding a lipid peroxyl radical which in turn abstracts a hydrogen atom from an adjacent PUFA, yielding a lipid hydroperoxide and a new PUFA radical. It is the latter reaction that carries the lipid peroxidation chain. If no chain termination took place, a single initiating event could convert all LDL. The precise length of the chain, i.e., the number of PUFAs oxidized per one initiating radical depends on many factors especially on the antioxidants. The antioxidants of LDL compete with chain propagation by very efficiently scavenging lipid peroxyl radicals.

Lipid peroxidation can be measured in a laboratory setting by a variety of methods. Oxidized lipid extracts is measurable in spectrophotometer technique. Recent methods of analysis includes the free oxygen radicals monitor (FORM) system (Garelnabi et al, 2008), Electron Spin Resonance Spin Trapping Techniques (ESRT), and several other traditional techniques. Peroxidation of fatty acids containing three or more double bonds will produce malondialdehyde (MDA). Malondialdehyde produced by peroxidation can cause cross-linking and polymerization of membrane components (Nielsen, 1981). This can alter intrinsic membrane properties such as deformability, ion transport, enzyme activity, and the aggregation state of cell surface determinants. Because MDA is diffusible, it will also react with nitrogenous bases of DNA (Bruce & James 1982). Increased formation of MDA has been associated with arachidonic acid metabolism and platelet aggregation (Marie, 1979; Macfarlane et al., 1977; Garelnabi et al. 2008; Garelnabi et al. 2010). Experimental studies have shown that free radicals promote platelet aggregation and thrombosis and chain breaking antioxidants, such as vitamin E, inhibit or delay arterial thrombogenesis (Ikeda et al., 1994; Jourdan et al., 1995).

Scavenger receptor	Expression	LDL-related substrates	Other substrates	Effect of knockout in mouse
Class A: SR-AI, SR-AII	Tissue macrophages, arterial endothelial cells, smooth muscle cells	Acetylated LDL, lower affinity for OxLDL; recognize modified ApoB	Apoptotic cells, beta-amyloid peptide, anionic phospholipids, advanced glycation end-products, Gram-negative and Gram-positive pathogen-related molecules	Controversial results on atherosclerosis development in knockout of both SR-AI and SR-AII genes (Msr-/-) in Apo-/- or Ldlr-/- mice
Class B: SR-B1 (and another minor splice variant of the same gene SR-B2)	Liver, macrophages; adrenal glands, ovaries, and testes - reverse cholesterol transport	OxLDL	Native LDL, HDL, apoptotic cells, beta-amyloid, anionic phospholipids, advanced glycation end-products, amyloid	Srb1 knockout in Apoe-/- or Ldlr-/- mouse promotes atherosclerosis
Class B: CD36	Macrophages, dendritic cells, endothelial cells	Moderately oxidized LDL, POV-PC (1-palmytoyl-2-(5-oxovaleryl)-snglycero-3-phosphocholine) ; does not bind acetylated LDL or extensively oxidized LDL	Native LDL, HDL, apoptotic cells, beta-amyloid, anionic phospholipids, advanced glycation end-products, thrombospondin-1, collagen, fatty acids, protozoan and bacterial peptides and lipopeptides	Knockout of Cd36 in Apoe-/- mouse partly protects from atherosclerosis
Class E: LOX-1	Endothelial cells, macrophages, SMC	OxLDL		Lox1 knockout inhibits atherosclerosis in Ldlr-/- mouse (Mehta et al,. 2007)

Less studied scavenger receptors such as MARCO, SRCL (Class A), CD68 (Class D), SREC-1 (Class F), SR-PSOX/CXCL16 (Class G) are not included in the table. The table is based on review (Moore & Freeman, 2006)

Table 1. Scavenger receptors involved in atherosclerosis

The autoxidation of polyunsaturated lipids is an irreversible destructive process; and in tissues it may be associated with accelerated cell aging and premature cell death. Because such biological autoxidation is essentially slow process, the quantitative measurement of susceptibility to oxidation requires standard experimental stress conduction (Dildar et al., 1998).

4. Cellular defenses against ROS

The biochemical defenses that protect organism from the ROS include both small molecules (low molecular weight compounds such as antioxidants and free radical scavengers) and complex enzyme systems. These defenses serve to lower concentrations of free radical species such as superoxide ($O_2^{\bullet -}$), nitric oxide ($^{\bullet}NO$) hydroxyl radical ($^{\bullet}OH$), lipid peroxyl radicals (L-$OO^{\bullet}$), and strong oxidants and precursors of free radicals such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$). If ROS generation exceeds defense capacity of the cell, ROS will cause excessive damage to cell components. ROS scavengers have also been used to characterize the production, nature, and toxicity of free radical species in *in vitro* and *in vivo* systems.

4.1 Lipid soluble scavengers

A variety of molecules that preferentially partition into membranes function by reducing lipophilic free radical species to less toxic forms. Vitamin E (a series of isomers of tocopherol) will reduce superoxide ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), singlet oxygen ($^1O_2$), lipid peroxy radicals, and other radical species. Ascorbate is proposed to have similar properties and may serve to maintain tocopherols in the reduced active form. Ascorbate serves as a water-soluble reductant and radical scavenger (Bruce & James 1982). The ascorbate-glutathione pathway represent an avenue through which ascorbate consumed in H2O2 reduction get recycled at the expense of NADPH. In the first step of this pathway, H_2O_2 is reduced to water by ascorbate peroxidase (APX) using ascorbate as the electron donor. The oxidized ascorbate (monodehydroascorbate) is regenerated by monodehydroascorbate; a radical and if not rapidly reduced it disproportionates into ascorbate and dehydroascorbate. Dehydroascorbate is reduced to ascorbate by dehydroascorbate reductase at the expense of GSH, yielding oxidized glutathione GSSG which is reduced by glutathione reductase (GR) using NADPH as electron donor (Fig 2), (Blokhina and Fagerstedt KV, 2010; Palma et. al, 2009; Halliwell, 2009). Enzymatic ROS scavengers: Catalase and peroxidases lower the steady state concentration of H_2O_2 which is a precursor of potent radical species. Thus, the cytotoxic potential of H_2O_2 is in large part a function of intracellular catalase and peroxidase activities that scavenge H_2O_2, and concentration of free ions of transition metals that promote generation of $^{\bullet}OH$ from H_2O_2. Three glutathione peroxidase (GPx; EC1.11.1.9) isozymes are known, cellular GPx, extracellular GPx, and phospholipid hydroperoxide GPx, and each contains a selenocysteine in its catalytic center. Cellular GPx; the most characterized form, can react with hydrogen peroxide and organic peroxides but not lipid hydroperoxide (Michio et al., 1995). Platelet GPx has been shown to influence the platelet arachidonic acid metabolism by stimulating lipoxygenase and inhibiting cyclooxygenase, since oxidative stress enhances the arachidonic acid metabolism and thereby creates greater demands on the regulatory systems (Malmgren et al., 1990). Phospholipid hydroperoxide

glutathione peroxidase (PHGPx) is an intracellular antioxidant selenoenzyme which interacts directly with peroxidized phospholipids and cholesterol and cholesteryl esters (Imai and Nakagawa 2003) . Selenium (Se) is an essential micronutrient for animals and humans that exerts its biological functions through selenoproteins. These proteins contain Se in the form of selenocysteine (Sec), Phospholipid hydroperoxide glutathione peroxidase (PHGPx or GPx4, E.C. 1.11.1.12) is characterized by the presence of selenocysteine at the active site, and belongs to the important family of glutathione peroxidases (GPx). Since the discovery of PHGPx, a number of studies have demonstrated that this seleno-enzyme is essential to organisms. However on the other hand glutaghione-S-transferase possessing glutathione peroxidase activity toward lipid peroxides, but not having selenocysteine in its active site (Ursini et al. 1982; Yagi et. al 1996)

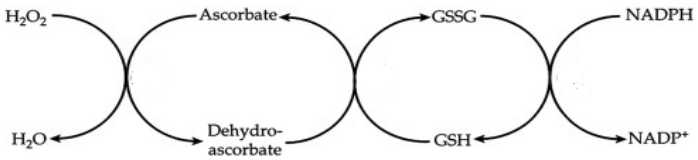

Fig. 2. The glutathione-ascorbate cycle.

Superoxide dismutases (SOD; EC 1.15.1.1) are metalloproteins that catalyze dismutation of superoxide anion radical to H_2O_2. Several types of SOD have been discovered. Mn-SOD (MW 85,000) has been found in mitochondria matrices and CuZn-SOD (MW 33,000) is contained in cellular cytosol. However, Mn-SOD and CuZn-SOD have been found also in extracellular fluids (Wesiger & Fridovich, 1973; Marklund et al., 1982). The superoxide radical has been reported as being produced from stimulated platelets (Levine et al., 1981) but its biological value in platelet function is not clearly understood (Violi et al., 1985). A decrease in cytosolic SOD the main defense against superoxide, could lead to increased cellular peroxides. Role of diet in the activity of Cu,Zn-SOD in platelets was studied and found to be influenced by the availability of Cu in diet (Catherine et al., 1993). Furthermore insufficiency in dietary copper was found to increase platelet thromboxane production, which in turn significantly correlated with endogenous lipid hydroperoxides. Evidence obtained from *in vitro* experiments indicates that superoxide dismutase may also inhibit platelet aggregation. That is, SOD given as adjuvant therapy with thrombolysis may both blunt free radicals mediated reperfusion injury and limit the incidence of spontaneous reocclusion after restoration of blood flow (Karin & Robert, 1993). Superoxide dismutase may protect endogenous $^{\bullet}NO$ from inactivation by scavenging superoxide anion. *In vitro* the inhibitory action of $^{\bullet}NO$ on platelet aggregation as well as their adhesion to endothelium induced by thrombin is potentiated by SOD consistent with its preventing inactivation of endothelium-derived $^{\bullet}NO$ (Meng et al., 1995).

Nitric oxide derived reactive nitrogen species (RNS) such as nitrogen dioxide ($^{\bullet}NO_2$) and peroxynitrite ($ONOO^-$) are indicated in the mediation of oxidative damage.Nitric oxide reacts very rapidly with oxygen radicals. Thus $^{\bullet}NO$ reacting with $O_2^{\bullet-}$ generates peroxynitrite (IUPAC–recommended name is oxoperoxonitrate O=N–O–O^-). The peroxynitrite anion ($ONOO^-$) is relatively stable but its acid form (ONOOH) decays to nitrite with a half life of at most 1 sec at physiological pH and temperature (Ducrocq et al., 1999). Peroxynitrite mediates several of the cytotoxic effects of $^{\bullet}NO$ such as the destruction

of FeS centres in enzymes. Persistent blockade of cytochrome c oxidase by $^{\bullet}NO$ may lead to the release of free calcium ions (Ca^{2+}) from the mitochondrial matrix into the cell cytosol. Nitric oxide also reacts with lipophilic peroxyl radicals, important propagating species in biological chain reaction of lipid peroxidation, to generate alkyl peroxynitrites (LOONO). These appear far more stable than $ONOO^{-}$. If LOONO derivatives can be metabolised without the release of toxic free radicals then the reaction of $^{\bullet}NO$ with peroxyl radicals is potentially beneficial because it allows $^{\bullet}NO$ to stop lipid peroxidation. $^{\bullet}NO$ inhibits platelet and phagocyte adhesion to the endothelium. However, in atherosclerotic lesions excess production of $O_2^{\bullet-}$ may cause loss of the modulatory action of $^{\bullet}NO$ and at the same time yield $ONOO^{-}$ which is pro-aggregatory and so could commit platelets in this environment to thrombus formation (Roger et al., 1994).

Protective mechanism: Several antioxidants can scavenge $ONOO^{-}$, a molecule responsible for irreversibly oxidation of thiols to higher oxidation states, but nitrosothiols can also form, and later may act as $^{\bullet}NO$ donors. Indeed, when isolated vascular tissues are exposed to $ONOO^{-}$ vasorelaxation occurs by a mechanism characteristic of release of $^{\bullet}NO$ from a carrier molecule such as nitrosothiol (Liu et al., 1994). Repeated exposure to $ONOO^{-}$ results in a progressive decrease in the efficiency of the vasorelaxing effect.

4.2 Benefits of antioxidants against lipid peroxidation

There are a vast number of studies on the role of anti-oxidants particularly in the area of atherosclerosis and CVD. These studies are controversial, and do not provide clear evidences on the benefits of antioxidants for prevention or treatment of the diseases. Supplementation of antioxidant vitamins such as α-tocopherol, ascorbic acid and β-carotene used alone or in combination had long been considered to be cardio protective. However, controlled clinical trials using antioxidant vitamin supplements to prevent CVD have yielded conflicting results (Raghavamenon et al., 2009). While some secondary prevention interventions have been shown with α-tocopherol supplementation alone or in combination with ascorbic acid is reported to reduce CVD risk, other studies have shown no effect of α-tocopherol supplementation in both primary and secondary prevention.

Vitamin E (α-tocopherol) is found in plant oils (Honarbakshsh & Schachter, 2009). This vitamin is extensively studied as a possible antioxidant agent against oxidation-induced cardiovascular diseases. Administration of 1000 IU/day α-tocopherol has been shown to reduce LDL oxidation (Princen et al., 1992). A human study shown that α-tocopherol supplementation of 150 IU/day to 1200 IU/day increases it level in plasma and in LDL in concentration-dependent manner. *In vitro* oxidation of LDL was partly inhibited in LDL with higher tocopherol content (Dieber-Rotheneder et al., 1991). α-Tocopherol is reported to reduce plasma OxLDL levels at 25 IU/day in both men and women, and the effect rises with increased supplementation until 800 IU/day (Princen et al., 1995). Tocopherol accumulation in monocytes decreases stress-induced adhesion of monocytes to endothelial cells (Islam et al., 1998; Devraj et al., 1996; Faruqi et al., 1994; Zapolska-Downar et al., 2000), which in turn inhibit the formation of atherosclerotic lesions. Overall, a number of *in vitro* studies demonstrate anti-atherogenic effect of vitamin E by decreasing the production of ROS, lipid oxidation, monocyte endothelial cell adhesion and cytokines secretion. However clinical studies have not revealed anti-atherogenic effect in human (Yusuf et al., 2000).

Vitamin C (ascorbic acid) is principally found in citrus fruits, broccoli, red pepper, and cauliflowers, etc. Ascorbate acts in combination with vitamin E and beta–carotene to protect them from excretion and recycle them for further use. It is also reported to inhibit OxLDL formation indirectly by protecting vitamin E and beta-carotene (Jialal & Grundy, 1991; Kagan et al., 1992). Apart from this vitamin C is reported to inhibit endothelial apoptosis initiated by inflammatory cytokines *in vitro*, and reduces circulating apoptotic microparticles in human (Rössig et al., 2001). Adhesion proteins such as ICAM-1 can be involved in atherosclerosis. Ascorbate supplementation of subjects with low baseline level of this vitamin suppresses mRNA and protein expression of ICAM-1 in monocytes (Rayment et al, 2003). While these and other studies suggest that vitamin C might have anti-atherogenic effect, there is no conclusive clinical evidence of such effect.

β-Carotene is indicated in preventing oxidation of lipids which might decrease atherosclerotic lesions formation. β-Carotene is proposed to be efficient scavenger of singlet oxygen and it attenuates oxidative stress, however it does not directly inhibit lipid peroxidation (Briviba et al., 2004).

Polyphenols are another group of antioxidants which are abundant in vegetables and fruits and are found to reduce the risk of CVD (Naderi et al., 2003). They contain both hydrophilic and hydrophobic moieties (Woodman & Chan, 2004). Polyphenols are suggested to inhibit lipid peroxidation (Madrau et al, 2009). It has also been reported that flavonoids chelates copper and iron ions, rendering them inactive to participate in free radical generating reactions (Fernandez et al., 2002). Polyphenols are also known to inhibit enzymes responsible for generation of ROS such as NADPH oxidase, lipoxygenase, phospholipase A2, and xanthine oxidase (Rice-Evans et al., 1997). Indirectly inhibiting the formation of OxLDL, the benefits of flavonoids goes beyond the protection against LDL oxidation to protect the HDL-associated paraoxonase activity (Patel et al., 2007). The antiatherogenic effect of mulberry leaf extracts (MLE) and the polyphenolic extracts (MLPE), which contain polyphenols including quercetin (11.70%), naringenin (9.01%) and gallocatechin gallate (10.02%) was studied by Yang et al. 2011. Both MLE and MLPE inhibited the oxidation and lipid peroxidation of LDL, while MLPE was shown to be more potent.

5. Clinical studies: OxLDL and antioxidants

A number of studies have demonstrated an association of circulating OxLDL with atherosclerosis disease (Itabe & Ueda, 2007; Hulthe & Fagerberg, 2002). The size of LDL particles might have an effect on LDL oxidation. Smaller LDL was associated with higher level of OxLDL. However the association was observed in diabetic subjects, but not in non-diabetic subjects (Scheffer et al., 2003).

OxLDL level normalized to LDL or ApoB protein levels was increased in diabetic subject with macrovascular diseases compared to diabetic subjects without such diseases. Increased OxLDL normalized level was associated with TT genotype of 108C/T polymorphism in PON1 promoter with lower level of expression of the gene (Tsuzura et al., 2004; Brinkley et al., 2009) have demonstrated for the first time that plasma OxLDL levels are related to arterial stiffness in elderly men and women; suggesting that the oxidative modification of LDL may be associated with changes in the elastic properties of blood vessels. Their findings suggest that

while antioxidant supplementation trials have been found to be largely ineffective in preventing cardiovascular outcomes, other interventions including aerobic exercise training and pharmacological treatment with lipid and blood pressure-lowering medications may have significant antioxidant effects that are related to reductions in CVD risk. Another study have shown that oxidized lipoprotein(a) is significantly correlated with blood glucose level among healthy young women, suggesting that lipoprotein(a) may be oxidized with increased glucose concentration even within the normal glucose level (Kotani et al., 2010).

There is some controversy on the role of antioxidants on development of atherosclerosis. A number of clinical studies have demonstrated an anti-atherosclerotic effect of antioxidants while a group of other studies do not see any appreciable benefit of the use of antioxidants. The following are examples of these studies that have suggested an inhibiting effect of antioxidants on lesion development. Gey & Puska (1989) have reported that vitamin E and A concentrations in the plasma were inversely proportional to cardiovascular risks. A study of 667 cases of atherosclerosis-induced coronary disease developed in originally healthy (not diagnosed with coronary heart disease, diabetes, or hypercholesterolemia) 39,910 US men have shown a protective effect of vitamin E but not vitamin C. Carotene appeared to be protective in non-smoking men, however increased the risk of coronary disease among smokers (Rimm et al., 1993). A protective effect of vitamin E was observed in similar study of 87,245 women developed 552 cases of major coronary disease in eight years (Stampher et al., 1993).

However a large the Heart Outcomes Prevention Evaluation (HOPE) study did not show any anti-atherogenic effect of vitamin E (Yusuf et al., 2000). Subjects who were taking vitamin E and placebo developed atherosclerosis-related diseases such as myocardial infarction, stroke, unstable angina, congestive heart failure at the same rate. Potential explanation for the failure of antioxidants in clinical studies may include the type of dose, duration, time of introduction, i.e. stages of the disease at which the treatment/supplementation were introduced and the selection of an optimal doses of antioxidants. Also, most of the studies did not measure the oxidative stress markers in the plasma to take it into account (Parthasarathy et al., 2001).

Research has provided strong evidence that LDL oxidation plays an important role in the pathogenesis of atherosclerosis and cardiovascular diseases. The involvement of lipid peroxidation in the propagation of the disease is well supported by clinical and scientific research using cell culture and animal models; these studies clearly point that modification of the LDL and the accompanied oxidative damage trigger an inflammation response that mediate the development of the atherosclerosis. One may assume that antioxidants should inhibit the oxidative damage and slow the inflammation processes that lead to CVD and associated with metabolic disorders. However despite of some positive findings, antioxidant compounds did not consistently prove to be potent protective agents against atherosclerosis. In animal atherosclerosis, which is studied in the short term, the emphasis is on establishing the lesions. Thus, antioxidants, such as α-tocopherol, might affect predominantly the initial formation and progression of the lesion. In humans, particularly in those who already have clinically significant events, the early steps might have already occurred. In such cases, α-tocopherol and similar antioxidants could affect the conversion of aldehydes into carboxylic

acids. The latter, are presumed to be nonatherogenic and are easily degraded *via* fatty-acid degradation pathways (Raghavamenon et al., 2009). Based on these arguments it may be necessary for the scientific community to revisit the topic and investigate in well structured studies the type, dose, duration of the anitoxidants on a well defined population of subjects with various stages of CVD and its associated metabolic disorders such as diabetes, obesity and hyperlipidemia.

Anti-oxidants	Mechanism	References
β-carotene	Scavenging ROS and excellent trapper of singlet oxygen, acts against LDL oxidation	Honarbakshsh & Schachter, 2009. Princen et al., 1992
Vitamin C	Scavenging ROS, reactivating other anti-oxidants such as Vitamin E; inhibit formation of OxLDL, Il-1β secretion or chemokines and monocyte – endothelial cell adhesion and β-carotene which are anti-atherogenic	Dunstan et al., 2007 Honarbakshsh & Schachter, 2009. Jialal & Grundy, 1991. Kagan et al, 1992. Rössig et al, 2001. Gokce et al., 1999, Rayment et al., 2003. Heller et al., 1999
Vitamin E	Scavenging ROS, reported to inhibit formation of OxLDL, Il-1β secretion or chemokines and monocyte –endothelial cell adhesion	Dunstan et al., 2007 Honarbakshsh & Schachter, 2009. Princen et al., 1992. Islam et al., 1998. Devraj et al., 1996. Faruqi et al, 1994. Zapolska-Downar et al., 2000
Selenium	Cofactor for glutathione peroxidase. Has antioxidant capacity.	Michiels et al., 1994
Zinc	Cofactor for superoxide dismutase. Protects cells from oxidative damage.	Michiels et.al., 1994
Curcumin	Chelating of iron and copper ions, scavenging of ROS, inhibiting lipid peroxidation Protects anti-oxidant enzymes.	Wongcharoen & Phrommintikul, 2009
Quercetin	Scavenging of metals ions, and inhibition ROS. Activation of NF-κB, which is involved in development of atherosclerosis.	Cho et al., 2003
Resevetrol	Inhibits ROS production and lipid peroxidation	Ramprasath & Jones, 2010
Ergothioneine	Protects endothelial cells from oxidative damage by reactive nitrogen species.	Martin, 2010

The table describes the currently investigated antioxidants and their relation to markers of CVD.

Table 2. Role of Antioxidants in Cardiovascular Disease

Clinical study	Findings	No of patients	References
Department of Internal Medicine, Kochi Medical School, Kochi, Japan.	OxLDL increased in subjects with PON1 genotype that lead to decreased expression of PON1 protein	155	Tsuzura et al., 2004
AIR study	OxLDL role in atherosclerosis and inflammation	391	Hulthe & Fagerberg, 2002
CARDIA study	OxLDL indication metabolic syndrome and in abdominal obesity, hyperglycemia and hypertriglyceridemia	1889	Holvoet et al., 2008
Metabolic Laboratory, Department of Clinical Chemistry study, Netherlands	Smaller LDL are associated with higher level of OxLDL	116	Scheffer et al., 2003
HOPE Study	No effect of vitamin E on development of CVD	9541	Yusuf et al., 2000

Table summarizes some clinical studies measured OxLDL in plasma

Table 3. Clinical studies on OxLDL

6. Conclusions and perspectives

The low density lipoprotein oxidation hypothesis is pivotal to the explanation of the formation of fatty streak lesions. A wide range of atherogenic processes has been reported to be influenced by OxLDL and its components. The presence of OxLDL in lesions and plasma of patients with various forms of coronary artery diseases and other related metabolic disorder confirms the role of oxidized lipids in atherosclerosis. This conclusion led to numerous studies on the role of antioxidants in the prevention or treatment of atherosclerosis. However they did not yield uniformed outcome on the role of antioxidants in suppressing of the atherosclerotic process. Possible reasons might include discrepancies in experimental models, study designs, and schemes of treatment. Results shown in cell culture or animal models do not necessarily translate to similar results in human due to the major difference between the atherosclerosis development and stages in the animal models and human. Another factor that has not been tested yet is a possible inhibition of oxidation of OxLDL-released aldehydes by antioxidants. If oxidation of aldehydes is inhibited, they modify proteins and cause wide spectra of biological effects that exaggerate atherosclerotic processes. The future studies on the role of antioxidants in atherosclerosis should take in consideration these factors.

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9

Oxidatively Modified Biomolecules: An Early Biomarker for Acute Coronary Artery Disease

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1. Introduction

Cardiovascular disease is the worldwide major cause of mortality and morbidity. The 2009 annual report from World Health Organization **(**WHO**)** highlighted the mortality rate prediction of the population worldwide that, in 2030, cardiovascular disease will become the major cause of deaths, and the mortality rate will higher than other infectious diseases such as HIV, Tuberculosis, malaria infection (World Heatlh Organization ,2009). Moreover, this report also mentioned that, among cardiovascular diseases, ischemic heart disease and cerebrovascular disease, which were reported as top 2 cause of mortality in 2004, are expected to still be the major cause of death in next 20 years (World Heatlh Organization ,2009). Coronary artery disease is a sequence of pathophysiologic processes in coronary arteries, myocardial ischemia and infarction (Wudkowska et al.2010). Therefore, the early diagnostic of myocardial ischemia and infarction, will lead to the rapid and more effective of medical intervention, and safe the patients' life. The standard diagnosis of coronary artery disease focuses on clinical assessment such as history of chest pain associated with electrocardiogram (ECG) changes, and elevation of cardiac specific-biochemical markers (Maneewong K. et al.2011).

Determination of serum or plasma level of cardiac specific-biochemical markers is one of the most essential and effective way for diagnosing myocardial ischemia/infarction. The ideal cardiac markers should have high specificity, high sensitivity, rapidly released after the onset of the symptoms, abundant in cardiac tissue but less in other tissues, long half life in blood circulation, and capable of representing the prognosis and estimating the infarct size.

It has been known that coronary artery disease, especially myocardial ischemia and ischemia-reperfusion injury is the phenomenon that related to an oxidative stress (Buja2005), which is an imbalance and inadequate production of reactive oxygen species (ROS), subsequently resulted in biochemical modifications of major principle biomolecules such as proteins, lipids, and nucleic acids (Valko et al.2007;Sbarouni et al.2008a;Sbarouni et al.2008b;Sbarouni et al.2008c;le-Donne et al.2003a;le-Donne et al.2003c). Some oxidatively modified biomolecules such as Ischemia Modified Albumin or IMA, has been approved by US Food and drug Administration (FDA) and used as a rule-out marker for acute myocardial ischemia (Apple et al.2005;Bar-Or et al.2000;Sbarouni et al.2008c;Sbarouni et

al.2008a;Sbarouni et al.2008b;Van et al.2010). In addition, many of oxidatively modified biomolecules have been reported to correlate with the severity of coronary artery disease and possibly used as a marker for myocardial ischemia (Apple et al.2005;Bar-Or et al.2001c; Bar-Or et al.2000;le-Donne et al.2003b;Berlett & Stadtman1997;Kiyici et al.2010;Turedi et al.2010; Melanson & Tanasijevic2005;Van et al.2010;Shen et al.2010;Pantazopoulos et al.2009; Bhagavan et al.2003;Santalo et al.2003;Sinha et al.2003;Mutlu-Turkoglu et al.2005;Mocatta et al.2007; Beal2002;Docherty2010;Wudkowska et al.2010;Charpentier et al.2010;Maneewong K. et al.2011; Sbarouni et al.2008a; Sbarouni et al.2008b;Sbarouni et al.2008c;le-Donne et al.2003a; le-Donne et al.2003d).

In this chapter, studies of oxidatively modified biomolecules such as proteins, lipids, and nucleic acids, related to coronary artery diseases will be discussed. Moreover, clinical usefulness of determining these oxidatively modified biomolecules as a biomarker for coronary artery disease will also be addressed.

2. Oxidative stress

The term oxidative stress has been commonly mentioned or explained the underline pathophysiological mechanism of some diseases during the last thirty years (Hensley et al.2000). Oxidative stress is referred to an inadequate of free radicals generation and/or insufficient removal of the radicals by antioxidants, radical scavengers. Free radicals can also be defined as atoms or molecules containing one or more unpaired electrons on an open shell configuration (Lushchak2011), which generate the highly reactivity properties of the molecules. There are 2 major types of free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS).

2.1 Reactive Oxygen Species

Reactive oxygen species (ROS) are generated from oxygen metabolism include superoxide anion ($O_2^{\bullet -}$), peroxyl ($RO^{\bullet}_2$), hydroperoxyl ($HRO_2^{\bullet -}$), and hydroxyl radical ($^{\bullet}OH$). In addition, ROS can also be non-radical species such as hydrogen peroxide (H_2O_2) and hydrochlorous acid (HOCl). ROS can be generated from regular metabolic processes or from external sources such as X-ray exposure, air pollutants, cigarette smoking, and etc. The primary source of intracellular free radicals generated by the addition of one oxygen electron, which is resulted in superoxide ($O_2^{\bullet -}$) (equation 1).

$$O2 + e^- \longrightarrow O_2^{\bullet -} \quad (1)$$

Intracellular mechanism to balance the generation of superoxide is achieved by specific enzyme called superoxide dismutase (SOD), which catalyze the changing of superoxide to oxygen and hydrogen peroxide (H_2O_2) (equation 2).

$$2\, O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2 \quad (2)$$

This hydrogen peroxide (H_2O_2) that is generated from equation 2 has a property of being an oxidizing agent and serve as a major source of $^{\bullet}OH$, which is one of the very harmful reactive oxygen species to the cell. According to hydrogen peroxide is non-radical and weak

polar, it can penetrate through the lipid bilayer of cell membrane or mitochondrial membrane, and destroy some biological molecules such as proteins, lipids, and nucleic acids. Cellular balancing mechanism for H_2O_2 is the enzyme catalase, which convert two molecules of H_2O_2 to oxygen and water (equation 3)

$$2\,H_2O_2 \xrightarrow{\text{(Catalase)}} 2H_2O + O_2 \quad (3)$$

Another alternative mechanism generating ROS in the cell is the Haber-Weiss reaction, which is a chemical catalysis of superoxide and hydrogen peroxide by ferric ion (Fe^{3+}) to generate hydroxyl radical ($^{\bullet}OH$) (equation 4)

$$O_2^{\bullet -} + H_2O_2 + O_2 \xrightarrow{Fe^{3+}/CU^{2+}} OH^- + {}^{\bullet}OH \quad (4)$$

In addition, superoxide can reduce ferric ion to form ferrous ion (equation 5), the reaction called "Fenton reaction". The production of ferrous ion in first Fenton reaction can react with H_2O_2 in the second reaction and result in OH^- and $^{\bullet}OH$ generation (equation 6).

$$Fe^{3+} + O_2^{\bullet -} \longrightarrow Fe^{2+} + O_2 \quad (5)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \quad (6)$$

2.2 Reactive Nitrogen Species

Reactive nitrogen species (RNS) are generated from the reaction of nitric oxide (NO), which is enzymatically generated by nitric oxide synthetase (NOS). The NOS oxidized the amino acid L-arginine or L-citrulline. The member of RNS include nitric oxide (NO) and nitrogen dioxide ($NO^{\bullet}_2$), as well as non radicals nitrogen species e.g. peroxynitrite ($ONOO^-$), nitrousoxide (HNO_2), and alkyl peroxynitrates (RONOO). Among these RNS molecules, $^{\bullet}NO$, and $ONOO^-$ are the most investigated species, which have significant impact in cardiovascular complication (Kumar et al.2010).

2.3 Antioxidants

Antioxidants are either endogenous or exogenous compounds that prevent the generation of harmful free radicals, reduce the generated radicals, inactivate their harmful reactivity, and thereby block the chain reactions of these oxidants. The primary or chain breaking antioxidants so called "scavenger" which is neutralize the free radicals by donating one of their own electrons (Kumar et al.2010). The secondary or preventative antioxidants work by sequestration of transition metal ions or removal the peroxides by catalase and glutathione peroxidase. The tertiary antioxidants defense is the repairing of damaged molecules, in attempt to avoid the accumulative damages (Kumar et al.2010).

3. The oxidative modification of biomolecules

Reactive oxygen species readily attack a variety of important biomolecules, including carbohydrates, proteins, lipids, and nucleic acids. Interaction between ROS and these

biomolecules resulted in biochemical modifications, which alter the functions as well as the properties of these biomolecules (Figure 1).

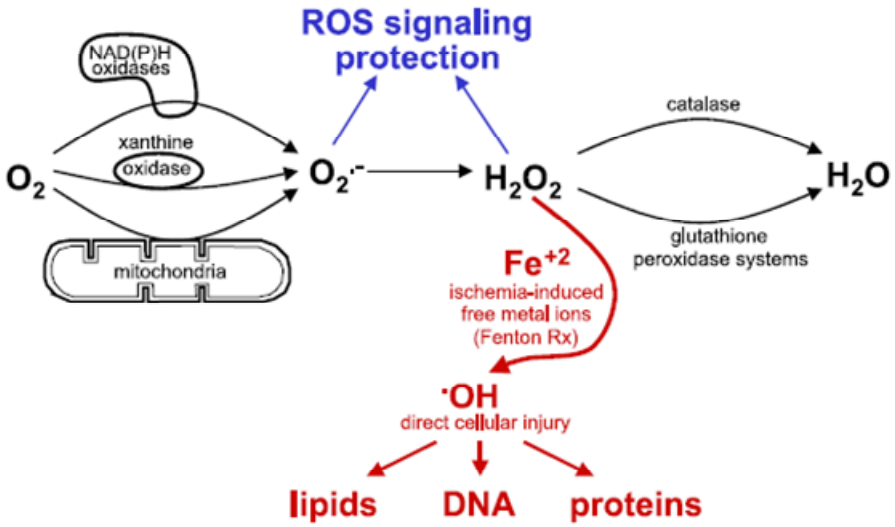

Fig. 1. Oxidative modification of biomolecules (Becker2004). In normal physiological conditions, superoxide and hydrogen peroxide are generated. Generation of intracellular ROS may activate the intracellular signaling pathways for cardiac protection, for example in ischemic preconditioning. The generated hydrogen peroxide, especially in myocardial ischemia, reacts with ferrous ion in Fenton reaction, which results in hydroxyl radicals. Over production of the hydroxyl radical causes oxidative modification of biomolecules, such as lipids, proteins, and nucleic acids.

3.1 Proteins

It has become manifested that proteins are also concerned as a target of free radical destruction. The mechanism involved in the oxidation modification of proteins is thought to occur at the monomeric level of amino acids, especially cysteine, tyrosine, phenylalanine, tryptophan, histidine, and methionine. The process of proteins oxidation creates new functional groups such as hydroxyl groups and carbonyl groups. These added up new functional groups can be generated by different mechanisms and can also indicate the degree of oxidative modification. The outcomes of the oxidative modification of proteins cause proteins fragmentation, cross-linking and unfolding, which may activate or hinder proteolytic and proteasome-mediated turnover. The biomarkers of oxidatively modified proteins include protein carbonyl, ischemia-modified albumin, and etc. Ischemia modified albumin (IMA) and protein carbonyl (PC) are oxidatively modified proteins found in many oxidative related disorders such as myocardial ischemia, renal ischemia, (Apple et al.2005;Pantke et al.1999;Bar-Or et al.2000;Kiyici et al.2010;Turedi et al.2010;Melanson & Tanasijevic2005). According to this, many studies suggested the ability of these two oxidatively modified proteins as the early biomarkers for diagnosis of coronary artery disease.

3.2 Lipids

Lipids are the basic biomolecules found throughout the cells, such as phospholipids component of the cell membrane. Therefore, lipids can be one of an oxidative modification targets, similar to proteins. Oxidative modification of lipids are the chain reactions, lead to the degradation of lipids or so-called "lipid peroxidation", which mediated by free radicals abstract electrons from lipid molecules such as aldehyde group e.g. Malonaldehyde (MDA). Polyunsaturated fatty acids are more sensitive to the lipid peroxidation according to these lipids contain multiple double bonds in between methylene- CH_2- groups that easily react with reactive hydrogen atoms (Lu et al.2010). The reactions of oxidative modified lipids consist of three major steps including; the initiation step, where is a fatty acid radicals are produced. The propagation is the direct reaction with oxygen molecules produced peroxyl fatty acid radials that react with another free fatty acid producing a different fatty acid radical and lipid peroxide or a cyclic peroxide and termination. The destruction of lipid molecules by lipid peroxidation can cause membrane permeability alteration, loss in fluidity, decreasing in electrical resistance, change in phospholipids bilayer membrane disruption, membrane-bound enzyme malfunction and loose of integrity ionic gradient, disruption or activation of enzyme function, and cellular injury (Ayres1984). Biomarkers of lipid peroxidation include malonaldehyde, F2-Isoprostanes, and etc.

3.3 Nucleic acid

Nucleic acid is one of the basic biomolecules that play many essential roles in the cell. The nucleic acids-DNA and RNA- are the principal informational molecules of the living cells. During aging processes, free radicals such as $^{\bullet}OH$ can be generated and can bind to DNA molecules. Association and reaction of free radical to DNA can lead to DNA bases damaging, both purines and pyrimidines, and result in DNA strand break (Valavanidis et al.2009). Alteration of purines and pyrimidines play a significant role in large variety of pathological stages such as cancer (Kasai1997). Biomarkers of oxidative modified nucleic acid include 8-hydroxy-2′-deoxyguanosine, 8-nitroguanin, and etc.

4. Oxidatively modified biomolecules as cardiac markers for coronary artery disease

4.1 Ischemia modified albumin

Generation of reactive oxygen species resulted in the modification of proteins, which introduce new functional groups such as hydroxyl groups and carbonyl groups (le-Donne et al.2003b;Berlett & Stadtman1997). Among these proteins, ischemia-modified albumin (IMA) was reported as an early biomarker in many pathological disorders (Kiyici et al.2010;Montagnana et al.2006;Abboud et al.2007;Gunduz et al.2009;Sharma et al.2007).

Ischemia Modified Albumin (IMA) is serum albumin that modified at the N-terminal portion, especially at aspartate-alanine-histidine-lysine sequences, by oxidative stress generated during ischemia (Bar-Or et al.2001b). This modification reduced the ability of albumin to bind with metal ions such as cobalt, copper, and nickel (Bar-Or et al.2001a) (Figure 2). The ischemic mechanism initiated with the insufficiency of oxygen supply during ischemia, which caused cardiomyocytes cellular anaerobic metabolism. Within a few seconds after occlusion of a major coronary artery tissue oxygen content decreases and

mitochondrial oxidative metabolism becomes inhibited. At this point, a compensatory increase in anaerobic glycolysis for ATP production leads to accumulation of hydrogen ions and lactate, resulting in intracellular acidosis and inhibition of glycolysis (Reimer & Ideker1987). An aerobic glycolysis cannot provide sufficient ATP to meet the demand of myocardium. The depletion of ATP also causes the interruption of cellular ion-pumps and calcium influx to the cells. The excess intracellular calcium activates calcium-dependent proteases such as calpain, calmodulin, generates $O_2^{\bullet-}$ and converts to H_2O_2. Blood consist of transition metals such as copper and iron, which can interact with $O_2^{\bullet-}$ and H_2O_2 and form the strong oxidant $^{\bullet}OH$, which lead to cellular destruction. Proteins, predominantly albumin, are damaged by free radicals especially at amino terminus (N-terminus), resulting in the albumin N-terminal derivatives. Human serum albumin, a major protein in circulation, consists of 585 amino acid residues with half life in circulation approximately 19 days. The metal binding properties of albumin depend on the three dimensional structure binding sites, which are distributed over the molecule (Bar-Or et al.2001b;Takahashi et al.1987). The modification of albumin during ischemia is independent on cell death, and can be an early biomarker for such an early stage of ischemia.

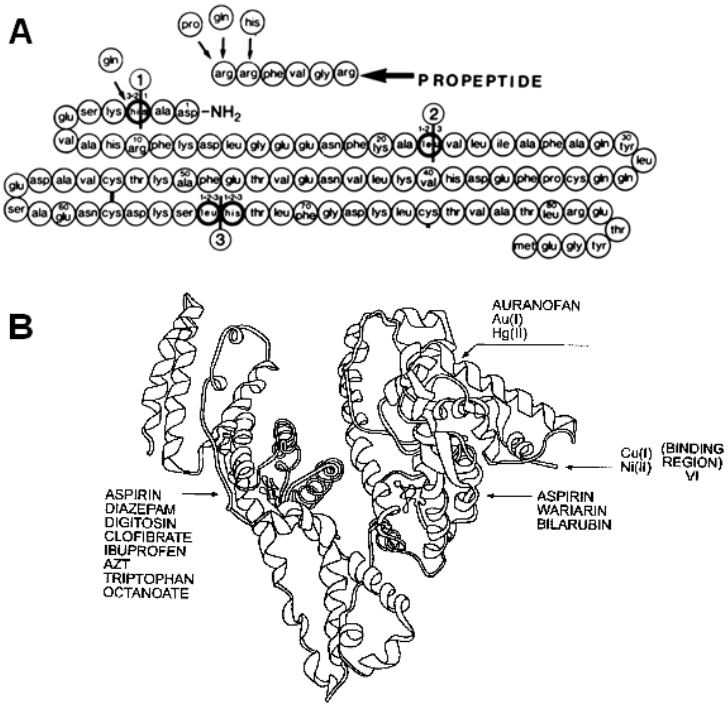

Fig. 2. The amino acid sequences (A) and molecular structure of albumin (B). This figure is modified from figure3 of Takahashi et al. (Takahashi et al.1987). Proteins albumin is oxidatively modified at NH_2 terminal of albumin.

Determination of serum or plasma IMA can be performed by Albumin Cobalt Binding (ACB) method. In 2003, US Food and drug Administration (FDA) approved that ACB

method is the diagnostic test for acute myocardial infarction (Van et al.2010). The principle of ACB method is based on the principle of free radicals, which is generated during ischemia, alters the metal ions binding capacity of serum albumin (Sbarouni et al.2008a) (Figure 3). The test is currently called IMA test instead of the ACB test. It has been reported that IMA test could be used for screening the patients with chest pain, who suspected AMI, at the emergency department, excluded patients from the other causes of chest pain (Bar-Or et al.2000). Many studies show that IMA levels increased in patients with acute coronary syndrome and also elevated in myocardial ischemia (Sbarouni et al.2008a). The analytical sensitivity of the test is 13 u/ml, with 98% recovery (Sbarouni et al.2008a). Moreover, there were reports mentioned of non-interfering effect from bilirubin, hemoglobin, cholesterol, total proteins, and number of cardiac drugs (Govender et al.2008). It was also reported that no biological variation of IMA regarding race and gender (Govender et al.2008).

According to the ability to detect IMA as a result of ischemia, the event of reduce oxygen supply prior to cardiomyocytes necrosis, make IMA test good enough to be an earlier cardiac marker (Sbarouni et al.2008a). It was reported that the serum IMA level increased and can be detected within 6-10 minutes after ischemia and returned back to baseline within 6 hours (Bhagavan et al.2003). This could be an advantage of IMA, in comparison to the other conventional cardiac markers such as cardiac troponin and CK-MB, which are necrotic marker and could not be detected until 4-6 hours after onset of chest pain/ischemia (Wu et al.1999). Determination of IMA has been used triage patient who suspected from cardiac ischemia. Low level of serum IMA, would estimate low risk for a cardiac ischemic event and make a rapid consideration to exclude or discharge patient. Low level of serum IMA perhaps indirectly predicts the low level of cardiac troponin (Sbarouni et al.2008a). So, it can make a clear distinct when the negative results from IMA, troponin, and ECG can exclude the patients from ACS (Bhagavan et al.2003). The assay method can be easily and rapidly performed by spectrophotometric method. It has been shown that IMA test has high method sensitivity more than troponin as it gave positive results in 84% of patient who suspect ACS while cardiac troponin could detect only 42% (Takhshid et al.2010). Combination with triple tests including ECG, IMA, and cardiac troponin could increase the negative predictive value for ACS to 96% (Takhshid et al.2010). Furthermore, IMA test might reduce in the number of diagnostic tests such as determination of serum high sensitivity C-reactive proteins (hsCRP), NT-proBNP elevation, and cTnT release (Kazanis et al.2009), invasive imaging, which have high cost (Keating et al.2006).

However, it seems like IMA test lack of specificity. High serum IMA level can also be detected in other diseases such as cancer, acute infections, end renal disease, and liver cirrhosis, (Kazanis et al.2009). Therefore, the negative results from cTnT test and IMA allow more confident to exclude the patients, who suspected AMI. However, a positive IMA alone still need further investigation. Moreover, it has been reported that serum IMA level can also be elevated in plasma from healthy subjects, 24-48 hours after exercise (Kim et al.2008). Therefore, utilization of IMA test as cardiac marker for coronary artery disease need to be further investigated to ensure the value of the test.

4.2 Proteins carbonyl

The carbonyl (CO) groups in proteins compose of aldehyde and ketone groups. Proteins Carbonyl groups (PC) is a product of oxidative modification on amino acid residues,

especially proline, arginine, lysine, and threonine from free radicals reactions, forming protein carbonyl groups [53]. In addition, protein carbonyl groups can be generated from an indirect mechanism of the hydroxyl radical-mediated oxidation of lipids (Figure 4). The product of lipid peroxidation, which will be described latter in this chapter, can diffuse across cell membrane, allowing the reactive aldehyde-containing lipids, which will covalently modified proteins in the cell (Grimsrud et al.2008). Proteins oxidation changes proteins functions by changing in pattern of proteins folding, which is important for their activity, decrease catalytic activity of enzyme, and finally breakdown of proteins by proteases (Almroth et al.2009). The cleavage of proteins may occur by either the amidation pathway or by oxidation of glutamyl side chain. Redox cycling cation such as Fe^{2+} or Cu^{2+} can bind to cation binding location on proteins. Free radical attack by H_2O_2 or $O_2^{\bullet -}$ can transform side chain of amine groups into carbonyls.

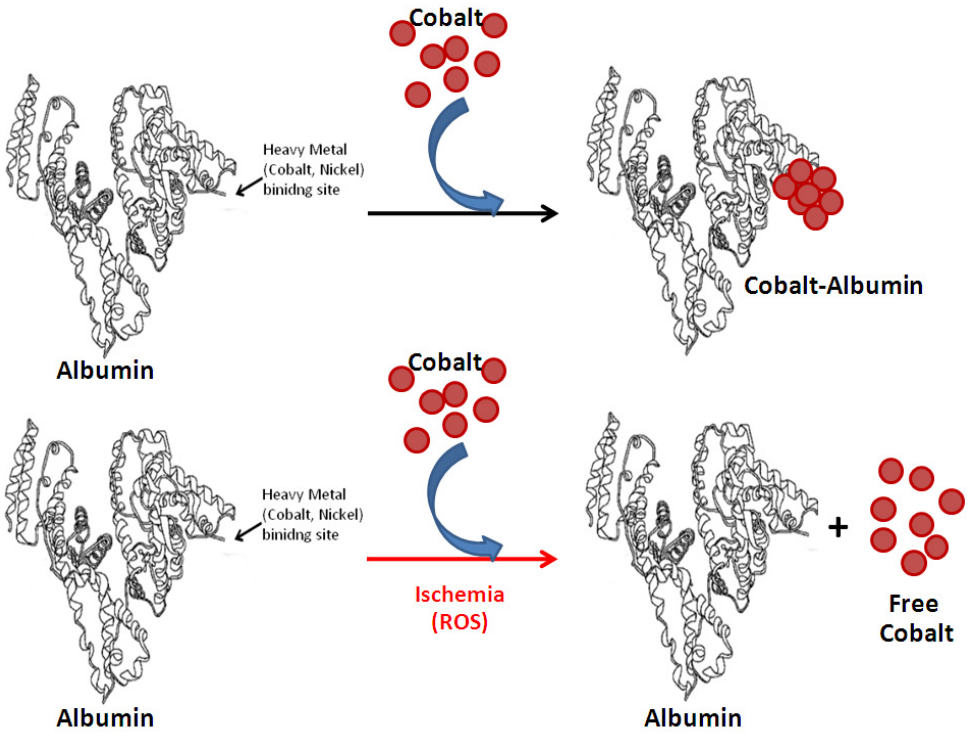

Fig. 3. Principle of Albumin cobalt binding (ACB) assay. In physiological conditions, albumin capable of binding with metal ions such as cobalt, copper, and nickel, at amino terminal end of the protein. During ischemia, N-terminal portion of albumin, especially at aspartate-alanine-histidine-lysine sequences, is modified and result in the reduction in albumin-metal ions binding ability.

Laboratory measurement of PC can be performed by variety of methods, for example, spectrophotometric assay, HPLC, ELISA, and immnunoblotting (le-Donne et al.2003a;le-

Donne et al.2003b;le-Donne et al.2006). Spectrophotometric assay for PC can be perform by 2,4-dinitrophenylhydrazine (DNPH spectrophotometric method), which based on the formation of a stable dinitrophenyl (DNP) hydrazone reacts in acidic pH solution. Spectrophotometric DNPH assay showed high sensitivity detection of carbonyl content level in purified proteins (le-Donne et al.2003b;Levine et al.1994). This method does not require any expensive or specialized equipments. It has been shown that the serum level of PC increased rapidly in blood stream and still remained at least 24 hours (Mutlu-Turkoglu et al.2005). Mutlu-Turkoglu *et al.* demonstrated that serum PC level increased in coronary artery disease, atherosclerotic lesion in human, and during ischemia-reperfusion (Mutlu-Turkoglu et al.2005). As it has also been reported that PC can be generated following the onset of myocardial infarction (Paton et al.2010) suggested the diagnostic value of PC and may be used as biomarker for coronary artery disease. The stability of this assay remained in hours and days, whereas lipid oxidation products can be removed within minutes (Mutlu-Turkoglu et al.2005). In AMI, serum PC level was significantly increased when compared with normal control (Paton et al.2010). Diagnosed value of PC in human can also be used in environmental studies, monitoring in subjects who exposed to the bunker oil (Almroth et al.2009). Although PC has been proven as a sensitive marker, but it was shown to has less specificity, similar to IMA. The increasing in serum level of PC can be detected in other human diseases such as Alzheimer' disease, cataract genesis, chronic hepatitis, diabetics, cigarette smoker, and after doing exercise (Mutlu-Turkoglu et al.2005). According to the time consuming and proteins precipitation is required in spectrophotometric method, this technique is inappropriate to determine the PC level in large number of clinical samples. Therefore, other techniques, for example ELISA, were developed. Recently, the findings from our study demonstrated that PC could be an early marker for myocardial ischemia. Serum PC level in non-ST elevation myocardial infarction (NSTEMI) was significantly higher than that in ST elevation myocardial infarction (STEMI) and healthy controls, suggesting that PC is an early marker. Moreover, combinatorial determination of PC with IMA helps to improve the diagnostic power of these two markers (Maneewong et al.2011).

4.3 8-hydroxy-2'-deoxyguanosine

As mentioned in the previous section, free radicals can attack DNA and cause molecular structural alterations of DNA and result in DNA strand break. The interaction of $^{\bullet}OH$ with the nucleobases of DNA, such as guanine, form the C8-hydroxyguanine (8-OHGua) or its nucleoside form deoxyguanosine (8-hydroxy-2'-deoxyguanine) or so called 8-OH-dG. The 8-OH-dG can be further oxidized and produced 8-oxo-7,8-dihydro-2'-deoxyguanine or 8-oxodG (Valavanidis et al.2009) (Figure 4). Although the other nucleic acids in DNA molecules can react with $^{\bullet}OH$ in the same manner, the 8-oxodG is the major form of oxidative modified nucleic acids in DNA, and known as a potential biomarker of carcinogenesis (Kasai1997). These days, the 8-OHdG can be a biomarker of oxidative stress, aging and cancer. This molecule can be measured and analyzed using high sensitivity by high performance liquid chromatography (HPLC), gas-chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry- mass spectrometry (LC-MS-MS), immunohistochemical methods, and single cell electrophoresis (Griffiths et al.2002;Halliwell & Whiteman2004;Collins et al.2004). There are many reports that the elevation of 8-OHdG related to some pathological disorders, for example, urinary 8-OHdG has been established as a marker to evaluate oxidative stress in carcinogenic exposure, environment pollutants

and cigarette smoking (Kiyosawa et al.1990;Asami et al.1996). Elevation of 8-OHdG has been found in the plasma and myocardium of the patients with heart failure (Kono et al.2006). Recently, Himmetoglu *et al*. reported that the plasma level of 8-OHdG increased in patients with myocardial infarction and the level of this molecule decreased after reperfusion therapy in patients with MI, suggested that 8-OHdG could possibly be biomarker for monitoring or determining the prognosis of the patients (Himmetoglu et al.2009). In addition, Nagayoshi *et al* demonstrated that the urinary levels of 8-OHdG were significantly higher in cardiac patients when assessed the serial alteration of oxidative stress of patients with AMI (Nagayoshi et al.2005).

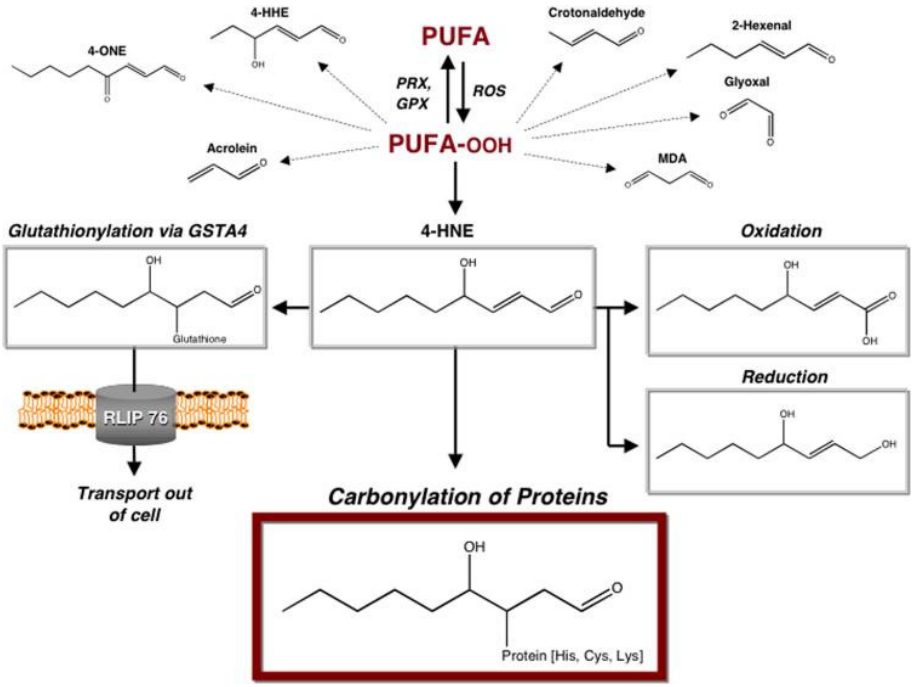

Fig. 4. Mechanism of ROS stimulates lipids peroxidation induced proteins carbonylation (Grimsrud et al.2008). Proteins carbonylation can be induced by the oxidative modification of polyunsaturated fatty acids (PUFA), which then undergo to lipid peroxidation reaction generating products such as α,β-unsaturated aldehyde 4-HNE. These molecules act as electrophiles in the covalently modification of proteins via non-enzymatic addition reactions.

It is known that reactive nitrogen species or RNS such as nitric oxide (NO) and peroxynitrite ($ONOO^-$) can modify the molecular structure of DNA (Ohshima et al.2006). The 8-nitroguanine is the example of nucleic acid in DNA, which can be oxidatively modified by RNS. There is overwhelm data showed that 8-nitroguanine is undetectable in normal tissues, which indicating that this molecule may be a candidate as a biomarker for DNA damage induced by RNS (Akaike et al.2003;Ma et al.2004;Horiike et al.2005;Pinlaor et al.2004). Several techniques have been developed for determining 8-nitroguanine in clinical

samples, for example, HPLC with electrochemical detection, HPLC with a UV detector, GC-MS, and immunohistochemistry (Halliwell & Whiteman2004;Ohshima et al.2006;Sawa et al.2006). Many studies showed that 8-nitroguanine increased in inflammation, carcinogenesis, and cigarette smoke (Hiraku2010). However, there are no any evidence of the increasing in 8-nitoguanine in coronary heart diseases. Therefore, further investigation of 8-nitoguanine in coronary heart diseases is still need to be further investigated.

4.4 MDA

One of the most frequently used biomarkers indicating lipid peroxidation is plasma concentration of malondialdehyde (MDA). This molecule is one of the end products of lipid peroxidation in the cell membrane or in low-density lipoproteins (LDL) (Ogino & Wang2007). Quantification of plasma MDA level can be performed by thiobarbituric acid (TBA) test (Nielsen et al.1997). TBA-reactive substances (TBARS) formed in plasma, urine, or tissue samples that need to be calibrated by sample pretreatment procedure, which forms a red adduct with 2 molecules of TBA (MDA-TBA2). The adducted compounds are separated by an HPLC method, which originally described by Wong *et. al.*(Wong et al.1987) and Carbonneau *et. al*. (Carbonneau et al.1991). The GC-MS method has been used to analyze the plasma MDA as well (Yeo et al.1994). It has been reported that the plasma from patients with coronary artery disease also had higher level of MDA than the healthy subjects, suggested that MDA could be one of the candidate biomarkers for coronary artery disease (Rajesh et al.2011;Rao & Kiran2011;Mogadam et al.2008;Pasupathi et al.2009). A recent report also suggested that serum levels of TBARS, which was determined by reverse-phase HPLC and spectrophotometric method, were a good predictive marker in patients with stable coronary artery disease (Walter et al.2004).

4.5 F_2-Isoprostanes

Isoprostanes are a complex family of compounds generated from arachidonic acid via a free radical's catalyzed mechanism. This compound was firstly discovered in 1990 by Morrow *et. al.* who discovered prostaglandin-F_2-like compounds, and termed this newly discovered compound as ***F_2-isoprostanes*** (Morrow et al.1990). The F_2 -isoprostanes can be generated by the oxidative induced peroxidation of arachidonic acid (Figure 5).

Determination of F_2-isoprostanes is similar to other techniques measuring the products from lipid peroxidation including GC-MS, which might be associated with an immunoaffinity extraction, GC-tandem MS, and LC-tandem MS (Halliwell2000). Although these techniques have high specificity, the budget cost of these techniques is the impediment of their routine use (Milne et al.2005). Determination of 15- F_{2t}-IsoP in urine samples, by radioimmunoassay, has been validated and easier alternative to GC-MS. In addition, the new technique is developed, for example enzyme-immunoassay for detecting F_2-isoprostanes (Milne et al.2005).

F_2-isoprostanes can be measured in varieties of clinical samples, for example urine, plasma, bronchoalveolar lavage fluid, bile, cerebrospinal, seminal and pericardial fluids (Iuliano et al.2001;Lindsay et al.1999;Delanty et al.1997;Cipollone et al.2000;Reilly et al.1997). In addition, F_2-isoprostanes can be detected in normal tissues, including umbilical cords (Chu et al.2003). The level of F_2-isoprostanes increased in cigarette smoking, similar to other oxidative modified molecules, which is known that the increasing in smoking can cause the

oxidative stress (Reilly et al.1996;Morrow et al.1995). The measurement of isoprostanes in biological fluids has prompted clinical investigations on the pathophysiological role of lipid peroxidation in cardiovascular diseases. In coronary artery disease, the quantified isoprostanes was mostly in 15- F_{2t}-IsoP and 5- F_{2t}-IsoP, which can be measured in urine samples (Haschke et al.2007). The urinary level of 15- F_{2t}-IsoP and 5- F_{2t}-IsoP was found to increase in, unstable angina, reperfusion following myocardial infarction and cardiopulmonary bypass, coronary angioplasty (Sakamoto et al.2002). These findings suggested that isoprostane could be biomarker for coronary artery disease.

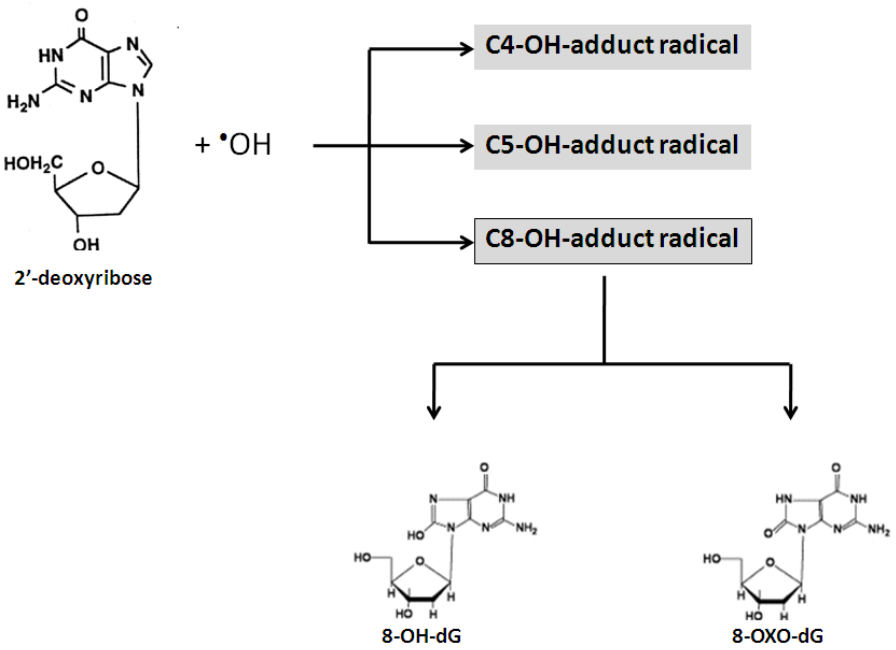

Fig. 5. The Chemical reaction of 2′-deoxyguanosine with hydroxyl radicals. The Oxidative modification reactions of 2′-doxyguanosine cause by hydroxyl radicals. This radical adducts are oxidized to 8-hydroxy-2′-deoxyguanosine (8-OHdG), or it tautomer 8-oxo-7-hydro-2′-deoxyguanosine (8-oxodG).

4.6 Advanced Glycation End-Produces (AGEs)

Advanced glycation end-produces (AGEs) are products of non-enzymatic glycation of proteins by reducing sugars (Zieman & Kass2004). AGEs was firstly discovered in early of 1900s by Louis Camille Mailard by the non-enzymatic chemical reaction between reducing sugars and amino groups on proteins to form protein-protein crosslink and complex yellow-brown pigments (Zieman & Kass2004). The Maillard reaction occurs when the reducing sugars, such as glucose, react with an amine groups, result in the formation of an unstable Shift bases (Figure 6). The produced unstable Shift bases that transform to an Amadori product, which can further rearrange to form advanced glycation endproducts (AGEs) capable of crosslinking proteins (Figure 7, 8).

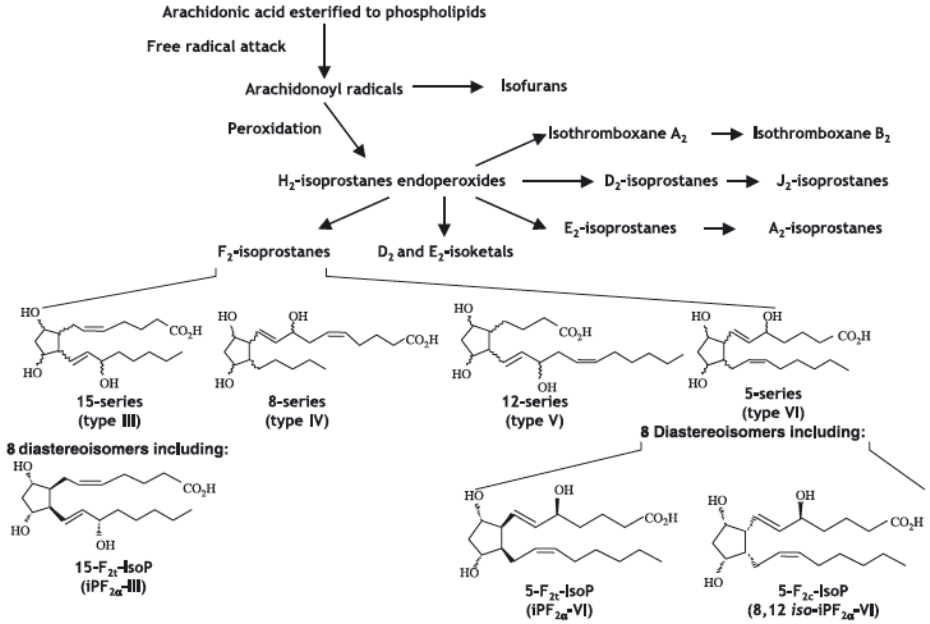

Fig. 6. Metabolic pathways of isoprostane (Cracowski & Durand2006). Free radicals interact with arachidonic acid produce arachidonyl radicals; these molecules were continue to the lipids peroxidation reaction and generated four types of prostaglandin-H_2-like compounds, which subsequently reduced to be 4 prostaglandin F2α.

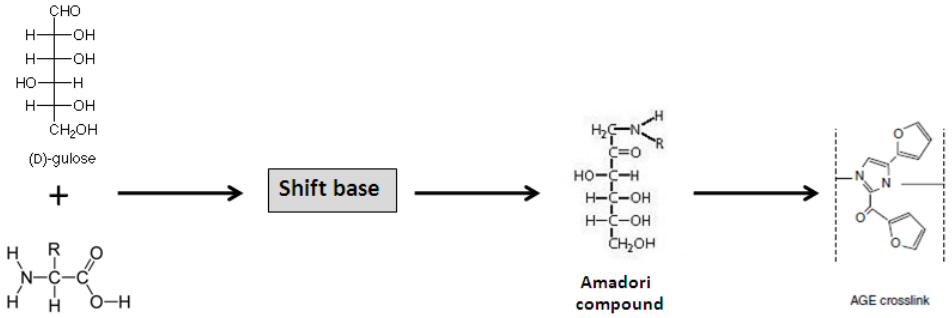

Fig. 7. The Maillard reaction is chemical reaction between a reducing sugar, such as glucose, and amino acid groups. The outcome from this reaction is the formation of unstable Schiff bases that can transform to an Amadori products, which can rearrange to form advanced glycation endproducts (AGEs) (Zieman & Kass2004).

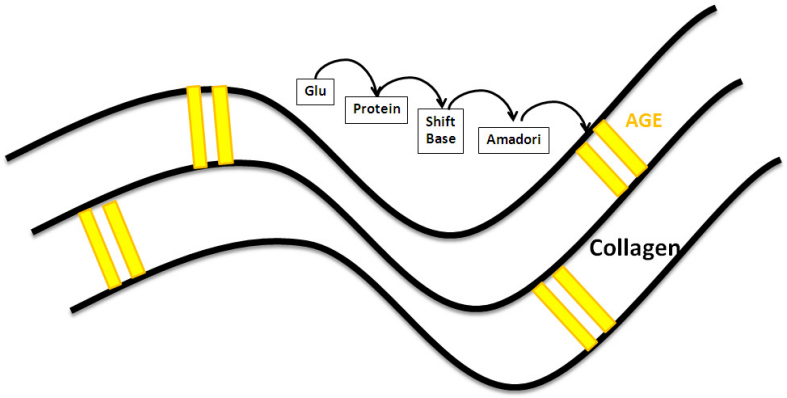

Fig. 8. The formation of collagen-collagen-AGEs crosslinking, this figure was modified from Zieman et. al. AGEs from the Maillard reaction can accelerate enzymatically crosslinking reaction of collagens strands (Zieman & Kass2004).

It has been known that AGEs play important role in the pathogenesis of diabetic vascular complications, as they lead to an abnormal leakage of proteins from the circulation and a progressive constriction of the luminal area of vessel (Brownlee et al.1988;Makita et al.1991;Ono et al.1998). Moreover, AGEs have been recognized as factors in the pathogenesis of other diabetic complications, such as nephropathy and retinopathy (Makita et al.1991;Ono et al.1998). In addition, the level of serum concentration of AGEs was associated with severity of coronary arthrosclerosis and development of this pathology in type 2 diabetic patients (Kiuchi et al.2001). Interestingly, it has been reported that the serum level of AGEs were elevated and correlated significantly with oxidized LDL, especially in diabetic patients (Lopes-Virella et al.2011). A recent evidence of 18-year study showed that the serum level of AGEs could predict the mortality from cardiovascular disease and coronary heart disease in non-diabetic women (Kilhovd et al.2005).

Determination of AGEs is similar to other techniques, used in determining other oxidative modified biomarkers, such as HPLC, GC-MS, ELISA, and immunochemistry (Ogino & Wang2007). The accuracy and reproducibility of these techniques have not been well examined according to lack of universally established unit of measurement, for comparing study findings from different laboratories (Ogino & Wang2007). Furthermore, AGEs has been reported to increase in cigarette smoking, similar to the findings found in other biomarkers (Nicholl & Bucala1998).

5. Early cardiac biomarkers for diagnostic acute coronary syndrome

The biochemical markers have been routinely used to assess myocardial damage, especially in patients suspected with ACS. World health organization criteria, formulated in 1979, have classically diagnosed in ACS patients if the patient present two (probable) or three (definite) diagnostic criteria of acute coronary syndrome. The criteria including clinical history of ischemic type chest pain lasting for more than 20 minutes, changes in serial ECG tracings, and elevation of serum cardiac biomarkers. Patients with ACS are subdivided into the

following 2 major categories based on the results from electrocardiogram (ECG) including those whose ECG show ST-elevation that is diagnostic of acute ST-elevation myocardial infarction (STEMI) and those who present other patterns of ECG change, but not categorized in STEMI, called non- ST elevation ACS (NSTEACS). The latter include unstable angina (UA) and non-ST-elevation myocardial infarction (NSTEMI) (Morrow et al.2007;Wiviott & Braunwald2004). The ECG in NSTEMI can be interpreted in the way that the artery is only partially blocked, or only transiently occlusive, and results in coronary ischemia without the appearance of ST-segment elevation. The ECG is the most readily available tool for diagnosing STEMI. However, the limitation of ECG is usually occur in acute chest pain, according to the low sensitivity of the baseline ECG, which is only 60% (Panteghini2002). Undetectable of ST-elevation of ECG lead to delay in final diagnosis and affect treatment and clinical outcome. Therefore, determination of high sensitivity, specificity and early ischemic biomarker is useful for diagnosis of acute myocardial ischemia, particularly in NSTEMI patients. There are many types of conventional cardiac biomarkers such as creatine kinase- MB isoenzyme (CK-MB), cardiac troponin I or T (cTnI, cTnT). These conventional cardiac markers are known to release in blood circulation as a result of cellular necrosis, not early enough to detect the early phase of myocardial ischemia that may not excess the reference range of biochemical markers of myocardial necrosis. Determination of plasma/serum cardiac biomarkers in patients, who has arrived hospital after the onset of symptoms, may not be detected. Therefore, screening method, for measuring the early cardiac biomarkers that actually reflect the early phase of cellular injury, is extremely useful. The more rapid diagnosis, the more effective intervention and treatment, the less cost for hospital stay, secondary prevention and reduce effective budget for screening test to exclude myocardial infarction patients (Figure 9)

Creatine kinase (CK) is an enzyme responsible for transferring a phosphate group from ATP to creatine. The molecular weight of this enzyme is approximately 80,000 dalton. It is composed of M and/or B subunits build up at least 3 isoenzymes including CK-BB, CK-MB, and CK-MM or CK-1, CK-2 and CK-3, respectively. Moreover, there are one more isoenzymes that have been reported e.g. mitochondrial isoenzyme. CK-2 or CK-MB is sometime called the cardiac isoenzyme as it is predominant isoenzyme in myocardium, whereas there is only 2-5% in skeletal muscle. CK-MB can be found in large amount of infarcted myocardium and can rise up in the circulation within 3-6 hours after ischemia, peaks in 10-48 hours, and returns to normal within 72 hours (Wu et al.1999). However, an elevated serum CK-MB may occur in people with severe skeletal muscle damage (such as in muscular dystrophy, accident) and renal disease (Green et al.1986). In such cases, the ratio of CK-MB per total CK, or CK index, is very helpful. If the index is under 4%, a non-myocardial source of high CK-MB should be concerned. One of the limitations of determining serum CK-MB is undetectable of minimal myocardial injury, late rise in the setting of AMI.

Cardiac Troponin is a useful cardiac marker, localized in myofibrils. Troponin consists of 3 subunits including inhibitory subunit (cTnI), calcium binding subunit (cTnC), and tropomyosin binding subunit (cTnT). The troponin complex is located on the thin filaments of the contractile muscles and regulates the calcium mediated interaction of myocardial myosin and actin filaments. The specificity, sensitivity, and reliability of troponin assay for diagnosed myocardial necrosis make cardiac troponin be an ideal cardiac marker. In addition, the minimal concentration in serum cardiac troponin, from healthy people without

cardiac disease, cannot be detected (Adams, III et al.1993). Among those 3 forms of cardiac troponin, troponin C cannot be used as cardiac marker according to non-specific expression in various tissues, not only the heart (Adams, III et al.1993). Cardiac troponin I (cTnI) and cardiac troponin T (cTnT) are 2 forms that normally used as cardiac marker. It has been known that, after myocardial ischemia, elevated cTnT last for 10 days to 2 weeks. The advantages of cTnT include highly sensitive for detecting MI, cTnT level may also help to risk stratify afterward, qualitative test run in 10 minutes. In contrast, the disadvantage of using cTnT assays as cardiac markers due to non specificity of cTnT, which can be found in unstable angina, chronic renal failure (Wood et al.2003). The cTnI is also being an ideal marker for ACS, according to high sensitivity and specificity of this marker in AMI. However, these two markers could not counted as the early marker, as it need increase around 6 hours after ischemia, until the level of cTnT is significantly higher than normal level.

The oxidatively modified markers such as PC and IMA have been proven as potential cardiac marker for diagnosis of ACS. However, determining of oxidative modified markers is not specific for myocardial ischemia. For example, it has been reported that the plasma level of IMA can be increased in cerebral, gastrointestinal intestinal and skeletal muscular ischemia as well as myocardial ischemia (Matthews et al.1990;Siegel et al.1995). Therefore, it is recommended that the interpretation of a positive IMA finding should be combined with other clinical indices (Shen et al.2010). Recently, our study showed the usefulness of determining serum IMA and PC content level to identify acute myocardial infarction, particularly in STEMI. The level of both serum IMA and PC content were significantly higher in STEMI compared to healthy control and determination of serum IMA level in combination of serum PC content level improved test performance (Kumphune et al.2010). However, the results from our recent study reported that diagnosis of NSTEMI was not improved by combination of serum IMA and PC level, in contrast, individual determination of serum PC content showed a good area under ROC curve and high PPV for NSTEMI diagnosis (Maneewong et al.2011). Charpentier *et. al.* also demonstrated in a large cohort study of patients admitted to an emergency department for chest pain that IMA did not provide valuable information for ACS diagnosis (Charpentier et al.2010). The possible explanation is NSTEMI patients did not have major myocardial necrosis, unlike in patients with STEMI. Therefore, the minor myocardial damage possibly has less degree of ROS mediated proteins oxidation.

Another limitation of using oxidative modified biomarkers is the interpretation in elder patients. It has been reported that oxidative modified forms of proteins were accumulated during aging (Berlett & Stadtman1997). For example, increases in proteins carbonyls occur in rat hepatocytes, drosophila, brain, and kidney of mice and in brain tissue of gerbils (Beal2002). In humans proteins carbonyls increase with age in brain, muscle, and human eye lens (Beal2002). The carbonyl content of human fibroblasts also increases as a function of age of the donor (Beal2002).

There are some reports determining other oxidative modified molecules, such as 8-OH-dG, isoprostane, and AGEs, in ACS. However, those reports were indicated only the incidences of elevated markers in ACS, but not the efficiency of the test. Therefore, determination of analytical method efficiency of those markers is challenge and need to be further investigated.

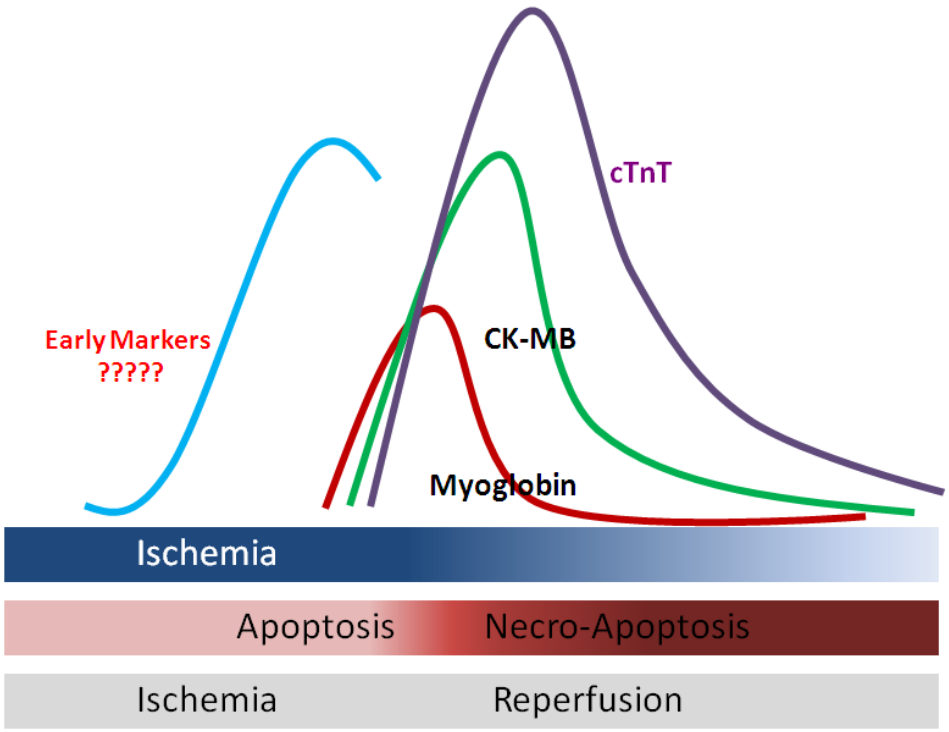

Fig. 9. The kinetics curve of conventional biomarkers. The rise and fall pattern of conventional cardiac biomarkers such as myoglobin, CK-MB, and cTnT. These markers release to circulation many hours after the onset of chest pain, post ischemia. Novel early markers, probably oxidatively modified markers, which release into blood stream, right after the onset of ischemia, will helpful.

6. Conclusion

Early cardiac biomarkers are essential for diagnosis of acute coronary syndrome. Conventional markers might not early enough to detect the early phase of cellular injury according to ischemia. Many oxidatively modified biomolecules were studied and known to have potential as cardiac markers. Further intensive investigation of these cardiac markers, especially the diagnostic power, is very helpful and can be used in real clinical investigation of coronary artery disease.

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Section 4

Diabetes Mellitus

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Oxidative Stress in Diabetes Mellitus: Is There a Role for Hypoglycemic Drugs and/or Antioxidants?

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1. Introduction

The most recent statistics indicate that the global prevalence of diabetes mellitus, estimated as 366 million in 2011, will increase to 522 million by 2030 (Whiting et al., 2011). Diabetes mellitus is a metabolic disorder of multiple etiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion and/or insulin action (ADA, 2011). There are basically two types of diabetes mellitus: type 1 and type 2 diabetes mellitus. Type 1 diabetes mellitus, an autoimmune disease, is characterized by the loss of pancreatic β-cells resulting in absolute insulin deficiency. It accounts for about 5-10 % of all newly diagnosed diabetes mellitus (ADA, 2011). On the other hand, type 2 diabetes mellitus is characterized by insulin resistance and β cell dysfunction. It remains the most common form of diabetes mellitus and constitutes about 90-95 % of all diabetes cases (ADA, 2011). In spite of the availability of different classes of oral hypoglycemic drugs, the incidence of microvascular complications (nephropathy, retinopathy and neuropathy) and macrovascular complications atherosclerosis, coronary artery disease, peripheral arterial disease and stroke continues to rise unabated in diabetic patients, even with treatment (Roglic and Unwin, 2010).

Pharmacological agents with different mechanisms of action are often combined to achieve optimal glycemic control (Turner et al., 1999). However, despite the use of multiple drugs, a lot of diabetic patients do not achieve the optimal glycemic goal (Turner et al., 1999). The Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) have demonstrated that intensive treatment of hyperglycemia reduces risk of developing microvascular and macrovascular complications (DCCT, 1993, UKPDS, 1998). However, recent findings indicate that intensive treatment of hyperglycemia is associated with higher incidence of weight gain, hypoglycemia and mortality than conventional therapy (Ismail-Beigi et al., 2010). These findings suggest that intensive therapy is not only detrimental, but limited and may not be the best. Besides, these findings may suggest that it is high time "hyperglycemia alone" was made the "culprit" in the management of diabetes mellitus. Generally, hypoglycemic drugs are incapable to prevent

pancreas degeneration, worsening of glycemic control and diabetic complications (DCCT, 1993, UKPDS, 1998, Turner et al., 1999, Ball et al., 2000). All these have been linked to increased oxidative stress in diabetes mellitus (Figueroa-Romero et al., 2008, Giacco and Brownlee, 2010). The aim of this chapter is to shed more light on the prospective of managing diabetes mellitus more effectively by targeting both "hyperglycemia and oxidative stress simultaneously". The data presented in this chapter convincingly suggest that the current management of diabetes mellitus may be improved upon by targeting hyperglycemia and oxidative stress as two potential therapeutic targets in diabetes mellitus.

2. General overview of oxidative stress

Considering the aim of this chapter, the general concept of oxidative stress will be discussed only in brief. Oxidative stress can defined as an "imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage" (Sies, 1991). According to Halliwell, "oxidative stress refers to a serious imbalance between reactive species production and antioxidant defenses" (Halliwell and Gutteridge, 2007). It occurs due to an increased generation and/or reduced elimination of reactive species (RS) by the antioxidant defense system. Oxidative stress is usually associated with oxidative damage, which is defined as "the biomolecular damage caused by attack of RS upon the constituents of living organisms" (Halliwell and Gutteridge, 2007). Most of the biologically relevant RS are either reactive oxygen species (ROS) or reactive nitrogen species (RNS). ROS include free radicals such as superoxide ($O_2^{\bullet -}$) and hydroxyl ($^{\bullet}OH$), and non-free radicals such as hydrogen peroxide (H_2O_2). Reactive nitrogen species include free radicals such as nitric oxide ($^{\bullet}NO$) and nitrogen dioxide ($NO_2^{\bullet -}$), and non-free radicals such as peroxynitrite ($OONO^-$) (Sies, 1991, Halliwell and Gutteridge, 2007). The generation of RS by aerobic organisms may occur as by products of metabolism (e.g. during operation of electron transfer chains), intentionally (e.g. during inflammation), or as a result of accidents of chemistry (such as the autoxidation of unstable biomolecules, e.g. dopamine) (Halliwell, 2011). Of all the RS, significant roles of $O_2^{\bullet -}$, $^{\bullet}NO$, and $OONO^-$ have been implicated in diabetic cardiovascular complications (Johansen et al., 2005). In order to prevent oxidative damage, it is important that excess RS is eliminated from the cells. Oxidative damage to cellular components impairs cellular functions. Besides their toxicities, ROS are also required in certain conditions and for physiological functions. For instance, during inflammation, the phagocytes release ROS which kill invading bacteria. ROS generated during mild or moderate exercise constitute part of the mechanism of exercise- or training-induced adaptation (Sachdev and Davies, 2008).

The ability of cells or tissues to withstand oxidative stress is largely dependent on the efficiency of the overall antioxidant defense system to scavenge excess RS, without compromising the physiological roles of ROS (Halliwell, 2011). The antioxidant defense system consists of endogenously-synthesized antioxidants which include antioxidant enzymes, glutathione, vitamins, small molecules and micronutrients (Sies, 1991, Halliwell and Gutteridge, 2007). An antioxidant is defined as "any substance that delays, prevents or removes oxidative damage to a target molecule" (Halliwell and Gutteridge, 2007). Antioxidant enzymes are enzymes which scavenge or eliminate a variety of RS including those generated during biological processes (Sies, 1991, Halliwell and Gutteridge, 2007). The main antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT) and glutathione

peroxidase (GPx) (Halliwell, 2011). SOD maintains the cellular levels of $O_2^{\bullet -}$ within the physiological concentrations by converting $O_2^{\bullet -}$ to H_2O_2, a more stable ROS (Halliwell and Gutteridge, 2007, Fukai and Ushio-Fukai, 2011). CAT metabolizes H_2O_2 to O_2 and H_2O (Halliwell and Gutteridge, 2007). CAT exerts two enzymatic activities, depending on the concentrations of its substrate (H_2O_2) (Scibior and Czeczot, 2006). It elicits a catalytic function at high concentrations of H_2O_2, whereas it produces a peroxidatic effect at lower concentrations of H_2O_2 (Scibior and Czeczot, 2006). GPx enzymatically reduces H_2O_2 to O_2 and H_2O using a hydrogen donor, glutathione (GSH) which is oxidized to glutathione disulfide (GSSG) (Lubos et al., 2011). Unlike CAT, GPx has a broader spectrum of substrates, detoxifying organic hydroperoxides and lipid peroxides (Lubos et al., 2011). However, CAT compared to GPx has a higher K_M for H_2O_2 and thus can protect against a higher concentration of H_2O_2 (Halliwell and Gutteridge, 2007).

The other antioxidant enzymes include glutathione reductase (GR), glutathione S-transferase (GST), peroxiredoxin, thioredoxin and thioredoxinreductase (Andreyev et al., 2005). GR scavenges $O_2^{\bullet -}$ and $^{\bullet}OH$ non-enzymatically or by serving as an electron donor to certain enzymes involved in the metabolism of ROS (Andreyev et al., 2005, Slonchak and Obolens'ka, 2009). GR helps to regenerate GSH via reduction of oxidized glutathione (GSSG) (Slonchak and Obolens'ka, 2009). GST comprises a family of multifunctional phase II biotransformation enzymes with a broad spectrum for a variety of substrates including epoxides, carcinogens, mutagens, 4-hydroxy-2-nonenal and malondialdehyde (MDA) (Andreyev et al., 2005, Slonchak and Obolens'ka, 2009). It catalyzes the conjugation of many electrophilic compounds with GSH (Andreyev et al., 2005). These enzymes work co-operatively together in order to scavenge RS and xenobiotics and thereby protect cells against oxidative damage (Halliwell, 2011). Generally, antioxidant enzymes differ from one another in terms of structure, tissue distribution, co-factor requirement, function, substrate specificity and affinity. The uniqueness of the antioxidant defense system lies in its capability to maintain the RS at certain steady-state levels thereby create and maintain a balance between the beneficial and injurious effects of RS (Lushchak, 2010). More detailed information on oxidative stress including free radicals, ROS, RNS, antioxidant enzymes, antioxidants, antioxidant defense system, and markers of oxidative stress can be obtained from following references (Sies, 1991, Johansen et al., 2005, Halliwell and Gutteridge, 2007, Halliwell, 2011).

3. Oxidative stress and its sources in diabetes mellitus

Under normal conditions and in most diseases including diabetes mellitus, mitochondria are the main source of RS and oxidative stress. Under physiological conditions, e.g. during mitochondrial oxidative metabolism, the bulk of oxygen (O_2) utilized is reduced to H_2O, while less than 2% of O_2 consumed is converted to $O_2^{\bullet -}$ (Brand, 2010). $O_2^{\bullet -}$ is an important ROS because it may be converted to other RS including H_2O_2, OH and $ONOO^-$ (Andreyev et al., 2005). Within physiological conditions, the body is protected from the detrimental effects of these free radicals by a network of antioxidant defense system. However, this defense system becomes impaired in diabetes mellitus and is further exacerbated by chronic hyperglycemia which generates ROS, resulting in oxidative stress. Some of the various sources of ROS and oxidative stress in diabetes mellitus include:

3.1 Hyperglycemia

Chronic hyperglycemia is the hallmark of diabetes mellitus. Evidence implicates mitochondrial generation of $O_2^{\bullet -}$ as a significant source of ROS (Andreyev et al., 2005). With persistent hyperglycemia, disproportionate amounts of glucose are delivered to the cells. This results in enhanced glucose flux through glycolysis and the tricarboxylic acid (TCA) cycle (Drews et al., 2010). This leads to an overdrive of the mitochondrial electron transport chain, which generates greater amounts of $O_2^{\bullet -}$ more than mitochondrial SOD can dismutase (Wiernsperger, 2003, Brand, 2010). This tilts the normal delicate balance between mitochondrial ROS production and mitochondrial ROS degradation in favor of mitochondrial ROS generation, and oxidative stress ensues (Brand, 2010). Evidence indicates that hyperglycemia-induced excessive mitochondrial $O_2^{\bullet -}$ production plays an important role in generating other RS in diabetes mellitus (Nishikawa et al., 2000b). Furthermore, glucose autooxidation produces ROS in the presence of transition metal ions (Johansen et al., 2005). Elevated glucose in diabetes may also react with lipids, resulting in the generation of RS (Johansen et al., 2005). Through non-enzymatic glycation reaction, glucose can react with proteins to produce several intermediate products such as Amadori and Sciff base products before generating advanced glycosylation endproducts (AGEs) (Johansen et al., 2005). Evidence indicates ROS are generated at each step of these reactions. Moreover, with excessive glucose in diabetes, it has been shown that glucose is diverted to other pathways such as sorbitol and hexosamine pathways where glucose is metabolized and ROS are generated (Johansen et al., 2005, Figueroa-Romero et al., 2008, Giacco and Brownlee, 2010).

3.2 Impaired antioxidant defense system

Impaired antioxidant defense system, such as reduced levels of endogenous antioxidants, reduced/enhanced antioxidant enzyme activities and increased levels of oxidative stress markers such as MDA, is very common in diabetes mellitus (Maritim et al., 2003, Johansen et al., 2005, Rahimi et al., 2005, Erejuwa et al., 2010a). The insufficient scavenging of RS as a result of impaired antioxidant defense system in diabetes may contribute to increased oxidative damage. The mechanisms for the impaired antioxidant defenses in diabetes mellitus remain poorly understood. It may be due to non-enzymatic glycation of these enzymes by hyperglycemia and thereby impairs their individual functions. Furthermore, evidence indicates that the antioxidant enzymes produce optimal protection when they function together (Michiels et al., 1994). Thus, glycation of any of these antioxidant enzymes may impair the efficiency of the entire antioxidant defense system or network. For instance, if SOD activity is impaired, this may result in $O_2^{\bullet -}$ build-up. On the other hand, if SOD activity is up-regulated, this may result in increased levels of H_2O_2. In either case, this may affect the activity of CAT or GPx. Similarly, if GR activity is impaired, there may be increased steady-state GSSG levels which will prevent regeneration of GSH, an endogenous antioxidant.

3.3 Increased activity of ROS-generating enzymes

The activities of many ROS-generating enzymes such as cyclooxygenase, xanthine oxidase, lipoxygenases, myeloperoxidase, NADPH oxidases and eNOS are augmented in diabetes (Wiernsperger, 2003). Besides, evidence implicates a role of endothelial NO synthase (eNOS). The eNOS produces NO which scavenges $O_2^{\bullet -}$ in non-diabetic subjects. However,

the eNOS has been found to be uncoupled in diabetic blood vessels where it produces O_2•− instead of •NO (Wiernsperger, 2003). Hyperglycemia might play an important role in the upregulation and uncoupling of ROS-generating enzymes.

3.4 Hyperinsulinemia

Insulin resistance characterized by hyperinsulinemia is frequent in the majority of the individuals with type 2 diabetic mellitus. Insulin induces the release of H_2O_2 when activating its receptors (Wiernsperger, 2003). Even though H_2O_2 is a non-free radical, it is membrane permeable and can diffuse to other sites, different from its site of production (Sies, 1991, Halliwell and Gutteridge, 2007). Chronic hyperinsulinemia together with the impaired antioxidant defenses in diabetes will lead to inefficient scavenging of H_2O_2. In the presence of transition metals such as copper and iron, H_2O_2 undergoes Fenton reaction to generate •OH which is implicated in the initiation and propagation of lipid peroxidation. Thus, hyperinsulinemia via H_2O_2 formation may increase RS and contribute to oxidative stress and damage in diabetes mellitus. Besides, insulin stimulates the release of many neurotransmitters, via activation of sympathetic nervous system. Many of these neurotransmitters are known to generate ROS and induce oxidative stress (Wiernsperger, 2003).

3.5 Insulin deficiency

Insulin deficiency is frequently observed in the diabetic patients. In some type 2 diabetic patients, insulin deficiency may be so severe such that the injection of exogenous insulin is required to control hyperglycemia (Turner et al., 1999, Cook et al., 2005). Insulin deficiency augments the activity of fatty acyl coenzyme A oxidase. Fatty acyl coenzyme A oxidase is an enzyme which is responsible for the oxidation of fatty acids, resulting in the increased generation of H_2O_2 (Schonfeld et al., 2009). H_2O_2 is well recognized for its role in exerting deleterious effects on cellular components such as proteins, nucleic acid and lipids including polyunsaturated fatty acids (PUFAs) (Sies, 1991, Halliwell and Gutteridge, 2007). These injurious effects of H_2O_2 can be mediated directly or indirectly through •OH formation or reaction with transition metals (such as copper or iron) to form toxic aldehydes, which are highly susceptible to free radical attack (Sies, 1991, Halliwell and Gutteridge, 2007). This sets up a chain reaction which further propagates the formation of more free radicals or RS and thereby contributes to or exacerbate oxidative stress.

3.6 Other sources of ROS and oxidative stress in diabetes mellitus

Diabetes mellitus is characterized by lipid abnormalities such as elevated LDL and cholesterol (ADA, 2011). These abnormalities are further exacerbated by the increased oxidizing environment which enhances the formation of oxidized LDLs (oxLDLs), glycated LDL and oxysterols (formed from the oxidation of cholesterol) (Johansen et al., 2005). These oxidized lipid products bind to specific receptor proteins or activate inflammatory proteins which generate ROS (Johansen et al., 2005). The import of oxLDLs in the vascular wall is the main mechanism by which ROS and oxidative stress induce atherosclerosis (Sies, 1991, Wiernsperger, 2003, Halliwell and Gutteridge, 2007). Evidence indicates that the levels of certain pro-oxidants such as ferritin and homocysteine are elevated in diabetes (Penckofer et

al., 2002). Free iron can increase ROS generation and the oxidation of LDL cholesterol. Similarly, homocysteine can generate ROS in the presence of transition metals which may enhance the oxidation of LDL cholesterol (Penckofer et al., 2002). Another source of ROS in diabetes mellitus is leptin. Elevated levels of leptin are associated with insulin resistance and diabetes mellitus. Evidence implicates a role of leptin in inducing ROS and oxidative stress in aortic endothelial cells in a dose-dependent manner while it produces additive effects with those of glucose (Wiernsperger, 2003). Other potential sources of ROS include aging, menopause, diet and physical activity (Penckofer et al., 2002). Increased ROS is reported in older people. Menopause may also enhance ROS production in older women. The levels of oestrogen, an antioxidant which decreases the oxidation of LDL cholesterol, usually decline during menopause (Penckofer et al., 2002). Aging and menopause may exacerbate oxidative stress since a large number of type 2 diabetics are older men and women. Consumption of foods rich in carbohydrates and fats, as opposed to antioxidant-enriched diets, may also enhance ROS formation. Sedentary lifestyle may predispose to increased ROS generation. This is corroborated by evidence which indicates mild or moderate, but not strenuous, exercise induces antioxidant defenses (Sachdev and Davies, 2008).

4. Role of oxidative stress in the pathogenesis of diabetes mellitus

Evidence implicates the role of oxidative stress in the different stages of the development of diabetes mellitus, starting from the pre-diabetes state, impaired glucose tolerance, postprandial hyperglycemia, mild diabetes and finally to overt diabetes mellitus (Ceriello et al., 1998). Loss of β-cell function, resulting from impaired secretory capacity and increased apoptosis, is a main occurrence in the pathogenesis of both types of diabetes (Drews et al., 2010). Besides β-cell dysfunction, insulin resistance is also a major characteristic feature of type 2 diabetes mellitus (Evans et al., 2003). Oxidative stress plays an important role in the pathogenesis of both β-cell dysfunction and insulin resistance (Evans et al., 2003, Drews et al., 2010).

4.1 Role of oxidative stress in β-cell dysfunction

The β-cells express low level of antioxidant enzymes such as SOD, CAT and GPx and thereby increase their susceptibility to oxidative stress (Tiedge et al., 1997). Increased mitochondrial ROS production in the β-cells results from enhanced glucose or fatty acid flux through glycolysis and the TCA cycle (Drews et al., 2010). This generates excess $O_2^{\bullet -}$ which gives rise to other ROS and RNS. The insufficiency of antioxidant enzymes to scavenge these ROS leads to oxidative stress. Besides mitochondria, NADPH oxidases, nitric oxide synthases, and phagocytes are other key sources of ROS in the β-cells (Drews et al., 2010). In type 1 diabetes mellitus, evidence implicates the role of ROS in impaired β-cell function caused by autoimmune reactions, cytokines and inflammatory proteins (Drews et al., 2010). Similarly, in type 2 diabetes mellitus, the role of ROS is implicated in the β-cell dysfunction as well as insulin resistance (Drews et al., 2010). The pancreas is highly susceptible to oxidative stress as evidenced by studies which show that H_2O_2 impairs insulin secretion in pancreatic β-cells (Maechler et al., 1999) and products of oxidative stress inhibit glucose-stimulated insulin secretion (Miwa et al., 2000). Other evidence shows that overexpression of antioxidant enzymes in islets or transgenic mice and antioxidants such as N-acetyl-L-cysteine (NAC) protect against ROS-induced β-cell toxicity (Tiedge et al., 1998, Drews et al.,

2010). Research within the last decade has recognized the role of glucotoxicity as the main causal determinant of β-cell dysfunction (Drews et al., 2010). The role of glucotoxicity in β-cell dysfunction is demonstrated by studies which indicate increased glucose concentrations impair insulin release in non-diabetic subjects (Marchetti et al., 2008). In contrast, improved glycemic control results in improved insulin secretion in patients with type 2 diabetes (Marchetti et al., 2008). The fact that antioxidants reduce or prevent the toxicities of elevated glucose on the expression of insulin mRNA, insulin content and secretion lends support to the role of oxidative stress in mediating the toxic effects of glucotoxicity (Tanaka et al., 1999). Besides glucotoxicity, lipotoxicity may also play a role in impaired β-cell function. Free fatty acids have been shown to uncouple mitochondrial oxidative phosphorylation and increase ROS formation in rat pancreatic islets (Carlsson et al., 1999), deplete pancreatic β-cell insulin content (Bollheimer et al., 1998) and inhibit glucose-induced insulin secretion and biosynthesis (Zhou and Grill, 1994). Both glucotoxicity and lipotoxicity may be involved in β-cell dysfunction in diabetes mellitus (Marchetti et al., 2008). The role of oxidative stress is also implicated in β-cell deficit, as well as increased apoptosis observed in humans with type 2 diabetes (Drews et al., 2010).

4.2 Role of oxidative stress in insulin resistance

Insulin resistance precedes the onset of diabetes mellitus and is influenced by factors such as genetic make-up and environmental factors including increased calorie intake, sedentary lifestyle, obesity, pregnancy and abnormally elevated levels of certain hormones (Evans et al., 2003). The role of oxidative stress is implicated in the pathogenesis of insulin resistance (Kim et al., 2006). Both pyruvate and fatty acids can serve as energy substrate in muscle and adipose tissue. Pyruvate is derived from glucose and other sugars, while fatty acids originate from fats. Once transported across the inner mitochondrial membrane, these fuel substrates are converted to acetyl CoA by mitochondrial enzymes. During citric acid cycle, oxidation of the carbon atoms of the acetyl groups in acetyl CoA generates CO_2. Besides CO_2 formation, the citric acid cycle generates high-energy electrons which are carried by NADH and $FADH_2$. The increased uptake of energy substrate in the muscle and adipose tissue enhances citric acid cycle activity. This in turn generates mitochondrial NADH more than required. This leads to an overdrive of the oxidative phosphorylation which increases the mitochondrial transmembrane proton gradient. These high-energy electrons are then transferred to O_2 leading to production of $O_2^{\bullet -}$ which is further converted to other ROS. This sets the stage for oxidative stress (Talior et al., 2003). A recent study showed that the skeletal muscle of high-fat diet-induced insulin-resistant rats liberated more mitochondrial H_2O_2 and had impaired ability to maintain normal redox balance compared to the data obtained in the skeletal muscle of the insulin-sensitive control rats (Anderson et al., 2009). Similar findings were observed in insulin-resistant, morbidly obese human subjects (Anderson et al., 2009). This study provides strong evidence in support of a role of mitochondrial ROS production and oxidative stress in the pathogenesis of insulin resistance.

Various mechanisms by which oxidative stress contributes to insulin resistance have been identified. These include oxidative stress-impaired insulin-induced GLUT4 translocation in adipocytes (Rudich et al., 1998), oxidative stress-induced impairment in insulin stimulation of protein kinase B and glucose transport in adipocytes (Rudich et al., 1999), oxidative stress-induced interactions between the PI3-kinase-dependent signaling pathway and

activation of p38 MAPK (Kim et al., 2006), and interruption of insulin-induced cellular redistribution of insulin receptor substrate-1 and phosphatidylinositol 3-kinase in adipocytes (Tirosh et al., 1999). Other studies indicate that oxidative stress can directly induce considerable insulin resistance in skeletal muscle by interfering with insulin signaling, glucose uptake and glycogen synthesis (Dokken et al., 2008). This oxidative stress-induced insulin resistance is mediated in part through reduced insulin-modulated suppression of glycogen synthase kinase-3 (GSK-3beta) (Dokken *et al.*, 2008; Henriksen, 2010). Evidence has also implicated a role of inducible nitric oxide synthase and •NO donor in the degradation of insulin receptor substrate-1. Increased S-nitrosylation of certain molecules or pathways involved in insulin signaling such as insulin receptor, insulin receptor substrate-1, and protein kinase B/Akt in skeletal muscle have also been reported to play an important role in insulin resistance (Carvalho-Filho et al., 2009).

In order to protect against increased glucose-induced oxidative stress (Talior et al., 2003), cells tend to respond by limiting more energy fuel or substrates from gaining access into the cells. Cells may utilize various mechanisms, including inhibition of α-ketoglutarate dehydrogenase, to limit the amount of NADH available for the oxidative phosphorylation. Therefore, to limit insulin-dependent nutrient uptake into the cells, the insulin receptors become less sensitive to the action of insulin. This marks the onset of insulin resistance - a phenomenon whereby normal amounts of insulin can no longer activate the glucose transport system in insulin-sensitive tissues such as skeletal muscle and adipose tissue. This results in enhanced steady-state glucose levels in the blood. It is suggested that, in this setting, insulin resistance may be a compensatory mechanism employed by the cells to prevent further uptake of insulin-stimulated glucose and fatty acids (Hoehn et al., 2009). This compensatory mechanism may result in reduced formation and accumulation of RS leading to reduced oxidative stress and damage. With persistently increased blood glucose levels, hyperglycemia ensues leading to overt type 2 diabetes mellitus.

4.3 Could antioxidants play a role in preventing diabetes mellitus and/or its progression?

In view of evidence which implicates a role of oxidative stress in β-cell dysfunction and insulin resistance, the question that arises is: could antioxidants play a role in preventing diabetes mellitus and/or its progression? As discussed earlier, there is a possibility for such a role of antioxidants. Antioxidants such as vitamin C, vitamin E, β-carotene, α-lipoic acids and honey have been shown to ameliorate hyperglycemia through increased β-cell mass and insulin secretion. However, at the moment, the evidence to recommend or prescribe antioxidants for the prevention of diabetes mellitus is very weak. Instead, efforts should be made to create and increase the awareness on (1) the role of ROS and oxidative stress in the pathogenesis and/or progression of diabetes mellitus and (2) the importance of increased consumption of antioxidant-enriched diets including fruits and vegetables, as opposed to increased calorie intake. The recommendation for increased consumption of fruits and vegetables is very important because even in the developed countries, majority of the population do not meet these requirements. The importance of exercise should also not be left out. These could be achieved by incorporating into educational curricula - at all levels of education. In addition, those who are already diabetic should be enlightened on the importance of maintaining good glycemic control in order to prevent or delay the progression or complications of diabetes mellitus. Diabetic patients also need to be informed

on the need for adherence to dietary regimens and compliance to prescribed medications. These recommendations could also be an important component of training received by healthcare providers.

5. Role of oxidative stress in the complications of diabetes mellitus

There is strong evidence implicating a role of oxidative stress in diabetic nephropathy, retinopathy and neuropathy which constitute the microvascular complications (Figueroa-Romero et al., 2008, Giacco and Brownlee, 2010, Brownlee, 2005). Similarly, a role of oxidative stress is implicated in the macrovascular complications (coronary artery disease, peripheral arterial disease and cerebrovascular disease) (Giacco and Brownlee, 2010, Brownlee, 2005). This section highlights the different mechanisms by which hyperglycemia causes diabetic complications.

5.1 Hyperglycemia-enhanced polyol pathway

The polyol pathway comprises two enzymes: aldose reductase (AR) and sorbitol dehydrogenase (SDH) (Brownlee, 2005). AR reduces a broad spectrum of substrates such as glucose, galactose, methylglyoxal, glucosone, deoxyglucosone and lipid-derived aldehydes (Petrash, 2004). Under euglycemic conditions, glucose is not reduced by AR. The bulk of glucose is normally phosphorylated by hexokinase to produce glucose-6-phosphate, a substrate for glycolysis and pentose phosphate pathway. However, under hyperglycemic conditions, AR reduces glucose to sorbitol (Brownlee, 2005). Sorbitol is oxidized to fructose by SDH (Giacco and Brownlee, 2010). With chronic hyperglycemia, the AR pathway becomes enhanced leading to increased formation of sorbitol. As a result of activated AR pathway, there is increased consumption of NADPH (as an obligate co-factor) by AR (Figueroa-Romero et al., 2008). The GR also requires NADPH, as co-factor, for the regeneration of GSH, an endogenous scavenger of ROS. Therefore, increased utilization of NADPH caused by enhanced activity of AR reduces intracellular concentration of GSH. Reduced levels of GSH will impair the activity of GPx which utilizes GSH as a hydrogen donor (Halliwell and Gutteridge, 2007). Taken together, decreased NADPH impairs GR activity, reduces GSH level and impairs GPx activity. This impairs the antioxidant defense network and increases cellular susceptibility to oxidative stress. NO• has been shown to inhibit AR activity in diabetes (Chandra et al., 2002). In view of evidence which indicates ROS can decrease NO• bioavailability, therefore, increased oxidative stress in diabetes will further augment AR activity. Thus, hyperglycemia-enhanced polyol pathway will exacerbate oxidative stress in diabetes mellitus.

Besides, the increased levels of sorbitol caused by enhanced AR activity increase the osmolality of intracellular milieu. Sorbitol, myoinositol, glycerophosphorylcholine, betaine and taurine are physiological osmolytes which help to maintain homeostasis in cells such as renal medullary cells (Yancey and Burg, 1989). Thus, as a compensatory mechanism, this leads to efflux of intracellular osmolytes (Figueroa-Romero et al., 2008). Some of these osmolytes e.g. myoinositol play a vital role in signaling transduction, while others such as taurine are endogenous antioxidants (Figueroa-Romero et al., 2008). Hence, increased sorbitol formation reduces endogenous antioxidants and other important osmolytes. This further impairs cellular function and increases intracellular susceptibility to oxidative stress. In contrast, the SDH pathway generates fructose from sorbitol. Similar to AR pathway,

chronic hyperglycemia increases the activity of SDH which results in the formation of high amounts of fructose (Figueroa-Romero et al., 2008). Increased SDH activity result in an increased NADH:NAD^+ ratio, which may inhibit oxidation of triose phosphates (Nishikawa et al., 2000a). Accumulation of triose phosphates increases *de novo* synthesis of diacylglycerols (DAG) which activate protein kinase C. Increased levels of triose phosphate may also increase generation of methylglyoxal, a potent AGE-precursor. Besides, increased fructose levels may enhance glycation and further contribute to reduced levels of NADPH, impaired antioxidant defenses and formation of AGEs.

5.2 Hyperglycemia-enhanced formation of advanced glycation end products

Advanced glycation end products (AGEs) are adducts formed non-enzymatically by the reaction between reducing carbohydrates and proteins, DNA, or lipids (Ahmed, 2005). AGEs also include products that are formed non-enzymatically from the reaction between AGE precursors, glycated sugars or oxidized products of fatty acid (in arterial endothelial cells) and protein (Giacco and Brownlee, 2010). They are produced through three major pathways: (1) convertion of glucose to glyoxal; (2) degradation of Amadori products to 3-deoxyglucosone; and (3) conversion of glyceraldehyde-3-phosphate to methylglyoxal (Nishikawa et al., 2000a, Ahmed, 2005, Figueroa-Romero et al., 2008). In diabetes mellitus, the levels of AGEs become elevated as a result of chronic hyperglycemia (Duran-Jimenez et al., 2009). The formation of AGEs occurs in different stages. During these stages of AGE formation, a number of highly reactive intermediates and cross-linkers, which enhance the binding affinity of AGEs to proteins, are also formed (Ahmed, 2005). AGEs can inhibit the antiproliferative effects of nitric oxide (Maritim et al., 2003). AGEs bind to and modify intracellular proteins thereby altering their functions (Giacco and Brownlee, 2010). In the vasculature, AGEs interact with cell surface protein or extracellular matrix components resulting in the formation of cross-linked proteins which enhance stiffening within the arterial vessel (Ahmed, 2005). AGEs can also modify plasma proteins which in turn activate receptor for advanced glycation end products (RAGE) on cells such as macrophages, vascular endothelial and smooth muscle cells (Giacco and Brownlee, 2010). AGEs can bind directly to and activate the receptors for AGEs.(Griesmacher *et al*., 1995, Ahmed, 2005).

The binding of AGEs or AGE-modified plasma proteins to RAGE induces the release of ROS (Nishikawa et al., 2000a; Ahmed, 2005; Giacco and Brownlee, 2010). The ROS activate the expression of several genes and proteins that are involved in inflammatory cascade and implicated in the pathogenesis of diabetic cardiovascular disease. These genes and proteins include nuclear factor kappa β, tumor necrosis factor α, interleukin-1 and granulocyte-macrophage colony-stimulating factor (Nishikawa et al., 2000a, Ahmed, 2005, Giacco and Brownlee, 2010).

5.3 Hyperglycemia-activated protein kinase C pathway

Protein kinase C (PKC) is an enzyme that modulates the functions of other proteins through their phosphorylation. PKC is activated by the elevated level of DAG, derived from enhanced formation of triose phosphate via hyperglycemia (Giacco and Brownlee, 2010). Hyperglycemia may also increase DAG content through phosphatidylcholine hydrolysis (Nishikawa et al., 2000a). In diabetes, elevated levels of triose phosphate occur through increased *de novo* synthesis due to inhibition of glycolytic enzyme glyceraldehyde phosphate

dehydrogenase (GAPDH) by increased ROS (Giacco and Brownlee, 2010). Besides DAG, evidence indicates that AGEs can also activate PKC pathway to increase the expression of vascular endothelial growth factor (VEGF) (Ahmed, 2005). Similarly, increased ROS levels in vascular endothelial cells may also enhance PKC pathway (Nishikawa et al., 2000a). Enhanced PKC activity induces several cytokines and protein signals including plasminogen activator inhibitor (PAI-1), NF-κB, NAD(P)H oxidases, endothelin-1, transforming growth factor β (TGF-β) and extracellular matrix (ECM) (Nishikawa et al., 2000a). These pathological alterations have been implicated in basement membrane thickening, vasoconstriction, altered capillary permeability, hypoxia and activation of angiogenesis (Nishikawa et al., 2000a, Figueroa-Romero et al., 2008).

5.4 Hyperglycemia-enhanced hexosamine pathway

Evidence has implicated the role of hexosamine pathway in the toxic or adverse effects of hyperglycemia in diabetes mellitus (Schleicher and Weigert, 2000). Under physiological conditions, a small quantity of fructose-6 phosphate derived from glycolysis is diverted to the hexosamine pathway. Glutamine: fructose-6 phosphate amidotransferase (GFAT) then converts fructose-6 phosphate to glucosamine-6 phosphate, which is converted to uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) (Schleicher and Weigert, 2000). The enzyme O-GlcNAc transferase then utilizes UDP-GlcNAc as a substrate, fixing O-GlcNAC to protein residues of transcription factors such as Sp1 and thus modify their expression (Figueroa-Romero et al., 2008). Similar to the other pathways, with chronic hyperglycemia, the hexosamine pathway becomes enhanced (Figueroa-Romero et al., 2008, Schleicher and Weigert, 2000). This leads to increased formation of UDP-GlcNAc and increased activity of O-GlcNAc transferase, with consequent alterations in gene expression (Figueroa-Romero et al., 2008). This pathway is implicated in the hyperglycemia-mediated increases in the transcription of TGF-α and TGF-β_1. Over-expression of these transcription factors such as TGF-β_1 is known to activate the proliferation of collagen matrix, basement membrane thickening and inhibition of mesangial cell mitogenesis, thus contributing to microvascular complications such as nephropathy (Schleicher and Weigert, 2000).

5.5 Hyperglycemia-activated Poly-ADP Ribose Polymerase (PARP) pathway

Poly(ADP-ribose) polymerase (PARP) is a family of enzymes that detect single- and double-stranded DNA and repair damaged DNA (Virag and Szabo, 2002). Activation of PARP is a direct cellular response to metabolic- or chemical-induced DNA damage. Once PARP senses and identifies a damaged DNA, it binds to the DNA and forms homodimers and catalyzes the cleavage of nicotinamide adenine dinucleotide (NAD^+) into nicotinamide and ADP-ribose (Virag and Szabo, 2002). It then uses ADP-ribose to synthesize a poly(ADP-ribose) chain (PAR) which serves as a signal for several DNA repairing enzymes such as DNA ligase III and DNA polymerase beta (polβ). In hyperglycemic environment, there is increased ROS formation leading to oxidative damage which activates PARP. As a result of NAD^+ utilization, this depletes cellular NAD^+ stores and induces a progressive ATP depletion. This further increases the vulnerability of cells to oxidative stress and damage as well as cell death (Figueroa-Romero et al., 2008). Available evidence suggests that PARP's catalytic activity may cause altered gene expression, increased oxidative stress and diversion of glycolytic intermediates to other pathogenic pathways (Figueroa-Romero et al.,

2008). A recent study demonstrated the beneficial effects of PARP inhibition in diabetic complications (Lupachyk et al., 2011).

5.6 Hyperglycemia-induced mitochondrial $O2^{\bullet-}$overproduction

Recent data indicates that hyperglycemia-induced mitochondrial $O_2^{\bullet-}$ overproduction is the sole underlying mechanism (directly or indirectly) by which hyperglycemia induces cellular damage (Giacco and Brownlee, 2010). During mitochondrial oxidative phosphorylation, $O_2^{\bullet-}$ is generated due to leakage of electrons from electron transport chain (ETC) on molecular oxygen. In euglycemic environment, about 0.2–2% of O_2 utilized by the mitochondria is reduced to $O_2^{\bullet-}$ (Bashan et al., 2009, Brand, 2010). The antioxidant defense network maintains the mitochondrial level of ROS within physiological concentrations (Andreyev et al., 2005, Brand, 2010). However, in hyperglycemic environment, enhanced glucose flux through glycolysis and TCA causes an overdrive of the mitochondrial ETC resulting in mitochondrial dysfunction and increased ROS formation (Bashan *et al.*, 2009; Brand, 2010). Elevated levels of ROS lead to oxidative stress and damage. Oxidative damage to DNA can activate Poly(ADP-ribose) polymerase (PARP) pathway (Wei, 1998).

Besides this pathway, evidence suggests that hyperglycemia, via $O_2^{\bullet-}$ overproduction , inhibits G6PDH. G6PDH is the rate-limiting enzyme of the pentose phosphate pathway necessary for generating reducing equivalents to the antioxidant defense system (Brand, 2010). Inhibition of G6PDH leads to increased levels of glycolytic intermediates resulting in increased flux into these pathways. For instance, glyceraldehyde 3-phosphate can non-enzymatically be converted to methylglyoxal which activates AGEs pathway (Giacco and Brownlee, 2010). Similarly, glyceraldehyde 3-phosphate is a precursor of DAG which can activate PKC pathway. Increased levels of fructose 6-phosphate will increase flux through the hexosamine pathway (Giacco and Brownlee, 2010). Furthermore, reduced levels of G6PDH result in increased glucose concentrations which further enhances flux through the polyol pathway. The aldose reductase pathway becomes intensified leading to increased formation of sorbitol. This sets up a chain reaction that continuously activates one pathway or the other generating ROS which may cause DNA damage. This causes activation of PARP pathway. Thus, virtually all these pathways, namely polyol pathway, formation of AGEs pathway, PKC pathway, hexosamine pathway, and poly-ADP ribose polymerase (PARP) pathway, can be activated by hyperglycemia-induced mitochondrial $O_2^{\bullet-}$ overproduction.

6. Interrelation among glycemic control, oxidative stress and diabetic complications

So far, this chapter has identified the various sources of oxidative stress in diabetes mellitus. It has also presented evidence that indicates hyperglycemia enhances formation of ROS in diabetes mellitus. It has also shown that hyperglycemia-induced oxidative stress plays an important role in the activation of several pathogenic pathways implicated in the pathogenesis of diabetic complications. Generally, the mechanisms by which hyperglycemia causes cellular damage can be classified into two groups (Nishikawa et al., 2000a). The first group of mechanisms entails constant acute fluctuations in cellular metabolism which are reversible following restoration of euglycemia. The second group of mechanisms involves cumulative changes in long-lived macromolecules which are irreversible even after euglycemia is restored (Nishikawa et al., 2000a). Studies have demonstrated the beneficial

effects of reduced hyperglycemia or glycemic control on the risk of developing diabetic complications (DCCT, 1993, UKPDS, 1998). Nevertheless, recent findings suggest that treatment of chronic hyperglycemia to achieve optimal glycemic goal in diabetic patients is limited and detrimental (Ismail-Beigi et al., 2010). It is recommended that the consequences (higher mortality rate, hypoglycemia and weight gain) should be weighed against the benefits of intensive therapy (Ismail-Beigi et al., 2010). Besides, available evidence indicates that achieving and/or maintaining optimal glycemic control in diabetic patients is difficult (Turner et al., 1999, Cook et al., 2005). The difficulty in maintaining optimal glycemic control is attributed to deterioration of pancreatic β-cell function, which is linked to hyperglycemia-induced oxidative stress (Drews et al., 2010). Interestingly, even in diabetic patients given pancreatic transplants, diabetic complications such as nephropathy continued to deteriorate at least five years after their diabetes had been cured (Fioretto et al., 1998). This contradicts evidence that links hyperglycemia to diabetic complications. Therefore, this section attempts to explain the interrelation among glycemic control, oxidative stress and diabetic complications and possible role of hypoglycemic drugs (and/or insulin) and antioxidants in the management of diabetic complications by answering the following questions:

1. Does reduced or intensive therapy of hyperglycemia prevent induction or development of oxidative stress?
2. Does reduced or intensive therapy of hyperglycemia prevent diabetic complications?
3. Why does reduced or intensive therapy of hyperglycemia not prevent diabetic complications?
4. Could there be a role of antioxidants in the management of diabetic complications?
5. Could there be a role of hypoglycemic drugs (and/or insulin) and antioxidants in the management of diabetic complications?

6.1 Does reduced or intensive therapy of hyperglycemia completely restore or ameliorate oxidative stress?

Hyperglycemia induces oxidative stress in diabetes mellitus. However, does evidence indicate that reduced or intensive treatment of hyperglycemia completely prevent development of oxidative stress? This section aims to answer this important question by presenting both experimental and clinical data. In diabetic rats, after two months of poor glycemic control, re-institution of good glycemic control for seven additional months partially reduced the elevated caspase-3 activity, levels of NF-kβ, lipid peroxides and nitric oxides with no beneficial effect on nitrotyrosine formation. In contrast, after six months of poor glycemic control, re-institution of good glycemic control for seven additional months demonstrated no significant effects on the elevated caspase-3 activity, NF-kβ, and oxidative stress parameters (Kowluru, 2003, Kowluru *et al.*, 2004). In another follow up study, after six months of poor glycemic control, normalization of hyperglycemia for another 6 months also had no significant effect on retinal nitrotyrosine levels, neither did oxidative stress parameters improve (Kowluru et al., 2007). Other studies have also shown that reduced hyperglycemia does not completely restore redox status (Rahimi et al., 2005, Erejuwa et al., 2010a, Erejuwa et al., 2011b).

Evidence suggests that proteins (collagen) are likely to be glycated irrespective of blood glucose levels (Monnier et al., 1999). In patients with type 2 diabetes mellitus, insulin treatment only partially improved oxidative stress parameters (Seghrouchni et al., 2002). This is evidenced by the elevated levels of thiobarbituric acid reactive substances and reduced erythrocyte GSH (Seghrouchni et al., 2002). In type 2 diabetic patients, treatment

with gliclazide for 12 weeks ameliorated oxidative stress better than did glibenclamide (Fava et al., 2002). Available data from DCCT and Epidemiology of Diabetic Complications and Interventions (EDIC) Trial indicated that type 1 diabetic patients in the intensive therapy group still had increased levels of protein glycation products and AGEs despite intensive treatment (Genuth et al., 2005). However, protein glycation and AGE formation were less compared with those in the conventional treatment group (Genuth et al., 2005).

The data highlighted in this section reveal that reduced or intensive therapy of hyperglycemia does not completely prevent induction of oxidative stress. In other words, once hyperglycemia (either via mitochondrial $O_2^{\bullet -}$ overproduction or other mechanisms) activates any of these mechanistic pathways (especially the polyol pathway), intensive therapy or even normalization of hyperglycemia (which is even difficult with the current hypoglycemic drugs) would have limited effects on oxidative stress. Once oxidative stress is induced in diabetes mellitus, it can enhance the generation of ROS and initiate redox-chain reactions which may activate various inflammatory proteins and signaling pathways including all the abovementioned mechanistic pathways. Even if normal glucose level is restored, these inflammatory proteins and signaling pathways on their own could cause oxidative stress and/or sustain the oxidative stress originally or previously induced by hyperglycemia. Thus, with or without hyperglycemia, the resulting ROS and oxidative stress secondary to hyperglycemia would suffice to activate some of these pathways and set the stage for oxidative stress or exacerbate the already developed or existing oxidative stress. Besides, evidence implicates a role of oxidative stress in many neurodegenerative disorders such as hypertension, cancer and Alzheimer's disease. All of these disorders are characterized by euglycemia. Therefore, it means or suggests that oxidative stress can be an entirely independent phenomenon which can exist even with normalization of hyperglycemia.

6.2 Does reduced or intensive therapy of hyperglycemia prevent diabetic complications?

Data from animal and human studies indicate that intensive therapy may delay the progression of diabetic complications, but does not prevent diabetic complications. A study that investigated the effect of improved glycemic control on the progression of retinopathy in diabetic dogs found that diabetic dogs with 5-year poor glycemic control developed diabetic retinopathy while those with 5-year good glycemic control had no diabetic retinopathy (Engerman and Kern, 1987). The third group comprised diabetic dogs with 2.5-year poor glycemic control. It was observed that these diabetic dogs did not develop diabetic retinopathy. However, the dogs later developed diabetic retinopathy despite 2.5-year good glycemic control (Engerman and Kern, 1987). The study further revealed that the extent of pathology of diabetic retinopathy in the third group (with 2.5-year poor glycemic control + 2.5-year good glycemic control) was similar to that of the dogs with 5-year poor glycemic control (Engerman and Kern, 1987). Similarly, in sucrose-fed diabetic rats, cure of diabetes via islet transplantation at 12 weeks (but only at 6 weeks after the confirmation of diabetes) did not prevent lesions or progression of diabetic retinopathy (Hammes et al., 1993). Kowluru and colleagues (2007) also reported that after 6 months of poor glycemic control in rats, normalization of hyperglycemia for 6 months had no effect on the lesions or pathology of diabetic retinopathy.

In type I diabetic patients whose diabetes had been cured (through pancreas transplantation) but still had diabetic nephropathy, the thickness of glomerular and tubular basement membranes was still similar at 5 years versus baseline values. In contrast, it was significantly reduced only by the 10th year versus baseline values. The study further showed that while mesangial fractional volume had increased by the 5th year, it significantly decreased by the 10th year (Fioretto et al., 1998). In the DCCT, due to the considerable benefits of intensive therapy, patients in the conventional therapy group were transitioned to intensive therapy group (DCCT, 1993). A long-term follow-up study under the EDIC Trial (DCCT/EDIC, 2002, DCCT/EDIC, 2003, Pop-Busui et al., 2009) showed that patients who were formerly in the intensive therapy group had lower incidence of diabetic microvascular complications than the patients who were originally in the conventional treatment group, despite 8 years of similar and normalized glycemic control in the EDIC trial (DCCT/EDIC, 2002). It was further reported that the conventionally treated-DCCT patients who were transitioned to intensive therapy group in the EDIC trial still had greater incidence of macrovascular pathology or complications (Nathan et al., 2003, Nathan et al., 2005, Cleary et al., 2006, Patel et al., 2008). Evidence from clinical trials also suggests that the incidence or development of diabetic complications is more likely to depend on the intensity of oxidative stress than on the level of glycemic control (Monnier et al., 1999, Genuth et al., 2005). Besides, evidence indicates that the degree of insulin resistance correlates with the onset of diabetic complications, independently of glycemic levels (Chillaron et al., 2009). In type 2 diabetic patients, it was reported that gliclazide treatment delayed the progression of diabetic nephropathy only, whereas it produced no significant effect on the development or progression of retinopathy or macrovascular complications (Patel et al., 2008). These findings are very important because gliclazide is one of the few hypoglycemic agents with an antioxidant effect (Fava et al., 2002).

It is evident from the different aforementioned hyperglycemia-induced mechanistic pathways and the data (both experimental and clinical) presented in this section that the pathogenesis of diabetic complications is a complex process. It involves oxidative stress and ROS-activated pathways including several inflammatory proteins, cytokines and growth factors. It is clear that hyperglycemia exerts long-term injurious effects in patients with type 1 and type 2 diabetes mellitus. Similar long-lasting detrimental effects of hyperglycemia also occur in animals (dogs). In both animals and humans with diabetes, glycemic control only delays the development or progression of diabetic complications. It does not prevent or completely restore diabetic complications. This is understandable in view of the fact that these diabetic complications are not primary effects of hyperglycemia but are its sequelae (secondary effects). In fact, in few cases in which normalization of hyperglycemia or cure of diabetes mellitus do prevent diabetic complications, it has to be initiated at a very early stage of the disease and maintained for several years. Overall, these findings clearly show that intensive therapy or normalization of hyperglycemia does not prevent diabetic complications.

6.3 Why does reduced or intensive therapy of hyperglycemia not prevent diabetic complications?

Investigators have introduced different phrases to describe this concept or observation in which reduced or intensive therapy of hyperglycemia does not prevent diabetic complications. Some of these phrases include ''glycemic memory", "hyperglycemic memory", "metabolic memory'' or "lasting memory". Glycemic memory is a term that refers to the development of diabetic complications during post-hyperglycemic normoglycemia

(Nishikawa et al., 2000a). It is a phenomenon whereby early glycemic milieu or environment is remembered in many target organs such as heart, eye, nerve and kidney. Evidence suggests that oxidative stress may contribute to the inability of reduced or intensive treatment of hyperglycemia to prevent diabetic complications (Ceriello et al., 2009). It is proposed that glycemic memory could involve two stages: induction and perpetuation (Nishikawa et al., 2000a). During induction, hyperglycemia generates increased ROS levels. This may result from increased production of reducing equivalents formed from overdrive of the mitochondrial ETC. As a result of increased ROS, cellular dysfunction and mutations in mitochondrial DNA may occur (Wei, 1998). On the other hand, perpetuation, which is glycemic memory itself, could occur because ROS-induced mutated mitochondrial DNA would encode defective ETC subunits (Nishikawa et al., 2000a). This hypothesis of defective mitochondrial DNA subunits seems valid. Two years after this hypothesis, a study demonstrated that methylglyoxal modified mitochondrial proteins causing disturbances in mitochondria in kidney of rats (Rosca *et al.*, 2002). The study further showed that methylglyoxal produced an inhibitory effect on the tricarboxylic acid (TCA) cycle and the electron respiratory chain in kidney of rats. In another related study, it was shown that methylglyoxal-modified mitochondria considerably augmented $O_2^{\bullet -}$ production,, independently of the level of hyperglycemia, and were characterized by oxidative damage (Rosca *et al.*, 2005).

Furthermore, Ihnat and colleagues (2007) also provided evidence in support of the role of oxidative stress in mediating the hyperglycemic memory (Ihnat et al., 2007). The authors showed that in human endothelial and ARPE-19 retinal cells, the levels of protein kinase C-beta, NAD(P)H oxidase subunit p47phox, BCL-2-associated X protein, 3-nitrotyrosine, ffibronectin and poly (ADP-ribose) remained elevated for 1 week after the levels of glucose had normalized. The study showed that inhibition of ROS production using antioxidant alpha-lipoic acid prevented the induction of these glucose-induced oxidative stress markers (Ihnat et al., 2007). Similar findings were also reported in aortic endothelial cells both *in vitro* and in non-diabetic mice (El-Osta et al., 2008). The study showed that short-term hyperglycemic spikes produced long-lasting effects on vascular cells (El-Osta et al., 2008). This suggests that transient spikes of hyperglycemia may be an HbA1c-independent risk factor for diabetic complications (El-Osta et al., 2008). A recent study also indicated that elevated glucose levels caused continual changes in cell viability and apoptosis-related gene expressions even after recovery of normoglycemia (Wei et al., 2011). The changes were associated with increased ROS production (Wei et al., 2011). Besides, evidence implicates the role of protein glycation and formation of AGEs in metabolic memory (Genuth et al., 2005, Ceriello et al., 2009). AGEs may reduce NADPH and impair antioxidant system which in turn can generate more ROS (Ahmed, 2005). Data from the DCCT suggest that the tendency of collagen to be glycated is less dependent on the level of blood glucose (Monnier et al., 1999). It was also demonstrated that both conventional and intensive therapies did not prevent protein glycation and formation of AGEs (Genuth et al., 2005). The data further revealed that the incidences of retinopathy and nephropathy were significantly associated with the levels of protein glycation and AGEs (Genuth et al., 2005). This phenomenon termed "glycemic memory" has also been corroborated in several other studies (Nathan et al., 2005, Holman et al., 2008).

Recent findings indicate that elevated oscillating glucose concentrations may contribute to increased risk for cardiovascular diabetic complications (Ceriello et al., 2008). As

demonstrated by Ceriello and colleagues (2008), using a euinsulinemic hyperglycemic clamp, glucose at 5, 10, and 15 mmol/l was given in rising steps as a single "spike", and oscillating between basal and high levels over 24-hour in non-diabetic subjects and type 2 diabetic patients. The authors reported that glucose at 10 and 15 mmol/l led to a concentration-dependent fasting blood glucose-independent impaired endothelial function and increased intensity of oxidative stress in both non-diabetic subjects and type 2 diabetic patients (Ceriello et al., 2008). The study also revealed that oscillating glucose concentrations between 5 and 15 mmol/l every 6 hour for 24 hours caused further significant impairments in endothelial function and induced oxidative stress compared with continuous 10 or 15 mmol/l glucose (Ceriello et al., 2008). These findings are very relevant because the current management of diabetes mellitus (including intensive therapy) does not control postprandial hyperglycemia. Besides, both endothelial function and oxidative stress are implicated in the pathogenesis of diabetic micro- and macrovascular complications. Oxidative stress also plays an important role in endothelial dysfunction (Sies, 1991, Halliwell and Gutteridge, 2007). This study is also remarkable because it shows that oscillating levels of glucose result in endothelial dysfunction and induced oxidative stress even in non-diabetic subjects (euglycemia). These postprandial hyperglycemia fluctuations may contribute to the continuous deterioration of diabetic complications, despite restoration of euglycemia. Therefore, postprandial hyperglycemia-induced endothelial dysfunction and oxidative stress may explain the mechanism of glycemic or hyperglycemic memory.

The findings indicate that ROS-induced mutated DNA mitochondria may continuously generate RS (Nishikawa et al., 2000a). The data also reveal that AGEs can modify or glycate protein content of mitochondrial TCA cycle and the electron respiratory chain (Ceriello, 2009, Ceriello et al., 2009). Irrespective of the level of glycemic control, all these will generate RS and trigger cellular injury. Hence, it does suggest that oxidative stress in diabetes mellitus can exist as an independent entity. As explained previously, oxidative stress is also implicated in other disorders characterized by euglycemia. Since hyperglycemia enhances the pathogenic pathways of diabetic complications via oxidative stress, therefore, whether hyperglycemia is normalized or the recommended optimal glycemic goal ≤ 6.5% HbA1c is achieved and/or maintained, the already existing oxidative stress can activate these same mechanistic pathways and thus propagate glycemic memory (induce diabetic complications).

6.4 Could there be a role of antioxidants in the management of diabetic complications?

Since oxidative stress is implicated in the pathogenesis of diabetes and its complications, there ought to be a place for antioxidants in the treatment of diabetes mellitus. However, previous clinical trials using antioxidants have yielded both promise and inconsistent results. Even though the data from the large scale clinical trials are inconclusive, it is noteworthy that many of those clinical trials were characterized by inappropriate study designs or several limitations (Johansen et al., 2005, Penckofer et al., 2002, Wierzba, 2005, Robinson et al., 2006, Willcox et al., 2008). These include: trials did not address specific diabetic populations; some studies included both healthy and unhealthy subjects; no data establishing the occurrence of oxidative stress in the patients before treatment and comparing such data with those obtained after treatment; the short duration of treatment; most of the trials were performed with vitamins A, C and E without consideration for other

antioxidants; the use of vitamin E supplementation without the concurrent use of vitamin C (Johansen et al., 2005, Penckofer et al., 2002, Wierzba, 2005, Robinson et al., 2006, Willcox et al., 2008). Other limitations include: some trials are gender-specific (comprising either men or women); lack of pharmacokinetic data of these antioxidants, before and after treatment, so as to ascertain if these antioxidants reached the target cells/tissues in adequate concentrations; no data to show that effects of different doses of each antioxidant were investigated so as to obtain and select optimal dose; the suppression of gamma-tocopherol by alpha-tocopherol; vitamins inappropriately administered relative to meal ingestion; and poor patient compliance are some of the issues that may contribute to the failure of antioxidants in clinical studies (Johansen et al., 2005, Penckofer et al., 2002, Wierzba, 2005, Robinson et al., 2006, Willcox et al., 2008). Some endpoints that were not directly related to oxidative stress such as mortality were used in some trials (Johansen et al., 2005). Hence, limited research and findings are available on the effects of antioxidants in diabetic patients. However, available evidences in small or medium sample-sized diabetic studies, both experimental and clinical, suggest antioxidants might play a role in the management of diabetes mellitus.

In diabetic rats with neuropathy, α-lipoic acid supplementation ameliorated oxidative stress parameters and improved lesions of diabetic neuropathy such as conduction velocity of the digital nerve, deficits in nerve conduction and nerve blood flow (Coppey et al., 2001). Evidence suggests that antioxidants can inhibit some of the pathways of diabetic complications such as protein kinase C-signaled increases in TGF-β in mesangial cells (Scott and King, 2004). Similarly, antioxidant treatment prevented the elevation in the levels of protein kinase C-beta, NAD(P)H oxidase subunit p47phox, BCL-2-associated X protein, 3-nitrotyrosine, fibronectin and poly (ADP) ribose in the retina of diabetic rats (Ihnat et al., 2007). In the kidney of diabetic rats, honey supplementation considerably reduced hyperglycemia, attenuated antioxidant enzymes, ameliorated oxidative stress markers and reduced mesangial matrix expansion and glomerular basement membrane thickness (Erejuwa et al., 2010a, Erejuwa et al., 2010c).

In patients with type 1 diabetes, supplementation with vitamins E and/or C combination ameliorated oxidative stress and improved endothelium-dependent vasorelaxation (Johansen et al., 2005, Rahimi et al., 2005). A study found that supplementation with combined chromium (Cr) and vitamins C and E ameliorated oxidative stress, reduced fasting blood glucose, HbA1c and insulin resistance in type 2 diabetes (Lai, 2008). Similarly, a recent study reported that vitamin E supplementation significantly reduced malondialdehyde (MDA) levels and increased the concentrations of GSH and vitamin E in type 1 diabetic patients (Gupta et al., 2011). The study also found a negative correlation between oxidative stress marker (MDA) and antioxidants (vitamin E and GSH) and a positive correlation between exogenously administered antioxidant (vitamin E) and endogenous antioxidant (GSH) (Gupta et al., 2011). However, the study showed that vitamin E supplementation in type 1 diabetic patients did not produce significant effects in metabolic parameters (Gupta et al., 2011). In patients with type 1 diabetes mellitus, vitamins C and E supplementation ameliorated oxidative stress markers, improved vascular dysfunction, retinal blood flow and creatinine clearance (Scott and King, 2004). These studies indicate that antioxidants could play a role in the management of diabetes mellitus. However, considering that diabetes mellitus is a disorder with multiple etiology and metabolic derangements, antioxidant supplementation alone is likely to be less effective.

This is corroborated by findings of Gupta and co-workers (2011). That could also explain the failure of antioxidants in clinical trials.

6.5 Could there be a role of hypoglycemic drugs (and/or insulin) and antioxidants in the management of diabetic complications?

A closer look at the various mechanistic pathways implicated in the pathogenesis of diabetic complications indicates that hyperglycemia-enhanced polyol pathway (via depletion of intracellular NADPH) and $O_2^{\bullet-}$ inhibition of G6PDH (via hyperglycemia-enhanced $O_2^{\bullet-}$ overproduction) would play a major role in impairing antioxidant defenses. This will increase intracellular susceptibility to oxidative stress during diabetes. As regards evidence, very few studies have investigated the effects of combined hypoglycemic agents and antioxidants in diabetic rodents or patients. Interestingly, all these studies found beneficial effects of combination of these two agents in both animals and human with diabetes mellitus. A study that investigated the effects of 4-week insulin and/or antioxidant (vitamin E and C) supplementation in diabetic rats found that the antioxidant treatment improved some of the oxidative stress parameters whereas insulin treatment prevented weight loss and ameliorated the activities and expression of antioxidant enzymes. In contrast, the combination of insulin and antioxidants resulted in normalization of all measurements including oxidative stress parameters (Sindhu et al., 2004).

Similarly, comparison of the effects of glibenclamide alone or combined with honey in pancreas of diabetic rats indicated that, even though glibenclamide reduced hyperglycemia, it only partially ameliorated oxidative stress parameters (most of the data were insignificant) (Erejuwa et al., 2011b). However, the combination of glibenclamide and honey significantly reduced hyperglycemia and ameliorated oxidative stress parameters in pancreas of diabetic rats (Erejuwa et al., 2011b). A similar study also showed that a combination of glibenclamide and metformin produced a limited antioxidant effect compared to when they were combined with honey in pancreas of diabetic rats (Erejuwa et al., 2010b). In the kidney of diabetic rats treated with metformin and/or glibenclamide, impaired antioxidant defenses were reported. In contrast, metformin and/or glibenclamide combined with honey significantly ameliorated oxidative stress parameters and restored the activities of antioxidant enzymes in the kidney of diabetic rats (Erejuwa et al., 2011a).

In type 1 and type 2 diabetic patients, a study found that while optimal glycemic control reduced the levels of MDA and increased the levels of GSH and vitamin E, it did not normalize the oxidative stress parameters (Chugh et al., 1999). However, after 4 weeks of vitamin E supplementation, the levels of oxidative stress markers were further reduced while those of endogenous antioxidants were increased compared to the optimal control values (without antioxidant treatment) (Chugh et al., 1999). A similar beneficial effect of vitamin E supplementation and optimal glycemic control was also reported in type 2 diabetic patients (Sharma et al., 2000). A number of other studies have also shown that antioxidants reduce glucose levels, improve insulin secretion and insulin resistance during diabetes (Penckofer et al., 2002). Moreover, a study found that in type 1 diabetic patients, normalization of glucose levels did not ameliorate hyperglycemia-induced endothelial dysfunction (Ceriello et al., 2007). The authors reported that, neither insulin nor vitamin C was able to ameliorate oxidative stress or normalize endothelial dysfunction (Ceriello et al., 2007). On the contrary, combination of insulin and vitamin C significantly decrease the

intensity of oxidative stress and normalized endothelial dysfunction in type 1 diabetic patients (Ceriello et al., 2007).

A critical analysis of the mechanistic pathways of hyperglycemia-induced diabetic complications indicates that they are all characterized by increased formation of ROS and impaired antioxidant defense network, which would further exacerbate oxidative stress and damage. In other words, these pathways begin and end with oxidative stress. Besides, evidence indicates that postprandial hyperglycemic fluctuations can cause endothelial dysfunction and induce oxidative stress in diabetic subjects and even in euglycemic subjects. Hence, findings from these studies clearly indicate that it is oxidative stress all over in diabetes mellitus and its complications. Moreover, the data from experimental and clinical studies indicate that there is a role for co-administration of hypoglycemic drugs or insulin and antioxidants in diabetes mellitus. It is possible that normalization of hyperglycemia may be achieved with hypoglycemic drugs, insulin, their combinations or even via pancreatic transplant. However, in patients with diabetic complications, whether euglycemia is achieved and/or maintained, oxidative stress (and oxidative stress-induced sequelae) becomes an independent entity. Thus, oxidative stress, as a possible independent entity, in diabetes mellitus necessitates antioxidant therapy. On account of these observations, findings and data, there seems little doubt that antioxidant therapy or other therapeutic intervention of oxidative stress in combination with hypoglycemic drugs or insulin should result in better management of diabetes mellitus. This should also prevent or reduce ROS-linked diabetic complications.

6.6 Other potential beneficial effects of hypoglycemic drugs (and/or insulin) and antioxidants in diabetes mellitus

Diabetes mellitus is characterized by impairments in renal and hepatic function as well as impaired metabolism of glucose, lipid and protein. Lipid abnormalities and induction of oxidative stress enhance the oxidation and glycation of low-density lipoproteins (LDLs), thereby exacerbate endothelial dysfunction (Penckofer et al., 2002). Studies have shown that antioxidants and some hypoglycemic drugs can prevent oxidation of LDL (Fava et al., 2002, Maritim et al., 2003, Rahimi et al., 2005). Besides, evidence indicates that antioxidants can ameliorate lipid abnormalities (Rahimi et al., 2005, Erejuwa et al., 2011d). The beneficial effects of antioxidants on glycemic control (blood glucose, fructosamine and glycosylated hemoglobin) in diabetes have also been documented (Rahimi et al., 2005, Lai, 2008, Erejuwa et al., 2010a; 2011d). Furthermore, antioxidants improve C-peptide and insulin levels as well as insulin resistance in diabetes mellitus (Rahimi et al., 2005, Lai, 2008, Erejuwa et al., 2011d). Co-administration of hypoglycemic drugs (glibenclamide or metformin) and antioxidant (honey) considerably improved glycemic control and lipid parameters in diabetic rats more than the effects produced by individual hypoglycemic drug (Erejuwa et al., 2011d). It is worth mentioning that other non-antioxidant constituents of honey, such as fructose and oligosaccharides, might contribute to this improved glycemic control and lipid parameters. In addition, antioxidants ameliorated and improved impaired renal function which has been documented in diabetes mellitus (Slyvka et al., 2009, Erejuwa et al., 2011d) or in combination with hypoglycemic drugs may produce synergism (Erejuwa et al., 2011d).

In the duodenum and jejunum of diabetic rats, a number of alterations in the brush border membrane (BBM) fluidity, non-enzymatic glycation, oxidative stress and damage have been

reported (Bhor and Sivakami, 2003). Similarly, increased protein glycation and lipid peroxidation might exacerbate diabetes-related alterations in BBM fluidity (Watala and Winocour, 1992). Therefore, antioxidants may ameliorate intestinal oxidative stress and improve BBM fluidity, thereby promote healing and enhance gastrointestinal tract health in diabetes mellitus. This might impact positively on glycemic control. Antioxidants may also augment bioavailability of essential macronutrients or co-administered hypoglycemic drugs (Faria et al., 2009). Furthermore, antioxidants may help to ameliorate liver damage and hepatic oxidative stress which are common in diabetes mellitus (Dias et al., 2005, Erejuwa et al., 2012b). Considering the role of liver in glucose homeostasis and the fact that some hypoglycemic agents (e.g. glibenclamide) mediate their effects via liver, these hepatic effects of antioxidants combined with those of hypoglycemic drugs may enhance liver functions and contribute to improved glycemic control. Moreover, evidence suggests that the use of antioxidants is associated with reduced weight gain (Razquin et al., 2009, Erejuwa et al., 2012a), therefore, co-administration of antioxidants and hypoglycemic agents, especially glibenclamide, may be beneficial in type 2 diabetic patients, majority of whom are obese. Majority of diabetic patients end up developing hypertension which further increases the risk of developing diabetic complications including cardiovascular events. Interestingly, oxidative stress is also implicated in the pathogenesis and/or complications of hypertension. Therefore, a combination of hypoglycemic drugs and antioxidants, via improved glycemic control and amelioration of oxidative stress, may help to prevent or delay the development of hypertension and diabetic complications (Erejuwa et al., 2011c, 2012a). Besides, the combination of both agents may help to minimize the adverse effects or toxicities of hypoglycemic agents. The use of antioxidants may necessitate lower doses of hypoglycemic agents to achieve the same therapeutic effect, thereby limiting the side or adverse effects of these drugs.

7. Conclusions

These studies indicate that hyperglycemia exerts long-term injurious effects in patients with type 1 and type 2 diabetes mellitus. Similar long-lasting detrimental effects of hyperglycemia also occur in diabetic animals. In both animals and humans with diabetes, glycemic control only delays the development or progression of diabetic complications. It does not prevent or completely restore diabetic complications. In few cases in which good glycemic control or cure of diabetes does prevent diabetic complications, it has to be initiated at a very early stage of the disease and maintained for several years. This is probably impracticable in the larger diabetic population. Evidence indicates it is not easy to achieve and/or maintain glycemic control in many diabetic patients close to the physiological range commonly observed in their healthy counterparts. Together with recent findings which demonstrate the deleterious effect of intensive therapy of hyperglycemia, it can be inferred that any therapeutic option that target hyperglycemia alone is not only limited and ineffective but may also be detrimental in diabetic patients. In view of the alarming rate of global prevalence of diabetes mellitus and the associated complications, morbidity and mortality, there is an urgent need for a better or new therapeutic management. While efforts are being made by researchers and scientists to unravel the main cause(s) of diabetes mellitus, it is high time clinicians, physicians and diabetologists began to look for an alternative and/or a complementary therapy to the current management of diabetes mellitus.

At the moment, one of such options or alternatives is the prospective of managing diabetes mellitus by targeting both hyperglycemia and oxidative stress simultaneously. As the data presented in this chapter have revealed, co-administration of oral hypoglycemic drugs (and/or insulin) and antioxidants might prove to be a better therapy in the management of diabetes mellitus. This is because, with the combination, hypoglycemic drugs (and/or insulin) will target hyperglycemia to improve glycemic control and reduce hyperglycemia-enhanced ROS production. In addition, administration of antioxidants will help to scavenge or eliminate RS including those generated in the various pathways highlighted in this chapter. Besides, many antioxidants have hypoglycemic effect which may also contribute to improved glycemic control. The co-administration of these two agents will help to minimize the level of oxidative stress in the vasculature and other targets of diabetic complications such as kidney (reducing diabetic nephropathy), retina (reducing diabetic retinopathy), nerves (reducing diabetic neuropathy) and heart (reducing diabetic cardiomyopathy). Moreover, evidence has shown that the pancreas and the liver are also target of oxidative stress in diabetes mellitus. Hence, co-administration of antioxidants will help to ameliorate oxidative stress in these tissues and organs (pancreas and liver) which play key roles in glucose homeostasis in diabetes mellitus.

In addition to improved glycemic control and amelioration of oxidative stress, evidence suggests that co-administration of hypoglycemic drugs and antioxidants may exert other beneficial effects on gastrointestinal tract, lipid profile, renal function and others which will contribute to better management of diabetic patients. Considering the limitations with antioxidants, coupled with the latest advances in our understanding of the various mechanisms involved in ROS formation, other interventions such as inhibition of the mitochondrial ROS overproduction, developing mitochondria-targeted antioxidants, blockage of hyperglycemia-induced mechanistic pathways are viable therapeutic options. This chapter has shown that the prospective of managing diabetes mellitus more effectively by targeting both hyperglycemia and oxidative stress simultaneously holds much promise. This new therapeutic option is worth investigating in patients with diabetes mellitus. Hence, both small and large, well designed, randomized clinical trials that examine the effect of combination of hypoglycemic drugs (and/or insulin) and specific antioxidants in patients with type 1 or type 2 diabetes mellitus are recommended. This may revolutionize the management of diabetes mellitus, at least in the interim, while attempts are being made to discover its main cause(s) and develop more potent and effective antidiabetic drugs.

8. Dedication

This chapter is dedicated to the memory of my dad, Educator Sephaniah Adeyemi Erejuwa, who battled diabetes mellitus and later succumbed to its complications. It is also dedicated to millions of people globally who are suffering from this disorder and its complications

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11

Diabetes, Oxidative Stress, Antioxidants and Saliva: A Review

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1. Introduction

Diabetes mellitus is a devastating disease throughout the world. It has been estimated that the number of peoples affected with diabetes in the world will increase to 300 million by 2025 (1). Diabetes is associated with several mechanisms, one of which is oxidative stress. Increased oxidative stress is a widely accepted participant in the development and progression of diabetes and its complications (2,3). Oxidative stress is a general term used to describe the imbalance between the production and manifestation of reactive oxygen species and a biological system's ability to ready detoxify the reactive intermediates or to repair the resulting damage (4). Oxidative stress occurs when free radical production exceeds the body's ability to neutralize them. The imbalance may be due to either: decrease production of antioxidants; or excessive production of free radicals. In diabetes, free radicals are formed disproportionately by glucose oxidation, non-enzymatic glycation of proteins and the subsequent oxidative degradation of glycated proteins (5). Abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms can lead to damage of cellular organelles and enzymes, increased lipid peroxidation and development of insulin resistance (6). These consequences of oxidative stress can promote the development of complications of diabetes mellitus.

2. Sources of oxidative stress in diabetes

There are multiple sources of oxidative stress in diabetes including non enzymatic, enzymatic and mitochondrial pathway.

Non enzymatic sources of oxidative stress originate from the oxidative biochemistry of glucose. Hyperglycemia can directly cause increased Reactive Oxygen Species (ROS) generation. Glucose can undergo autooxidation and generate hydroxyl *OH- radicals (7). In addition, glucose reacts with proteins in a non enzymatic manner leading to the formation of advanced glycation end products (AGEs). ROS is generated at multiple steps during this process. In hyperglycemia, there is enhanced metabolism of glucose through the polyol (sorbitol) pathway, which also results in enhanced production of superoxides (*O2-) .

Enzymatic sources of augmented generation of reactive species in diabetes include Nitrous Oxide Species (NOS), NAD(P)H oxidase and xanthine oxidase (8-10). The mitochondrial

respiratory chain is another source of non enzymatic generation of reactive species. Hyperglycemia-induced generation of $*O_2^-$ at the mitochondrial level is the initial trigger of vicious cycle of oxidative stress in diabetes (11,12).

3. Saliva as diagnostic fluid

Saliva in humans is a mouth fluid possessing several functions involved in oral health and homeostasis, with an active protective role in maintaining oral health. It plays a role in the preliminary digestion of food, fascilitates taste perception, maintains teeth enamel mineralization, buffers the acid components of food, and antimicrobial functions. The assay of saliva is an increasing area of research with implications of basic and clinical purposes. Recently, the use of saliva has provided a substantial addition to the diagnostic armamentarium as an investigative tool for disease processes and disorders. In addition to its oral indications, the analysis of saliva provides important information about the functioning of various organs within the body. Saliva analyses have been used mainly in dentistry and for studies in oral disease to help assess the risk of caries, by measuring buffering capacity and bacterial contents (13). Oral fluid is mainly utilized for research and diagnostic purposes concerning systemic diseases such as diabetes.

The determination of the oxidative stress and antioxidants require sometimes invasive techniques such as venepuncture. Whole saliva is an important physiologic fluid that contains a highly complex mixture of substances. Variable amounts of blood, serum markers that accurately reflect the redox status of the body can be determined in saliva and may have great clinical interest. The assay of salivary oxidative stress parameters has brought substantial insight into the pathogenesis and evolution of many systemic diseases including diabetes.

4. Mechanisms for increased oxidative stress in diabetes

1. Advanced glycation end products (AGEs):

AGEs are products of glycation and oxidation (glycol-oxidation), which are increased with age, and at accelerated rate in diabetes (14,15). The formation of AGEs is an important biochemical abnormality that accompanies diabetes mellitus. AGEs initiate oxidative reactions that promote the formation of oxidized LDL. Interaction of AGEs with endothelial cells as well as other cells accumulating within the atherosclerotic plaque, such as mononuclear phagocytes and smooth muscle cells provides a mechanism to augment vascular dysfunction (16)

Nuclear magnetic resonance spectra of AGEs were determined in saliva of 52 consecutive patients with diabetes mellitus and 47 age-matched healthy control subjects. Resonance spectra showed specific peaks at 2.3, 7.3, and 8.4 ppm in saliva from patients with diabetes mellitus, indicating the presence of advanced glycation endproducts which was associated with approximal plaque index. (17).

In a study of Garay-Sevilla et al (18) who measured AGEs in skin, serum and saliva of diabetic patients with complications they concluded that the AGEs measurement in saliva is useful to evaluate diabetes complications.

2. Alteration in glutathione metabolism:

Reduced glutathione detoxify reactive oxygen species such as hydrogen peroxide and lipid peroxide directly or in a glutathione peroxidase (GPX) catalyzed mechanism. Glutathione reductase (GRD) catalyzes the NAD(P)H dependent reduction of oxidized glutathione, serving to maintain intercellular glutathione stores and a favorable redox status (19).

Blood GSH was significantly decreased in different phases of type 2 diabetes mellitus such as: glucose intolerance and early hyperglycemia (20) and poor glycemic control (21)

Measurement of salivary GPX and GRD activities and GSSG/GSH ratio, provide a non-invasive method to assess the degree of oxidative stress in pathophysiologic status, such as diabetes (22).

The decrease in salivary reduced-glutathione levels in patients with type 1 DM may have a role in periodontal tissue destruction by predisposing tissues to oxidative stress.(23). Our previous study (24) identified GSH activity in serum and saliva of patients with type 2 diabetes which was significantly low when compared with control group. This finding was explained on the basis that oxidative stress may consumes some naturally occurring local antioxidants such as reduced glutathione and this reflects the overwhelming adaptive response to the challenge of oxidative stress in the diabetic state with or without complications

3. Impairment of SOD and catalase activity:

SOD and catalase are also major antioxidant enzymes, SOD exists in 3 different isoforms; Cu,Zn-SOD is mostly in the cytosol and dismutate superoxide to hydrogen peroxide, Extracellular SOD is found in the plasma and extracellular space and Mn-SOD is located in mitochondria. Catalase is H_2O_2 decomposing enzyme mainly localized to peroxicomes or microperoxicomes. Superoxide may react with other reactive oxygen species such as Nitric Oxide to form highly toxic species such as peroxynitrite (25).

The major reason for the decreased SOD activity is the glycosylation of Cu,Zn-SOD which has been shown to lead to enzyme inactivation both in vivo and in vitro (26). Salivary SOD was measured in saliva (27) .Belce et al suggested that the main reason for the decrease of salivary SOD activity may be increased glycation of the enzyme and/or deleterious effect of increased free oxygen radicals by glycated proteins on SOD activity in diabetes which could lead to oral complications in diabetic patients.However; Al-Rawi study (24) demonstrated an increase in the level of SOD in serum and saliva of diabetic patients, this increase could be due to the existance or increased free radicals production which could enhance the antioxidant defense system that couter-balance the pro-oxidant environment.

4. Polyol Pathway:

The polyol pathway consists of two enzymes. The first enzyme, aldose reductase (AR), reduces glucose to sorbitol with the aid of its co-factor NADPH, and the second enzyme, sorbitol dehydrogenase (SDH), with its co-factor NAD^+, converts sorbitol to fructose. In animal models, treatment with AR inhibitors (ARI) was shown to be effective in preventing the development of various diabetic complications, including cataract, neuropathy, and nephropathy (28). The possibility of determination of sorbitol and fructosamine in saliva has been studied in healthy volunteers and patients with diabetes. It was concluded that saliva

sorbitol and fructosamine levels measurements may be used as diagnostic tests in diabetes and serve as indicators of efficacy of therapy in diabetes (29).

5. Lipid peroxidation and protein oxidation in diabetes

Lipid peroxidation: Lipid peroxidation end-products very commonly detected by the measurement of thiobarbituric acid reactive substance (TBARS). The use of TBARS as an index of lipid peroxidation has been increased in plasma of diabetic patients (30-35). Thiobarbituric acid reacting substances (TBARS) are produced during lipoperoxidation-oxidative stress-induced damage of lipids and are, thus, a widely used marker of oxidative stress (36,37). However, they represent a heterogeneous group of compounds – best known is malondialdehyde (MDA). TBARS is associated with parodontopathies when measured directly in the injured gingival tissue (38). In previous studies we have shown that TBARS can be found in measurable concentrations in saliva and that these levels are higher in patients with parodontopathies and their origin is unlikely to be plasma (39,40). Whether the difference in patients is caused by a rise of MDA or instead of and which other factors influence salivary TBARS levels is unknown.(40) thus, assume, that salivary TBARS may reflect the local oral oxidative stress, although the producer is still hidden (41). Salivary MDA levels are directly affected by sytemic oxidative stress, since MDA levels were also elevated in saliva of diabtetic patients without parodontopathies (24). Astaneie et al (42) have reported no difference in salivary versus serum MDA levels and presence of high Antioxidant activity (AOA) in type 1 diabetics. Studies conducted on diabetic rats have reported an increase in salivary and serum MDA with variable antioxidant activity (43). Celec et al (44) have found an increase in MDA levels in non diabetics which was attributed to age, altered periodontal status and smoking. Hodosy et al (45) suggest that MDA levels depend on the time of sampling and also are affected by factors like tooth brushing and antioxidative therapy received by the patients. Studies by Reznick et al (46)and Astaneie et al (42) have shown both salivary and serum antioxidants to increase depending on HbA1C levels and severity of diabetes. The AOA levels of both the groups did not show notable correlation with Fasting Plasma Glucosa (FPG) but a significant correlation existed between salivary MDA and FPG levels in the diabetic group.

6. Diabetes and antioxidants

Antioxidants are substances that inhibit the destructive eefects of oxidation. Some of the general antioxidants that are known are glutathione effects, glutathione peroxidase, vitamins A,C,E, catalase and SOD. The decreased efficiency of antioxidant defenses (both enzymatic and non-enzymatic) seems to correlate with the severity of pathological tissue changes in type 1 diabetes (47).

Administration of the antioxidants, for example, the vitamin C and free amino acids, get a better reaction to insulin and can supply extra benefit to the proposed reduction of oxidative stress in tissues (48,49). Experimental study on diabetic rats suggested that nutritional vitamin E supplementation helps fatty acids metabolism and lower lipid peroxidation in rat tissues (50). Oral vitamin C and vitamin E has the ability to lower the oxidative stress in eye (51) and the vascular endothelia function get better in type1 and not type 2 diabetes (52). Vitamin C and Vitamin E, probably have an important role in reducing the oxidative

damage produced by nitric oxide and other free radicals. The estimation of vitamin levels and other antioxidants in saliva could provide a good insight about the body function against oxidative stress and it can be used to monitor therapy.

7. Conclusion

The saliva matrix is an upcoming area of research for basic and clinical application purposes, with considerable potential for growth and progress. Nevertheless, to date salivary assays are still little used compared with plasma assays, even it is possible to have a quantitative estimate of oxidative stress markers and antioxidants in saliva.

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12

Oxidative Stress and Novel Antioxidant Approaches to Reduce Diabetic Complications

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1. Introduction

In 1995, the International Diabetes Federation estimated the prevalence of diabetes to be approximately 135 million patients worldwide. More recently in 2010, it was estimated that around 285 million people were diabetic and this number is predicted to reach 438 million by 2030, accounting for 7.7% of the population aged 20-79 (Shaw et al 2010). However, despite greater knowledge of the disease, approximately one-third of people with diabetes remain undiagnosed. Although intensive blood glucose and blood pressure control have reduced the risk of diabetes-associated microvascular (nephropathy, retinopathy, neuropathy) and macrovascular complications (atherosclerosis), diabetes remains a major risk factor for cardiovascular complications, cardiomyopathy, end-stage renal disease (ESRD), blindness and neuropathy. There is therefore an urgent need to develop more effective therapeutic strategies to prevent and/or halt the progression of diabetic complications.

Accumulating evidence suggests that oxidative stress plays a pivotal role in the aetiology of diabetic complications. Many biochemical pathways associated with hyperglycaemia increase the production of free radicals leading to oxidative stress, including glucose auto-oxidation, the polyol pathway, prostanoids synthesis, protein glycation and the protein kinase C (PKC) pathway (Giugliano et al 1996). Hyperglycaemia alters reactive oxygen species (ROS) production, particularly in the mitochondria, leading to increased intracellular ROS and activated stress-sensitive pathways such as nuclear factor κB (NFκB), p38 mitogen-activated protein kinase (MAPK), and the c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathways (Johansen et al 2005). Subsequently, PKC activity, advanced glycation end-products (AGE) and sorbitol levels increase and this can lead to more ROS generation in a positive regulatory feedback loop to chronically stimulate stress-sensitive pathways. ROS can also inflict direct damage upon cellular macromolecules which, in turn, result in further oxidative stress (Evans et al 2002).

Under physiological conditions, reactive oxygen and reactive nitrogen species (RNS) are produced and maintained at steady-state levels within a cell (Lushchak 2011). On the other hand, oxidative stress arises when an imbalance occurs between the production of ROS/RNS and the antioxidant defences that neutralise them, shifting the balance in favour

of enhanced ROS levels. The consequence of this shift is cellular damage to biologically important molecules and organelles (Sies 1997). Elevations in ROS/RNS levels are mainly caused by an imbalance between the activity of endogenous pro-oxidant enzymes, such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase or the mitochondrial respiratory chain, and the antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), heme oxygenase (HO), thioredoxin (Trx) peroxidase/peroxiredoxin, catalase and paraoxonase (Forstermann 2008). ROS include the superoxide anion ($^{\bullet}O_2^-$) and the hydroxyl radical ($^{\bullet}OH$), as well as non-radical species such as hydrogen peroxide (H_2O_2). RNS include the free radicals nitric oxide ($^{\bullet}NO$) and non-radical species such as peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO_2)(Johansen et al 2005). In a hyperglycaemic milieu, $^{\bullet}O_2^-$ increases through the enhanced activity of enzymatic sources, including NADPH oxidase and xanthine oxidase, and non-enzymatic sources such as the mitochondrial respiratory chain, glucose autoxidation, AGE formation and activation of the polyol pathway. In addition, antioxidant defences are known to decrease in a hyperglycaemic milieu, shifting the balance away from steady-state levels of ROS towards an environment of oxidative stress.

2. Oxidative stress and diabetic complications

2.1 Diabetes-associated atherosclerosis

Atherosclerosis is a major cause of mortality and morbidity in patients with diabetes (Beckman et al 2002). The buildup of fat and cholesterol along the walls of arteries is progressive; it thickens and hardens, forming calcium deposits, and may eventually block the arteries. Blockage of the arteries and/or rupture of vulnerable plaques is a common cause of heart attack and stroke. Diabetes has been shown to accelerate the clinical course of atherosclerosis in the coronary arteries (coronary artery disease, including myocardial infarction), lower extremities (peripheral arterial disease) and extracranial carotid arteries (cerebrovascular disease, including stroke) (Beckman et al 2002).

An understanding of the underlying mechanisms that accelerate diabetes-associated atherosclerosis is important in the search for treatments to protect against or retard the progression of this disease. The abnormal metabolic state associated with diabetes, which includes chronic hyperglycaemia, dyslipidemia and insulin resistance can alter the function of multiple cell types including endothelial cells, smooth muscle cells and platelets. The single layer of endothelial cells that line the vessels of the circulatory system, provide a metabolically active interface between the blood and the underlying tissue to facilitate blood flow and nutrient delivery. Disruption of the integrity of the endothelium leads to inflammation, activation of platelets, coagulation, and thrombosis (Cines et al 1998). To protect against this, the endothelium synthesises important bioactive substances such as endothelial-derived NO (EDNO), prostaglandins, endothelin (ET) and angiotensin II (Ang II) that regulate blood vessel function and structure (Beckman et al 2002). It is now known that hyperglycaemia-mediated dysregulation of these vasoprotective agents either enhances the intensity of oxidative stress directly or is affected by oxidative stress. The consequences of hyperglycaemia-driven enhanced ROS/RNS on the vasculature will be discussed below.

2.1.1 The role of ROS in diabetes-associated atherosclerosis

Due to its vasorelaxation, anti-inflammatory and anti-proliferative properties, EDNO is often viewed as vasculoprotective. Diabetes is associated with an attenuation of EDNO bioavailability, which is lowered by either decreased formation or enhanced removal of $^{\bullet}NO$. One way in which hyperglycaemia attenuates the level of EDNO is by blocking the function of endothelial NOS (eNOS) synthase in endothelial and vascular smooth muscle cells (De Vriese et al 2000). However, evidence now points most strongly at the prevention of EDNO reaching its molecular target, rather than its attenuated production as being critical for the loss of EDNO bioavailability in diabetes. An increase in ROS, such as $^{\bullet}O_2^-$ within the endothelium is one of the most significant factors known to decrease EDNO (de Haan & Cooper 2011). Increased $^{\bullet}O_2^-$ can reduce EDNO bioavailability due to its propensity to react with $^{\bullet}NO$, producing the highly reactive oxidant, $ONOO^-$ (Beckman 1996). Loss of functional EDNO causes impaired relaxation of the vessel wall and inhibition of the proliferative effects of EDNO (Maritim et al 2003a). In addition, $ONOO^-$ can induce cell damage via lipid peroxidation, and the inactivation of enzymes and structural proteins by oxidation and nitration. Furthermore, $ONOO^-$ can activate matrix metalloproteinases (MMPs), trigger the release of pro-apoptic factors such as cytochrome c and induce DNA damage (Pacher & Szabo 2006). $ONOO^-$ is also involved in oxidising tetrahydrobiopterin (BH_4), an important cofactor of eNOS, thereby uncoupling eNOS which then produces $^{\bullet}O_2^-$ instead of EDNO (Maritim et al 2003a).

It is also known that a reduction in $^{\bullet}NO$, as evident in diabetes, stimulates endothelial angiotensin-converting enzyme (ACE) activity and the generation of Ang II and $^{\bullet}O_2^-$ (Schulman et al 2006). While EDNO inhibits the production of endothelin-1 (ET-1) which is a vasoconstricting peptide (Boulanger & Luscher 1990), increased Ang II can stimulate the endothelial cell to synthesise and release ET-1, thereby contributing to vascular smooth muscle dysfunction (Sasser et al 2002). A disruption in vascular smooth muscle function may lead to plaque destabilisation and rupture, with often fatal consequences (Beckman et al 2002). Since EDNO also limits inflammation by reducing leukocyte adhesion and migration (Chen et al 1998), lowering EDNO will also promote atherogenesis via accelerated pro-inflammatory pathways. Additionally, EDNO is also involved in the inhibition of platelet activation (Loscalzo 2001). A reduction in the bioavailability of EDNO in diabetes therefore potentiates platelet activation, adhesion and aggregate formation, leading to thrombosis. Hence, the increased presence of $^{\bullet}O_2^-$, as seen in diabetes, limits the protective effects of EDNO in the vasculature leading to increased inflammation, thrombosis, plaque destabilisation and plaque rupture (Fig.1).

The effects of hyperglycaemia-mediated increases in $^{\bullet}O_2^-$ levels in the mitochondria are numerous and mostly detrimental to the cells of the vasculature. For example, $^{\bullet}O_2^-$ has been shown to activate the nuclear enzyme poly(ADP-ribose) polymerase (PARP) which in turn leads to metabolic alterations that activate NFκB, AGE/receptor for AGE (RAGE) and the polyol pathways (Pacher & Szabo 2006). Upregulation of these pathways results in more $^{\bullet}O_2^-$ production, while NFκB activation increases the expression of many proinflammatory mediators, leading to endothelial dysfunction. Furthermore, it is well accepted that ROS such as $^{\bullet}O_2^-$ are involved in the oxidation of low-density lipoprotein (ox-LDL), which is not recognised by the LDL receptor and is preferentially taken up by scavenger receptors on macrophages, leading to foam cell formation and atherosclerotic plaques (Boullier et al 2001).

Finally, it is important to highlight that increased $^{\bullet}O_2^-$ via enzymatic antioxidant conversion (see Fig.2) gives rise to increased levels of H_2O_2, an important ROS implicated in pro-inflammatory processes that are further amplified as diabetes develops (Nicolls et al 2007). H_2O_2 has also been shown to upregulate vascular cell adhesion molecule-1 (VCAM-1), an important adhesion molecule that aids in the migration of leukocytes from the blood into the tissue (Cook-Mills 2006). It is therefore clear that elevations in hyperglycaemia-mediated ROS lead to cellular, molecular and functional vascular alterations that initiate, mediate and ultimately hasten cardiovascular complications associated with diabetes.

2.2 Diabetic cardiomyopathy

Diabetic cardiomyopathy is considered a distinct clinical entity first recognised by Rubler et al. (1972) in 4 diabetic patients with congestive heart failure without evidence of hypertension, coronary artery disease or congenital heart disease. The Framingham study showed that diabetic men and women had a 2- and 5-fold greater incidence of heart failure respectively, even after taking into account other common risk factors such as coronary artery disease, age, blood pressure, weight and cholesterol (Kannel et al 1974). Diabetic cardiomyopathy is characterised by early diastolic dysfunction and late systolic impairment, and is accompanied by a wide range of structural abnormalities and pathophysiological impairments (Hayat et al 2004). Despite intensive investigations, the aetiology of diabetic cardiomyopathy remains elusive.

2.2.1 The role of ROS in diabetic cardiomyopathy

Hyperglycaemia is known to upregulate the production of Ang II, which is the overt hormone of the renin-angiotensin system (RAS). This has a profound effect on the myocardium given that most of the cellular components of the RAS including angiotensinogen, renin and the angiotensin II type I (AT_1) receptor are found in myocytes (Fiordaliso et al 2000). Although Ang II is known to contribute to the development of diabetic cardiomyopathy through its hemodynamic vasoconstrictor effects and its ability to act as a proinflammatory mediator, it is now becoming increasingly clear that Ang II mediates its effects on the myocardium via its ability to enhance production of $^{\bullet}O_2^-$ (Cooper 2004). Indeed, Privratsky et al. (2003) found that hyperglycaemia induces cardiac myopathies via the AT_1 receptor, with activation of NADPH oxidase and increased ROS generation. Furthermore, $^{\bullet}O_2^-$ levels were attenuated with an ACE inhibitor (Fiordaliso et al 2006). However, other sources of ROS generation are known to contribute to the oxidative stress that accompanies diabetic cardiomyopathy. For example, treatment of diabetic mice with allopurinol, the xanthine oxidase inhibitor, improved type 2 diabetes-induced cardiac dysfunction by decreasing oxidative/nitrosative stress and fibrosis (Rajesh et al 2009).

The biochemical pathways described earlier for diabetes-associated atherosclerosis, including those leading to endothelial dysfunction and inflammation due to the overproduction of ROS, appear to play a causal role in the pathogenesis of diabetic cardiomyopathy (Fig.1). Several lines of evidence suggest that nitrosative stress and peroxynitrite-induced damage contribute to the pathogenesis of diabetic cardiomyopathies. In particular, Szabo et al. (2002) showed that a metalloporphyrin peroxynitrite decomposition catalyst, FP15, which neutralises $ONOO^-$, ameliorates cardiovascular

dysfunction in STZ-induced murine models of diabetes. Treatment with FP15 prevented loss of endothelium-dependent relaxant ability of blood vessels and improved both diastolic and systolic dysfunction of the diabetic heart, supporting the concept that neutralisation of $ONOO^-$ can be of significant therapeutic benefit. Furthermore, hyperglycaemia-mediated oxidative stress has been shown to cause abnormal gene expression, altered signal transduction, and the activation of pathways leading to myocyte apoptosis (Cai & Kang 2001). Myocyte death causes a loss of contractile units, compensatory hypertrophy of myocytes and reparative fibrosis, all characteristics of diabetic cardiomyopathy (Kang 2001). In a study by Frustaci et al. (2000), increased levels of nitrotyrosine were associated with apoptosis and necrosis of cardiac cells including myocytes, endothelial cells and fibroblasts in patients with diabetes, as well as in STZ-induced diabetic mice (Cai et al 2005). However, the mechanisms underlying hyperglycaemia-induced myocyte loss are still poorly understood.

Recently, it was shown that hyperglycaemia-induced oxidative stress results in the activation of the hexosamine biosynthetic pathway (HBP), leading to increased *O*-GlcNAcylation (endproduct of HBP) of the pro-apoptotic peptide, BAD. In this manner oxidative stress is linked to increased cardiac myocyte death (Rajamani & Essop 2010). Other known mediators of hyperglycaemia-induced cardiac cell death include AGEs (Li et al 2007), apoptosis signal-regulating kinase 1 (ASK1) (Thandavarayan et al 2008) and mitochondrial dysfunction (Duncan 2011). All of these pathways appear to induce myocyte apoptosis via an increase in oxidative stress in the diabetic heart. Myocyte loss and myocyte injury are believed to contribute to systolic dysfunction due to the impairment in the ability of the myocardium to develop force, resulting in reduced contractility, decreased pump function, and decreased ejection fraction (Fang et al 2004).

For destructive $ONOO^-$ to form within the diabetic myocardium, as detailed above, $^{\bullet}O_2^-$ must interact with and reduce the bioavailability of $^{\bullet}NO$. Since $^{\bullet}NO$ is one of the most important regulators of myocardial function, it is not surprising that hemodynamic studies have indicated that endothelium-dependent vasodilatory responses are impaired in the diabetic milieu (Farhangkhoee et al 2006). Indeed, the bioavailability of vasodilatory $^{\bullet}NO$ was found to be reduced with the progression of diabetic cardiomyopathy (Joffe et al 1999). In the heart, both eNOS and inducible NOS (iNOS) are the principal producers of $^{\bullet}NO$. Under physiological conditions, low levels of $^{\bullet}NO$ produced by eNOS increase diastolic relaxation and decrease oxygen consumption in cardiac myocytes, whereas high levels of $^{\bullet}NO$ produced by iNOS decrease the contraction of cardiac myocytes and induce apoptosis (Khullar et al 2010). Under pathological conditions, such as diabetes, both enzymes can produce highly reactive $^{\bullet}O_2^-$ and increase oxidative stress and inflammation (Razavi et al 2005). Additionally, loss of $^{\bullet}NO$ bioavailability in diabetes causes endothelial cell dysfunction, resulting in increased permeability of the vessel wall and reduced blood flow through the myocardium causing tissue ischemia. In response, endothelial cells release growth factors, such as transforming growth factor-β (TGF-β), resulting in increased basement membrane thickening, extracellular matrix (ECM) deposition and interstitial fibrosis (Farhangkhoee et al 2006).

Diabetes-mediated increases in ROS are also known to affect structural proteins pertinent to the integrity of the myocardium, as well as proteins that affect its function. ROS have been shown to cause alterations in the function of regulatory and contractile proteins such as the

sarcoplasmic reticulum Ca^{2+}-ATPase and Na^{+}-Ca^{2+} exchanger in the heart (Fang et al., 2004). This leads to diminished calcium sensitivity of proteins involved in the regulation of the cardiac actomyosin system, a reduction in the sarcoplasmic reticulum Ca^{2+}-ATPase and a decrease in the sarcoplasmic reticulum calcium (SERCA2a) pump protein (Abe et al., 2002). These deficits may all contribute to impaired LV function. Indeed, abnormal diastolic and systolic function was normalised in streptozotocin-induced diabetic rat hearts when SERCA2a was overexpressed (Trost et al., 2002).

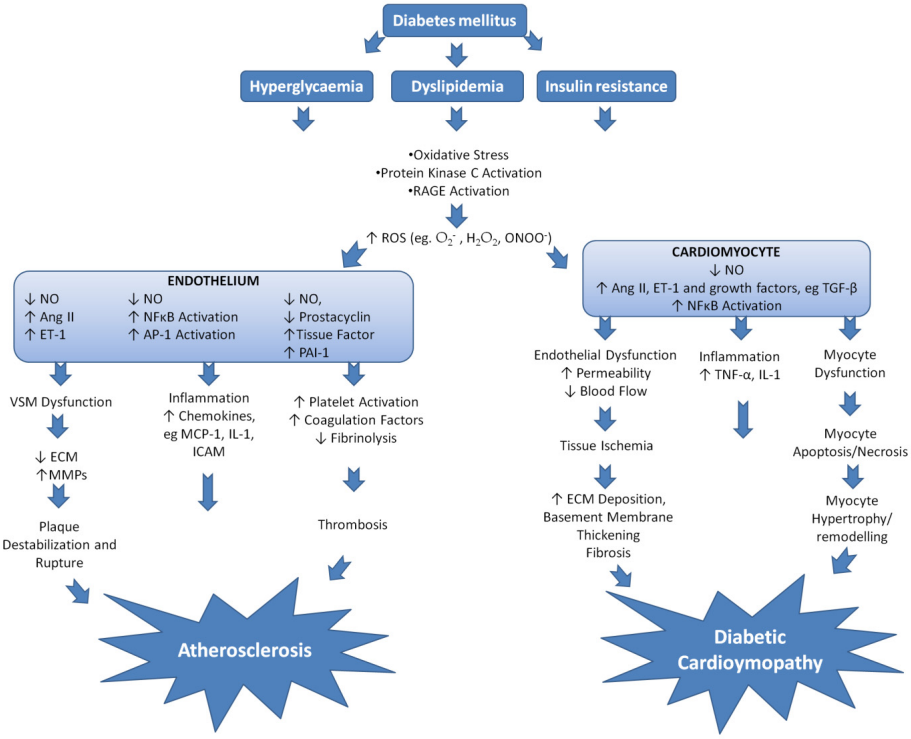

Fig. 1. Roles of reactive oxygen species (ROS) in the pathophysiology of diabetic macrovascular complications, namely atherosclerosis and diabetic cardiomyopathy.

2.3 Diabetic nephropathy

Diabetic nephropathy (DN) has become a worldwide epidemic, accounting for approximately one third of all cases of ESRD (Rossing 2006). DN is classically defined as the increase in protein excretion in the urine. The early stage of DN is characterised by a small increase in urinary albumin excretion (microalbuminuria), while overt diabetic nephropathy is defined as the presence of macroalbuminuria or proteinuria (Zelmanovitz et al 2009).

The earliest structural changes associated with diabetic nephropathy are the expansion of glomerular mesangial area, mesangial cell hypertrophy and thickening of the glomerular basement membrane (Gilbert & Cooper 1999), leading to a progressive reduction in the

filtration surface of the glomerulus, a process known as glomerulosclerosis (Kalant 1978). Furthermore, there is now compelling evidence to suggest that disruption of the tubulointerstitial architecture is as important, if not more important in contributing to kidney injury as glomerular damage (Nangaku 2004).

Despite intensive glucose control and blockade of the RAS (Barit & Cooper 2008; Keane et al 2006), DN continues to progress in a significant proportion of patients and often leads to organ failure and the need for dialysis and/or kidney transplantation. Therefore, the development of novel targeted therapeutics is warranted to reduce or eliminate kidney disease in diabetic patients.

2.3.1 The role of ROS in diabetic nephropathy

An upregulation of ROS in diabetes has been implicated in the pathogenesis of kidney injury (Forbes et al 2008). ROS activate a number of signalling pathways including PKC, p38 MAPK, p42/p44 MAPK and the transcription factor NF-κB, which leads to the increased activation of growth factors such as TGF-β that contribute to the pathogenesis of DN. In the diabetic kidney, enhanced glucose uptake occurs in many of the cell populations including glomerular epithelial cells, mesangial cells and proximal tubular epithelial cells, leading to the excessive production of intracellular ROS, making these cells particularly susceptible to the diabetic milieu (Forbes et al 2008).

Sufficient evidence exists, from both clinical and pre-clinical studies, to suggest that oxidative stress accompanies the progression of diabetic nephropathy. Hyperglycaemia has been shown to increase 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidative mitochondrial DNA damage in diabetic rat kidneys (Kakimoto et al 2002). In this study, intervention by insulin treatment normalised renal 8-OHdG level in diabetic rats, clearly linking the diabetic milieu and increased oxidative stress in this pre-clinical model (Kakimoto et al 2002). In type 2 diabetic patients, it was found that urinary 8-OHdG excretion was significantly higher than in healthy controls and furthermore, that this increase was proportional to the severity of the tubulointerstitial lesion observed in the kidneys of these patients (Kanauchi et al 2002). In addition, it was reported that the 24-hour urinary content of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), a product of oxidative DNA damage, strongly predicted the progression of DN in type 2 diabetic patients in a 5-year follow up study (Hinokio et al 2002).

Several studies have examined the pathways through which increased ROS may mediate its damaging effects on glomerular and tubular injury in the diabetic kidney. One study showed that ROS mediates high glucose-induced activation of PKC in mesangial cells, leading to an increase in TGF-β expression (Studer et al 1997). Ha et al. (2002) have demonstrated that by inhibiting ROS with a series of antioxidants, high glucose-induced activation of NFκB and NFκB-dependent monocyte chemoattractant protein-1 (MCP-1) expression was inhibited in mesangial cells. Furthermore, increased ROS led to accelerated glomerulosclerosis through TGF-β-mediated plasminogen activator inhibitor-1 (PAI-1) upregulation in mesangial cells (Jiang et al 2003b). Similarly, it was proposed that ROS mediate kidney fibrosis in renal cells through the upregulation of the transcription factors NFκB and activator protein-1 (AP-1), that in turn increase MCP-1, TGF-β and PAI-1, resulting in the increased accumulation of ECM (Lee et al 2003). Using several disparate antioxidants, another study found that the TGF-β-induced cellular ROS, the phosphorylation of Smad2, p38 MAPK and extracellular signal-

regulated kinase (ERK), as well as endothelial-mesenchymal transition (EMT) were inhibited, further suggesting an important role for ROS in TGF-β-dependent pathways in renal tubular epithelial cells (Rhyu et al 2005).

3. A role for antioxidant defence in diabetic complications

Evidence suggests that glucose alters antioxidant defences in endothelial cells (Ceriello et al 1996) and in patients with diabetic complications such as DN (Ceriello et al 2000; Hodgkinson et al 2003). Fibroblasts derived from type 1 diabetic patients susceptible to microvascular complications were unable to upregulate their protective antioxidative defences after exposure to high glucose compared with skin fibroblasts from normal subjects, suggesting a failure of antioxidant defences in diabetic patients with nephropathy (Ceriello et al 1996). In addition, the concentration of the antioxidant glutathione (GSH) is found to decrease in a range of organs including the liver, kidney, pancreas, plasma, and red blood cells of chemically induced diabetic animals (Maritim et al 2003b). Given that reduced GSH functions as a direct free-radical scavenger and a cosubstrate for GPx activity, as well as a cofactor for many enzymes, reductions in this antioxidant induced by the hyperglycaemic environment is likely to impact on the progression of diabetic complications. Thus, these findings suggest that increased ROS in diabetes is not only the result of their increased production, as detailed in section 2, but also a consequence of impaired antioxidant defences.

3.1 Glutathione peroxidase

Pre-clinical and clinical evidence are now mounting in support of an important role for GPx in the protection against diseases such as atherosclerosis, both in a non-diabetic and a diabetic setting. The selenocysteine-containing GPx family of antioxidant enzymes attenuates oxidative stress by utilising GSH to reduce hydrogen and lipid peroxides to water and their corresponding alcohol (Fig.2). Additionally, GPx also functions to remove harmful $ONOO^-$. Thus the major role for GPx in the protection against pathogenesis may reside in the fact that it is the only antioxidant enzyme that metabolises three major ROS, H_2O_2, lipid peroxide (LOOH) and $ONOO^-$ (Fig.2). Several isoforms of GPx have been identified and they are each encoded by separate genes, which vary in cellular location, substrate specificity and tissue-specific functions (Brigelius-Flohé 1999).

3.1.1 Different isoforms of GPx

GPx1, also known as cellular GPx, was first identified as an erythrocyte enzyme that protects haemoglobin from oxidative injury (Mills 1957). Its ubiquitous expression in almost all tissues, together with its abundant expression in organs such as the kidney and liver have meant that this isoform is one of the most well-characterised of the GPx family (Lei 2001). GPx2 is most prominent in the gastrointestinal tract and its role is mainly to protect intestinal epithelium from oxidative stress (Chu et al 1997; Esworthy et al 1998). GPx3 is secreted by the kidney and is the main source of plasma GPx; however GPx3 is also expressed in other tissues, for example in the heart (Reeves & Hoffmann 2009). GPx4 reduces phospholipid hydroperoxides (Conrad et al 2007; Thomas et al 1990) and is thought to play a protective role in oxidative stress-induced apoptosis, possibly through the mitochondrial death pathway (Nomura et al 1999; Seiler et al 2008).

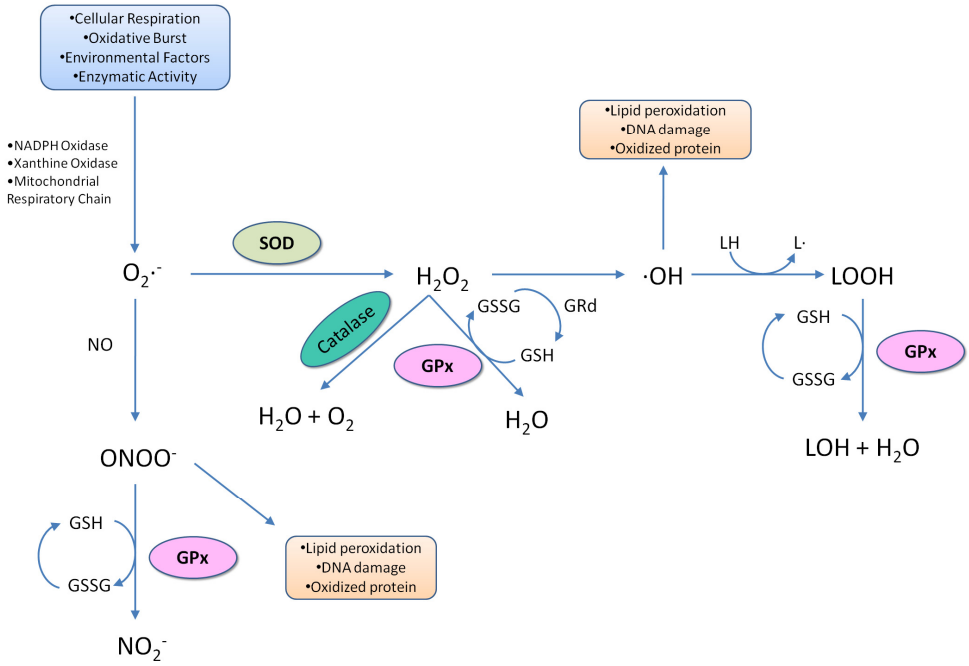

Fig. 2. Removal of reactive oxygen species (ROS) by antioxidant defence systems. Superoxide radical ($^{\bullet}O_2^-$) is generated in low levels under physiological states but its production is greatly enhanced under pathological situations via enzymes such as NADPH oxidase, xanthine oxidase and a dysfunctional mitochondrial respiratory chain. $^{\bullet}O_2^-$ is neutralised to water via a two-step process involving superoxide dismutase (SOD) in the first step, and glutathione peroxidase (GPx) or catalase in a second step. Increased production of $^{\bullet}O_2^-$ and/or impairment of antioxidant defence systems lead to a build-up of the intermediate hydrogen peroxide (H_2O_2). H_2O_2 forms the toxic oxygen species hydroxyl anion ($^{\bullet}OH$) via Fenton biochemistry, which is highly reactive and causes lipid peroxidation forming lipid hydroperoxides (LOOH). The functional importance of GPx resides in its ability to remove H_2O_2 and LOOH and neutralise these to water and lipid alcohol, respectively. Additionally, the increase in $^{\bullet}O_2^-$ also favours the formation of peroxynitrite ($ONOO^-$) which reduces the bioavailability of nitric oxide ($^{\bullet}NO$). GPx also functions to neutralise $ONOO^-$.

3.1.2 GPx in diabetes-associated complications

GPx activity is decreased in patients with type 1 diabetes, as well as in experimentally-induced diabetic rats (Chiu et al 2005; Dominguez et al 1998), although some studies have shown opposite results (Maritim et al 2003b). The decrease in GPx activity may contribute to the progression of diabetic complications due to the build-up of ROS such as H_2O_2 and $ONOO^-$, leading to lipid peroxidation and oxidative injury.

Clinical evidence has shown that diabetic patients with cardiovascular complications have significantly lower enzymatic antioxidant defences, including an impairment in GPx activity, with this defect being more pronounced in younger patients (Čolak et al 2005). GPx activity is also found to decrease in diabetic rats in the heart, kidney and brain, leading to enhanced oxidative stress and secondary organ damage (Aliciguzel et al 2003). In particular, glomerular expression of GPx is significantly reduced in both human and rats with diabetes (Chiu et al 2005). Furthermore, diabetic rats with reduced glomerular GPx expression were found to have more severe glomerulosclerosis and mesangial expansion (Chiu et al 2005). Moreover, patients with type 1 diabetes together with DN displayed a defective GPx defence mechanism, in contrast to patients with type 1 diabetes but without DN (Ceriello et al 2000). Several studies have linked selenium deficiency to a reduction in GPx mRNA expression and activity in the kidney together with elevated plasma glucose, albuminuria and glomerulosclerosis (Fujieda et al 2007; Reddi & Bollineni 2001). These changes may be mediated by the profibrotic growth factor, TGF-β, since inhibition of TGF-β with a TGF-β neutralising antibody, abrogated the reduction in GPx activity, as well as the increase in lipid peroxidation, albuminuria and glomerular injury in rats fed with a selenium-deficient diet (Reddi & Bollineni 2001).

Several clinical studies have linked reduced GPx1 levels with diabetes-associated atherogenesis (Čolak et al 2005; Hamanishi et al 2004). Polymorphisms identified within the GPx1 gene resulting in reduced GPx1 activity have been linked with increased intima-media thickness of carotid arteries and an increased risk of cardiovascular and peripheral vascular disease in type 2 diabetic patients (Hamanishi et al 2004). Moreover, a reduction in red blood cell GPx1 activity has been associated with an increased risk of cardiovascular events in a prospective cohort study assessing the extent of atherosclerosis (Espinola-Klein et al 2007; Winter et al 2003), while atherosclerotic plaques of patients with carotid artery disease have reduced GPx1 activity (Lapenna et al 1998). These evidence, although correlative, suggest that GPx1 is a key enzyme for the protection of vessels against oxidative stress and atherogenesis, particularly in the highly pro-oxidant diabetic environment (de Haan & Cooper 2011).

The roles of GPx in mediating diabetes-associated heart injury are less well understood. Current evidence suggest that the compensatory upregulation of antioxidant enzymes, including GPx1 is impaired in the heart of severely hyperglycaemic mice (Fujita et al 2005). More recently, GPx3 is reported to be upregulated in the heart of STZ-diabetic mice, suggesting that GPx3 is the major antioxidative enzyme of the heart in the cellular defence against oxidative stress under hyperglycaemia (Iwata et al 2006). However, these were short term studies of only 4 to 10 weeks of diabetes, therefore further studies are required to investigate whether diabetes has long term effects on the expression and/or activity of GPx3 in the heart.

3.1.3 The GPx1 knockout mouse: a model of enhanced intensity of oxidative stress

GPx1 knockout (-/-) mice, generated in our laboratory (de Haan et al 1998) and by others (Cheng et al 1997; Esposito et al 2000; Yoshida et al 1997) have become an important research tool to specifically study the protective role of GPx1 in the ROS-mediated progression and promotion of oxidative stress-mediated injury. Most studies investigating the role of GPx do so by limiting selenium intake which results in non-specific reductions of

all selenium-dependent enzymes (Reddi & Bollineni 2001; Rosenblat & Aviram 1998). Furthermore, studying GPx1 knockout mice allows us to draw meaningful conclusions about the protective role of this isoform of the GPx family, since standard assays do not discriminate between different isoforms (Lewis et al 2007). This specific knockout model also facilitates the distinction between the contribution of GPx1, catalase (a peroxisomal H_2O_2 metabolising enzyme) and thioredoxin reductase in the peroxidation of H_2O_2 to water (de Haan & Cooper 2011).

Forgione et al. first reported vascular functional changes in mice associated with both a heterozygous (Forgione et al 2002a) and homozygous deficiency of GPx1 compared to wildtype (WT) mice (Forgione et al 2002b). Mesenteric arterioles of GPx+/- and GPx-/- mice demonstrated paradoxical vasoconstriction to endothelium-dependent vasodilatory compounds such as acetylcholine and bradykinin, whereas WT arterioles showed dose-dependent vasodilation. Superfusion of GPx-/- vessels with an endothelium-independent vasodilator, sodium nitroprusside (SNP) resulted in dose-dependent arteriolar vasodilation that was similar in GPx-/- and WT vessels. These results suggest that GPx-/- mice may have a depletion of bioavailable EDNO. Furthermore, the observed endothelial dysfunction was accompanied by increased nitrotyrosine levels in the endothelial layer of the vessel wall, as well as elevated plasma isoprostanes, indicative that lack of GPx1 leads to oxidative stress in this tissue. By increasing intracellular thiol pools (GSH, cysteine) in the vascular tissue of GPx-/- mice using (L)-2-Oxothiazolidine carboxylic acid (OTC), endothelial dysfunction and oxidant stress was attenuated, further indicating the importance of GPx1 in maintaining normal endothelial function, as well as protecting the blood vessels from oxidative injury (Forgione et al 2002b).

Several isoforms of GPx are present in kidney, however GPx1 is the major isoform expressed in normal kidney, and accounts for >96% of the renal GPx activity (de Haan et al 1998). Protection against oxidative stress is therefore most likely due to lipid and H_2O_2 quenching effects of the GPx1 isoform in the kidney. Until recently, no study has directly linked GPx1 to the protection against DN (Chew et al 2010). Our initial studies using diabetic C57Bl/6J GPx-/- mice, surprisingly failed to show accelerated kidney injury compared with diabetic WT mice (de Haan et al 2005). Furthermore, an assessment of atherosclerosis, which is only possible in the aortic sinus after feeding mice diets rich in fats, cholesterol and choline, failed to reveal a protective role for GPx1 in G57Bl/J6 GPx-/- mice (de Haan et al 2006). As detailed below, the importance of GPx1 in limiting diabetes-associated atherosclerosis and diabetic nephropathy became evident in ApoE/GPx1 double knockout (dKO) mice, a murine model that encompasses three important risk factors, namely hyperglycaemia, hyperlipidemia and enhanced intensity of oxidative stress, seen in diabetic patients.

3.1.4 Diabetic ApoE/GPx1 double knockout mouse: a model of accelerated diabetes-associated atherosclerosis and diabetic nephropathy

Since the lack of GPx1 plays a major role in endothelial dysfunction (Forgione et al 2002b), which is known to be an important mediator of hyperglycaemia-induced atherosclerosis (Nakagami et al 2005), we hypothesised that a lack of GPx1 would accelerate atherosclerosis in a diabetic setting. Because rodents are more resilient than humans to the development of diabetic atherosclerosis, in order to generate a mouse model with similar disease progression and aetiology, we crossed our GPx1-/- mice with ApoE-deficient mice that

were also on a C57/BL6 background (Lewis et al 2007). We then compared aortic lesion formation and atherogenic pathways in ApoE-deficient and ApoE/GPx1 dKO mice after these mice were rendered diabetic using the diabetogenic agent, streptozotocin (STZ). STZ destroys the pancreatic β-islet cells, thus providing a robust model of insulin deficient diabetes (Wilson & Leiter 1990).

In our study, we demonstrated that atherosclerotic lesions within the aortic sinus region, as well as lesions within the arch, thoracic and abdominal region were significantly increased in diabetic ApoE/GPx1 dKO aortas compared with diabetic ApoE-/- aortas (Lewis et al 2007). This increase in aortic lesions was accompanied by an increase in macrophages, α-smooth muscle actin (α-SMA), RAGE and various proinflammatory (VCAM-1, MCP-1) and profibrotic mediators (vascular endothelial growth factor (VEGF), connective tissue growth factor (CTGF)). Gene expression analyses also revealed a concomitant increase in RAGE, VCAM-1, VEGF and CTGF in diabetic dKO aortas compared with diabetic controls. Furthermore, the oxidative stress marker nitrotyrosine was also significantly increased in the diabetic dKO aortas. These findings were observed despite upregulation of other antioxidants, suggesting that a lack of functional GPx1 accelerates diabetes-associated atherosclerosis via upregulation of proinflammatory and profibrotic pathways in ApoE-/- mice. Similar results were reported in a separate study using ApoE/GPx1-/- dKO mice, but in this instance atherosclerosis was induced by high fat diet (Torzewski et al 2007). In particular, these authors demonstrated increased atherosclerosis in their dKO mice, which was accompanied by increased cellularity in atherosclerotic lesions, as well as increased nitrotyrosine levels in the aortic wall and a lower level of bioactive $^{\bullet}NO$ (Torzewski et al 2007).

As previously discussed, diabetic GPx1-deficient mice on a C57Bl/J6 background did not show accelerated kidney injury. However, on examination of the kidneys of diabetic dKO mice, we observed increased albuminuria and renal pathological changes which included mesangial expansion of the glomeruli and upregulation of profibrotic (collagen I and III, fibronectin, TGF-β) and proinflammatory mediators (VCAM-1, MCP-1) (Chew et al 2010). Thus, we believe that in the diabetic C57Bl/J6 GPx1-/- mice, the significance of a lack of GPx1 may not have been properly revealed since lipid levels were unaffected in this model (de Haan et al 2005). Elevated lipid levels have been shown to be critical in accelerating DN since clinical observation suggests that hyperlipidemia is an important contributory factor to the progression of diabetic renal disease (Jenkins et al 2003; Tolonen et al 2009). Importantly, we show enhanced staining for nitrotyrosine, which is a marker of $ONOO^-$ damage in diabetic ApoE/Gpx1 dKO glomeruli and tubules of the kidney compared to diabetic ApoE-/- controls. We have therefore established a role for GPx1 in limiting and/or preventing DN in the pathophysiologically relevant milieu of increased lipids known to accompany diabetes (Chew et al 2010).

3.2 Other antioxidant defence systems

3.2.1 Superoxide Dismutase (SOD)

The SOD family of enzymes catalyse the conversion of $^{\bullet}O_2^-$ into H_2O_2 and oxygen in the first step of the antioxidant pathway (Fig.2), thereby performing an important role in the removal of $^{\bullet}O_2^-$. Three isoforms exists in humans, Cu/Zn-SOD (also known as SOD1), Mn-SOD (also known as SOD2) and SOD3 with distinct cellular localisation, namely cytosolic, mitochondrial and extracellular, respectively.

Diabetes is associated with a decrease in SOD activity in most animal studies (Brocca et al 2008; Fujita et al 2009; Fukuda et al 2010). Lowered SOD levels are reported in serum and urine of STZ-treated Sprague-Dawley rats (Luo et al 2010), and decreased SOD1 and SOD3 levels are suggested to play a key role in the pathogenesis of diabetic nephropathy (Fujita et al 2009). Manipulations of SOD, through the use of SOD knockout mice or SOD overexpressing mice, have shown the importance of these enzymes in the protection against diabetic complications. Indeed, SOD1 knockout mice have clearly shown the importance of protection by SOD1 against superoxide-mediated DN. These mice demonstrated significant mesangial matrix expansion, renal cortical malondialdehyde content and severe tubulointerstitial injury compared with diabetic controls (DeRubertis et al 2007). Importantly, these pathological changes were attenuated in the presence of the SOD-mimetic, tempol (discussed in more detail in section 4.3.2). The significance of SOD2 was revealed in eMnSOD-Tg mice where the overexpression of mitochondrial-specific SOD targeted to the endothelium prevented diabetic retinopathy (Goto et al 2008). Furthermore, the targeted overexpression of Mn-SOD significantly attenuated morphological changes in diabetic hearts and improved contractility in diabetic cardiomyocytes (Shen et al 2006). Collectively, these studies show that targeted removal of $^{\bullet}O_2^-$, leads to improved outcomes in diabetic nephropathy and retinopathy.

3.2.2 Catalase

Catalase is present mainly in the peroxisomes of mammalian cells as a tetrameric enzyme of four identically arranged subunits; each containing a heme group and NADPH at its active centre (MatÉs et al 1999). Similar to GPx1, the enzyme neutralise H_2O_2 to water and oxygen (Fig.2). A role for catalase in the protection against atherosclerosis comes from the analysis of mice overexpressing catalase. In these experiments, overexpression of catalase significantly reduced the severity of lesions in ApoE-deficient mice (Yang et al 2004). However, the role of catalase in diabetes is debatable; studies have shown that onset and progression of diabetes is accompanied by reductions in catalase activity (Ali & Agha 2009; Kamboj et al 2010; Pari et al 2010; Patel et al 2009), while others report an increase in the activity of catalase (Kakkar et al 1996; Kesavulu et al 2000).

More recently, mutations within the catalase gene have been suggested to contribute to the increased risk of diabetes (Góth & Eaton 2000). However, other studies report no such association with catalase gene polymorphisms and the development of diabetic complications (Letonja et al 2011; Panduru et al 2010). For example, one study reported that blood catalase activity was lowered due to the downregulation of catalase synthesis, rather than specific catalase gene mutations in type 2 diabetic patients. This was also associated with increased H_2O_2 levels and dysfunctional insulin receptor signalling (Góth 2008).

3.2.3 Thioredoxin system

The mammalian Trx system is ubiquitously expressed and consists of Trx, Trx reductase and NADPH. The antioxidant properties of Trx is exerted mostly through the antioxidant enzymes, Trx peroxidase (also known as peroxiredoxin), which uses sulfhydryl (SH) groups as reducing equivalents (Chae et al., 1994). Trx reduces oxidised peroxiredoxin, which then scavenges H_2O_2 to produce water (Kang et al., 1998), thus attenuating oxidative stress in cells. The role of the Trx system in diabetic complications has gained considerable interest

since thioredoxin-interacting protein (Txnip), which is an inhibitor of Trx activity, was discovered to be a highly upregulated hyperglycaemia-induced gene in both human and animal studies (Kobayashi et al 2003; Qi et al 2007; Shalev et al 2002). Txnip directly binds to the catalytic active site of Trx, thus inhibiting the reducing activity of Trx (Nishiyama et al 1999).

In vitro studies have shown Txnip expression to be significantly upregulated by glucose in mesangial cells (Kobayashi et al 2003), proximal tubule cells (Qi et al 2007) and distal tubule/collecting duct cells (Advani et al 2009; Kobayashi et al 2003). Overexpression of Txnip in STZ-treated diabetic rats was associated with an increase in ROS, as well as ECM accumulation in the kidney (Kobayashi et al 2003; Tan et al 2011). By reducing Txnip gene transcription with siRNA in kidney cells, high glucose-mediated increases in ROS production and ECM accumulation were attenuated, together with a restoration of Trx activity (Advani et al 2009). These results clearly delineate a role for Trx in maintaining redox homeostasis and highlight the importance of Txnip dysregulation in diabetic complications.

Txnip also plays an important role as a biomechanical effector of atherosclerosis (World et al 2006). In the endothelium of intact rabbit aorta, exposure to physiological fluid shear stress decreased Txnip expression and increased Trx activity, leading to a reduction in pro-inflammatory events mediated by the tumour necrosis factor (TNF)-ASK-1-JNK/p38 pathway, in association with a decrease in TNF-mediated VCAM1 expression (Yamawaki et al 2005). Furthermore, a calcium channel blocker promoted cardiac myocyte survival and improved cardiac function by reducing cardiac Txnip expression, suggesting a role for Txnip in mediating cardiac myocyte apoptosis in diabetic cardiomyopathy (Chen et al 2009).

4. Novel antioxidants to limit diabetic micro- and macrovascular disease

4.1 GPx1-mimetic ebselen

Ebselen (2-phenyl-1,2-benzisoselenazol-3[2H]-one) is a synthetic, lipid soluble, non-toxic seleno-organic compound (Sies & Masumoto 1997) with anti-inflammatory and antioxidant activities (Muller et al 1984). Ebselen eliminates hydroperoxides, including H_2O_2 and lipid peroxides due to its GPx mimetic activity (Maiorino et al 1988; Parnham et al 1987; Safayhi et al 1985). Additionally, ebselen also scavenges other ROS such as peroxyl radicals and $ONOO^-$ (Sies & Masumoto 1997). Several enzymes involved in inflammatory processes, including 5-lipoxygenases, NOS, NADPH oxidase, PKC and ATPase are also inhibited by ebselen (Sies & Masumoto 1997). Importantly, ebselen has shown tremendous potential in reducing injury in various experimental models. Ebselen conferred protection against endothelial dysfunction in stroke-prone hypertensive rats (Sui et al 2005) and improved cardiac function in a model of chronic iron overload (Davis & Bartfay 2004). Furthermore, ebselen partially restored endothelial dysfunction in Zucker diabetic rats (Brodsky et al 2004). Additionally, arterial lesions were reduced by ebselen in a superoxide-driven non-inflammatory transgenic murine model, suggesting a role for ebselen in reducing atherosclerosis (Khatri et al 2004). Importantly, in a clinical trial, ebselen has shown improved neurological outcomes in patients after cerebral infarction (Yamaguchi et al 1998) and a safety and pharmacokinetic profile of ebselen has been established in normal volunteers in a Phase I trial to determine whether ebselen is protective against noise-induced hearing loss (Lynch & Kil 2009).

4.1.1 Ebselen in experimental models of diabetes associated atherosclerosis and nephropathy

We hypothesised that ebselen, in its capacity to act as a mimetic of GPx, would attenuate oxidative stress and lessen diabetes-associated atherosclerosis (Chew et al 2009). Eight week-old male C57Bl/J6 ApoE-/- mice were rendered diabetic with STZ and assigned to ebselen-gavaged and non-gavaged groups. Ebselen was administered twice daily at 10mg/kg/day body weight starting at 10 weeks of age and continued for 20 weeks. Our analysis showed that ebselen reduced lesion formation in most regions of the aorta including the arch, thoracic and abdominal regions of diabetic ApoE-/- mice. In addition, ebselen attenuated aortic nitrotyrosine levels and the expression of the Nox2 subunit of NADPH oxidase. The cellularity of the aorta associated with a pro-atherosclerotic phenotype (increased α-SMA-positive cells and increased macrophage infiltration) was also decreased by ebselen, together with a reduction in the aortic expression of the pro-atherosclerotic mediators, RAGE and VEGF (Chew et al 2009). These data support the notion of Blankenberg et al. (2003) that bolstering GPx-like activity reduces atherosclerosis.

Furthermore, similar results were observed in our diabetic ApoE/GPx1 dKO mice where ebselen significantly reduced total aortic plaque, as well as regional plaque (arch, thoracic and abdominal) (Chew et al 2010). These reductions in plaque were also accompanied by a decrease in vascular oxidative stress (aortic 4-hydroxynonenal (HNE), nitrotyrosine, the Nox2 subunit of NADPH oxidase), as well as reductions in plasma hydroperoxides and urinary 8-isoprostanes. Additionally, the pro-inflammatory and pro-atherogenic mediators VCAM-1, MCP-1, CTGF and VEGF were attenuated by ebselen in diabetic ApoE/GPx1-deficient aortas. These data suggest that ebselen is able to replenish GPx1 activity in this model, thereby reducing atherosclerosis.

As mentioned in section 3.1.4, diabetic ApoE/GPx1 dKO mice demonstrated significant renal injury with an increase in albuminuria, hyperfiltration, mesangial expansion, oxidative stress (nitrotyrosine), pro-fibrotic (TGF-β, CTGF, collagen I, III and IV, fibronectin) and pro-inflammatory mediators (MCP-1, VCAM-1 and TNF-α) (Chew et al 2010). Ebselen significantly attenuated all of these parameters in diabetic dKO kidneys (Chew et al 2010). Our results are in agreement with Chander et al. (2004) where ebselen improved renal function and attenuated structural defects such as glomerulosclerosis, tubulointerstitial fibrosis and vasculopathy in the Zucker diabetic fat rat. Moreover, ebselen prevented the accumulation of lipid peroxidation products and 3-nitrotyrosine-modified proteins, and restored renal tissue levels of GSH (Chander et al 2004).

4.1.2 Mechanistic understanding of the actions of ebselen

Ebselen is well-characterised for its ability to act as a GPx mimic and can be catalytically maintained at the expense of GSH (Muller et al 1984; Sies & Masumoto 1997). Ebselen has been shown to reduce oxidative stress in several *in vivo* models; however this protective effect is unlikely to be solely due to the direct pro-oxidant interception by ebselen. Recently, ebselen has been reported to be an inducer of NF-E2-Related Factor 2- (Nrf-2)-dependent gene activation (Tamasi et al 2004). Nrf-2 is a member of the Cap'n'Collar family of basic region-leucine zipper (bZIP) transcription factors (Chui et al 1995). Genes upregulated by

Nrf-2 can be broadly classified into three separate classes; those that are involved in GSH synthesis (cystine membrane transporters), detoxication enzymes (rat glutathione S-transferase A2, a non-selenium-dependent glutathione peroxidase, and NAD(P)H:quinine oxidoreductase), and those directly involved in the amelioration of oxidative stress (heme oxygenase-1 (HO-1), peroxiredoxin MSP23, and Trx reductase) (Tamasi et al 2004). Tamasi et al. (2004) showed that ebselen can directly induce Nrf-2-dependent gene transcription, including the increase of intracellular GSH production, which acts as a substrate for GPx activity. These data showed that the activation of Nrf-2-dependent signalling by ebselen could indirectly augment cellular defences, independent of the direct interception of ROS by ebselen.

To further elucidate the mechanistic actions of ebselen observed in our diabetic experimental models, we examined the effect of ebselen on various signalling pathways implicated in atherosclerosis and nephropathy in human aortic endothelial cells (HAEC) (Chew et al 2009) and normal rat kidney (NRK) cells (Chew et al 2010). Pre-treatment of HAEC with 0.03 μM ebselen prior to exposure to 100 μM H_2O_2 reduced the H_2O_2-mediated increase in IkB-kinase (IKK) phosphorylation on critical activatory residues. Since IKK is a key regulator of NK-κB activation (Schmid & Birbach 2008), it is anticipated that by reducing IKK phosphorylation, ebselen anchors NF-κB in the cytoplasm thereby preventing the activation of pro-inflammatory genes. We further showed that ebselen reduced the H_2O_2-mediated increase in Nox2 expression; Nox2 is known to be regulated by NK-κB (Anrather et al 2006), further confirming the view that ebselen affects downstream targets of NF-κB.

The cytokine TNF-α is an important diabetes-associated pro-inflammatory mediator and is involved in the activation of NF-κB (Hacker & Karin 2006). Our *in vitro* data showed that H_2O_2-induced upregulation of TNF-α was reduced by ebselen. Our results support previous findings that ebselen inhibits TNF-α-induced pro-inflammatory responses in endothelial cells (Yoshizumi et al 2004) and other cell types (Sharma et al 2008; Tewari et al 2009). We also investigated the effects of ebselen on H_2O_2-mediated phosphorylation of JNK, a kinase involved in the activation of the transcription factor, AP-1. Our *in vitro* analysis showed that ebselen effectively attenuated H_2O_2-mediated phosphorylation of JNK, an important finding since phosphorylated JNK has been implicated in TNF-α-mediated endothelial activation (Min & Pober 1997) in particular through the interaction of AP-1 and NF-κB (Read et al 1997). Collectively, our results with ebselen have implications not only for inflammatory genes known to be regulated by these pathways, but also on the proatherosclerotic pathway itself, since inflammatory events are integrally linked with the development and progression of atherosclerosis.

Our *in vitro* studies in NRK cells further strengthen the notion that ebselen downregulates proinflammatory pathways in renal cells (Chew et al 2010). Similar to what we observed in HAEC, ebselen attenuated the phosphorylation of the pro-inflammatory mediator, IKK. Furthermore, stress-response kinases such as JNK and p38 MAPK phosphorylation were also attenuated by ebselen in NRK cells (Chew et al 2010).

4.2 Novel GPx1-mimetics

Novel GPx-mimetics, synthesised for their greater solubility and efficacy than ebselen are now available. Several laboratories have reported the synthesis and characterisation of novel

small selenium compounds as functional mimics of GPx, either by modifying the basic structure of ebselen or by incorporating some structural features of the native enzyme (Alberto et al 2010; Back 2009; Bhabak & Mugesh 2010). These synthetic mimics can be classified into three major categories: (i) cyclic selenenyl amides having a Se-N bond, (ii) diaryl diselenides, and (iii) aromatic or aliphatic monoselenides (Bhabak & Mugesh 2010). The most widely studied among the novel GPx-mimetics is diphenyl selenide.

In addition to their GPx-like activity, the antioxidant activity of diphenyl diselenides may also be attributed to their capacity to be a substrate for mammalian Trx reductase (De Freitas et al 2010). In a study where acidosis was used to mediate oxidative stress in rat kidney homogenates, diphenyl diselenide significantly protected against lipid peroxidation, whilst the protection afforded by ebselen was only minor (Hassan et al 2009). Furthermore, diphenyl diselenide was also effective in protecting against acute renal failure induced by glycerol in rats (Brandão et al 2009). In cholesterol-fed rabbits, where animals exhibited hypercholesterolaemia and oxidative stress, diphenyl diselenide significantly reduced both of these risk factors of coronary artery disease in these animals (De Bem et al 2009). More recently, diphenyl diselenide has also been reported to attenuate atherosclerotic lesions in LDLr-/- mice by lowering oxidative stress and inflammation (Hort et al 2011).

The clinical benefits of ebselen and/or these novel GPx-mimetics in attenuating diabetes-associated complications are yet to be reported. Our pre-clinical assessments, as well as those of others, have highlighted the attractiveness of this class of therapeutic for their clinical use to prevent or attenuate both diabetes-associated atherosclerosis and DN, two co-morbidities often present in diabetic patients.

4.3 Other novel antioxidants

4.3.1 NOX inhibitors

Increased NADPH oxidase (NOX) activity, which catalyses the production of ROS, has been implicated in the pathogenesis of diabetic complications (Kakehi & Yabe-Nishimura 2008). NOX4 has been shown to not only mediate the increase in ROS, but also activate profibrotic pathways in type 2 DN (Sedeek et al 2010). Additionally, inhibition of NOX1 suppresses neointimal formation in the prevention of vascular complications associated with diabetes (Lee et al 2009).

Several small molecule and peptide inhibitors of the NOX enzymes have been developed and are showing promise in experimental studies, but issues of specificity, potency and toxicity militate against any of the existing compounds as candidates for drug development (Kim et al 2011; Williams & Griendling 2007). In a recent study, apocynin, a proven NADPH oxidase inhibitor, attenuated albuminuria, improved kidney structure (glomerular and mesangial expansion) and reduced oxidative stress (urinary 8-OHdG and malondialdehyde) in aged Otsuka Long Evans Tokushima Fatty (OLETF) rats (Nam et al 2009). However, apocynin is considered to be non-selective in its mode of action as it also targets other enzymes such as Rho-kinase (Heumüller et al 2008).

One of the most specific NOX inhibitors developed so far is gp91 (ds-tat), an 18-amino acid peptide which interferes with NOX assembly and activation (Rey et al 2001). This peptide functions by mimicking the binding region of NOX2, and possibly NOX1, which interacts

with p47^{phox}. In doing so, it inhibits subunit assembly, resulting in the specific inhibition of $^{\bullet}O_2^-$ production from NOX and not from other oxidases such as xanthine oxidase. Gp91 (ds-tat) has shown promise in reducing vascular ROS associated with Ang II-mediated hypertension in mice (Rey et al 2001), as well as reducing endothelial dysfunction and vascular ROS in the Dahl salt-sensitive rat model (Zhou et al 2006). However, limited bioavailability of this peptide has hampered its usefulness as a therapeutic agent; at present, it can only be administered via intravenous injection (Williams & Griendling 2007). VAS2870 is a fairly new NOX inhibitor discovered via high-throughput screening and is specific for NADPH oxidase activity (ten Freyhaus et al 2006). It has been shown to attenuate platelet-derived growth factor (PDGF)-dependent smooth muscle cell chemotaxis *in vitro* via a mechanism that includes the complete abolition of NOX activation and ROS production (ten Freyhaus et al 2006).

Only limited *in vitro* and *in vivo* data are available for these novel compounds. Further testing in pre-clinical models is necessary to determine if these approaches represent feasible therapeutic strategies for diabetic complications.

4.3.2 SOD mimetics

As discussed in section 3.2.1, SOD is the first line of defence against both physiological and pathological ROS, by catalysing the dismutation of $^{\bullet}O_2^-$ to H_2O_2 (Fig.2). Protective and beneficial effects of SOD enzymes have been demonstrated in a broad range of superoxide-driven diseases, both pre-clinically and clinically (Muscoli et al 2003). When tested in humans, Orgotein (a bovine CuZn-SOD mimetic) showed promising results in acute and chronic conditions associated with inflammation; however, because of its non-human origin, the use of bovine native enzyme in the human context caused a variety of immunological disorders (Muscoli et al 2003). Since then, several synthetic, low-molecular weight mimetics of SOD have been produced with promising results in pre-clinical models.

The most well-characterised of the SOD-mimetics is tempol (4-hydroxy-2,2,6,6,-tetramethylpiperidine-*N*-oxyl). Tempol has been reported to protect animals and mammalian cells from cytotoxicity induced by oxygen radicals such as H_2O_2 and $^{\bullet}O_2^-$ (Mitchell et al 1990). One attractive attribute of tempol is its ability to penetrate cell membranes and hence react with ROS both intracellularly and extracellularly, as well as within important organelles such as the mitochondria (Simonsen et al 2009). Tempol has been shown to improve acetylcholine- and arachidonic acid-induced relaxation in skeletal muscle arteries and in coronary arteries from diabetic animals (Gao et al 2007; Xiang et al 2008). Furthermore, in an *in vivo* one-kidney one-clip hypertensive rat model, tempol improved endothelial function in small arteries exposed to high blood pressure (Christensen et al 2007). However, the use of tempol may be limited in some instances by its propensity to increase H_2O_2, thereby exacerbating the progression of disease. For instance, in an experimental model of glomerulonephritis, tempol increased proteinuria and crescentric glomerulonephritis with leukocyte infiltration, as well as accelerating mortality in the treated group (Lu et al 2010). Moreover, tempol upregulated p65-NFκB and osteopontin in the kidney and increased H_2O_2 levels in the urine. In another study, tempol could not prevent the development of hypertension in a hypertensive rat model induced by inhibiting renal medullary SOD with diethyldithiocarbamic acid (Chen et al 2003). These results may be due to the increased formation of H_2O_2, as a result of the dismutation of $^{\bullet}O_2^-$ by tempol, causing constriction of the medullary vessels, and counteracting the vasodilatory actions of tempol.

Manganese(Il) complex with a bis(cyclohexylpyridine)-substituted macrocyclic ligand (M40403) is another SOD-mimetic which has high catalytic SOD activity and is chemically and biologically stable *in vivo*. Injection of M40403 into rat models of inflammation and ischemia-reperfusion injury protected the animals against tissue damage, possibly by preventing the formation of $ONOO^-$, as well as assisting in the direct removal of $^{\bullet}O_2^-$ (Salvemini et al 1999). M40403 also reversed endothelial dysfunction in ApoE-/- aortas *ex vivo* by decreasing NADPH oxidase-dependent $^{\bullet}O_2^-$ levels (Jiang et al 2003a).

4.3.3 Mitochondrially-targeted antioxidants

The targeted delivery of antioxidants to the mitochondria is an attractive approach to effectively remove or attenuate pathogenic ROS produced by the mitochondria. Animal studies using transgenic mice over-expressing mitochondrially-targeted SOD and catalase have already shown the potential of this strategy, since these transgenic mice were associated with significant reductions in cardiac mitochondrial oxidative stress and improvements in left ventricular function after antiretroviral-induced cardiomyopathy (Kohler et al 2009). Indeed, the development of antioxidants that specifically target the matrix-facing inner surface of the mitochondrial membrane is hypothesised to protect against mitochondrial oxidative damage (Ross et al 2005). Because of the high negative membrane potential of the inner mitochondrial membrane, antioxidants conjugated with a lipophilic triphenylphosphonium (TPP) cation such as mitoquinone, mitovitamin E and mitophenyltertbutyline accumulate in the mitochondrial matrix at concentrations several-fold greater than cytosolic non-mitochondrially targeted antioxidants (Subramanian et al 2010; Victor et al 2009). In particular, MitoQ, which has a TPP modification added to coenzyme Q is 100 times more potent than idebenone, a coenzymes Q derivative. Furthermore, MitoVitE (vitamin E attached to a TPP cation) is 350 times more potent than Trolox, a water soluble version of vitamin E, in fibroblasts from patients (Victor et al 2009). *In vitro* experiments using both mitoquinone and mitovitamin E have shown promising reductions in peroxide-mediated oxidant stress and apoptosis whilst maintaining proteasomal function in bovine aortic endothelial cells (BAEC) (Dhanasekaran et al 2004). Moreover, Mito-carboxy proxyl (Mito-CP), a mitochondrial-targeted SOD, significantly diminished glucose/glucose oxidase-induced formation of intracellular ROS and apoptosis in BAEC, while the "untargeted" carboxy proxyl (CP) nitroxide probe did not (Dhanasekaran et al 2005). Furthermore, a mitochondrial-targeted form of the Gpx1-mimetic, MitoPeroxidase, which contains an ebselen moiety covalently linked to a TPP cation, decreases glucose and H_2O_2-mediated apoptosis in a rat basophilic leukemia cell line (RBL-2H3) (Filipovska et al 2005). Several studies have shown the accumulation of these compounds in the mitochondria of various tissues including brain, heart, liver and kidney, in mice fed mitrochondrially-targeted antioxidant compounds for several weeks (Smith et al 1999). However, to date, their therapeutic potential in militating against diabetic complications has not been explored.

4.3.4 Bolstering antioxidant defences via the transcription factor Nrf2

Nrf2 is a redox sensitive transcription factor which regulates the expression of important cytoprotective enzymes (Kensler et al 2007). Nrf2 plays an important role in endogenous defence against sustained oxidative stress by upregulating important detoxifying phase II

enzymes, such as NAD(P)H:quinine oxidoreductase (NQO1) and antioxidant proteins, such as HO-1, through an antioxidant response element (ARE)-dependent pathway. The protective role of Nrf2 in diabetes-mediated kidney injury has gained considerable attention recently. Diabetic Nrf2 knockout mice demonstrated increased glomerular ROS production and greater oxidative DNA damage and renal injury compared to control mice (Jiang et al 2010). In addition, in human renal mesangial (Jiang et al 2010) and coronary arterial endothelial cells (Ungvari et al 2011), high glucose induced ROS production and the enhanced expression of Nrf2 and its downstream genes, such as NQO1, glutathione S-transferase (GST), glutamate-cysteine ligase catalytic (GCLC) and HO-1. These effects of high glucose were significantly attenuated by silencing Nrf2 expression using siRNA or overexpression of kelch-like ECH-associated protein (Keap-1), which is an inhibitor of Nrf2 (Ungvari et al 2011). Furthermore, overexpression of Nrf2 inhibited the promoter activity of TGF-β1 in a dose-dependent manner, whereas knockdown of Nrf2 by siRNA enhanced TGF-β1 transcription and fibronectin production, suggesting that Nrf2 plays a protective role in attenuating diabetic nephropathy (Jiang et al 2010).

Hyperglycaemia is associated with the increased formation of AGE and enhanced oxidative stress, leading to the progression of diabetic cardiovascular disease (Thomas et al 2005). It was recently reported that Nrf2 is activated by AGE in BAEC, resulting in the induction of the antioxidant genes HO-1 and NQO1, thus confirming a protective role of Nrf2 against oxidative stress in diabetes (He et al 2011). Furthermore, to test the protective effects of Nrf2 under metabolic stress, which often occurs concurrently with diabetes, Nrf2-/- mice were subjected to high fat diet (Ungvari et al 2011). These mice failed to show significant increases in the gene expression of the Nrf2 downstream targets GCLC and HO-1. In addition, increased ROS and endothelial dysfunction was attenuated in Nrf2-/- aortas, in contrast to Nrf2+/+ controls, further confirming that an adaptive activation of the Nrf2/ARE pathway confers endothelial protection under diabetic conditions (Ungvari et al 2011).

Nonetheless, very few studies have directly demonstrated the therapeutic potential of increasing Nrf2 in pre-clinical models. Oltipraz, an Nrf2 activator, significantly prevented the development of insulin resistance and obesity in high fat diet (HFD)-induced C57BL/6J mice (Yu et al 2011). Control mice fed with HFD demonstrated reduced nuclear content of Nrf2 in adipose tissue, which was associated with increased Keap-1 mRNA expression and reduced HO-1 and NQO1; all of which were attenuated with oltipraz. Moreover, resveratrol, which is a polyphenolic phytoalexin that occurs naturally in many plant species, including grapevines and berries, was shown to attenuate STZ-induced diabetic nephropathy in rats, through the preservation of Nrf2 mRNA and protein expression, with an inhibitory effect on diabetes-induced upregulation of Keap-1 in diabetic kidneys (Palsamy & Subramanian 2011). As mentioned earlier, ebselen can directly upregulate the expression of Nrf2-dependent genes, in addition to its ability to quench H2O2 (Tamasi et al 2004). Furthermore, ebselen has been shown to modify Keap-1, thereby relieving the inhibitory effect of Keap-1 on Nrf2 (Sakurai et al 2006).

A recent clinical trial using the Nrf2 activator, bardoxolone methyl, (Pergola et al 2011) has generated particular interest in this type of drug to lessen the burden of DN. In this double-blind, randomized, placebo-controlled trial, bardoxolone significantly improved the estimated glomerular filtration rate (eGFR) in patients with type 2 diabetes and impaired renal function. Current interventions appear to slow the decline in renal function by less

than 1 ml/min/1.73 m² per year, therefore (Brenner et al 2001), improvements with bardoxolone methyl of between 5-10 ml/min/1.73 m² are seen as a major advance over standard therapies. These results may lead to further pre-clinical and clinical activity to identify additional Nrf2 activators with possibly even greater efficacy. Indeed, the strategy of bolstering antioxidant defences by manipulating Nrf2 may represent a new class of therapy with potentially major advances over conventional therapy in the treatment of diabetic complications such as diabetic nephropathy.

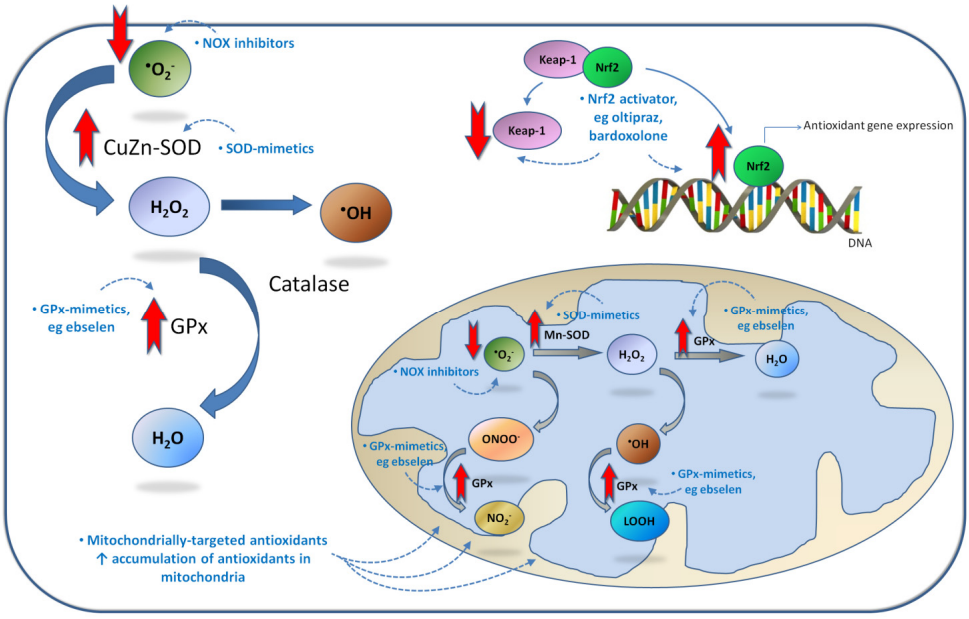

Fig. 3. Novel antioxidant strategies to attenuate increased cellular ROS production and/or increase the activity of endogenous antioxidant defence systems in diabetes-associated complications.

5. Conclusion

Increasing evidence has implicated a role for oxidative stress in mediating diabetes-associated complications. Despite this, very few therapies are currently available in clinical practice to effectively target oxidative stress and lessen the burden of diabetic complications. The use of vitamins in clinical trials have been mostly disappointing, showing no overall benefit for major cardiovascular events and in some instances, even increasing cardiovascular mortality (McQueen et al 2005). Failure of vitamins in the clinic may be due to their lack of specificity in not correctly targeting the ROS responsible for pathogenesis; conversely, total ablation of ROS could be detrimental as ROS are essential for basic cell signalling and homeostasis. Thus, the challenge for developing an effective antioxidant therapy for diabetes-associated complications would be to target either the production of specific ROS involved in diabetes-mediated injury or to eliminate ROS now appreciated to contribute to diabetes-associated-atherosclerosis such as hydrogen peroxide. A further

challenge would be the maintenance of steady-state levels of ROS important for cellular processes including cell signalling. This is by no means an easy task; however, as highlighted in this review (Fig.3), several approaches are currently being investigated in pre-clinical models to lower the levels of those ROS involved in pathogenesis, or to activate specific antioxidant defences to lessen the diabetes-driven enhanced intensity of oxidative stress. The ultimate aim of these investigations is the clinical translation of novel targeted antioxidant therapies to lessen the burden of diabetic complications.

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13

Evaluation of Oxidative Stress and the Efficacy of Antioxidant Treatment in Diabetes Mellitus

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1. Introduction

Studies on the efficacy of antioxidant treatment in type 1 diabetes mellitus is an interesting, actual research subject. Reactive oxygen species (ROS) are continuously produced and eliminated by living organisms normally maintaining ROS at certain steady-state levels. Under some circumstances, the balance between ROS generation and elimination is disturbed leading to enhanced ROS level causing oxidative stress [Lushchak, 2011]. Oxidative stress is involved in the development of several important diseases (cancer, ulcer, atherosclerosis, autoimmune diseases, ischaemia-reperfusion injury, emphysema, inflammation, etc.).

Oxidative stress due to increased production of reactive oxygen species and/or impaired antioxidant capacity of the body plays a special, important role in the development of type 1 diabetes mellitus and its complications [Baynes, 1991], [Giugliano et al., 1995], [Krippeit-Drews et al., 1994], [Nakazaki et al., 1995], [Nomikos et al., 1989], [Raes et al, 1995], [Shinn, 1998].

Under normal conditions, oxidative tissue damage is prevented by enzymatic and non-enzymatic antioxidants. Superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) are three enzymes involved in detoxification of reactive oxygen species (superoxide radical, hydroxyl radical, and hydrogen peroxide). Pancreatic cells have a poor antioxidant defence system, so they are very vulnerable to oxidative stress, especially to superoxide mediated radical damage [Dejica, 2000].

Evaluation of oxidative status can be made by various methods, most of them very sophisticated, requiring laboratories with modern equipment, and are used almost exclusively for research purposes.

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Exploring the body oxidative status can be made through the following ways:

1. Free radical measurement by absorption spectroscopy with electronic spin resonance (ESR) and electronic paramagnetic resonance (EPR) [Olinescu, 1994].
2. Measurement of chemical uptake (chemical trapping) by quantitative determination of the elimination of specific derivatives of salicylic acid, hydroxylated or nitrosylated compounds.
3. Measuring the antioxidant capacity of each antioxidant in part or total plasma antioxidant capacity.
4. Determination of antioxidant enzyme activites (SOD, CAT, GPX) and non-enzymatic antioxidants (tocopherols and tocotrienols, vitamin C, ubiquinone, glutathione, carotenoids and vitamin A, bilirubin, melatonin, uric acid, ceruloplasmin, vitamin K, lipoic acid) [Dejica, 2000, 2001].
5. The measurement of biological compounds resulting from oxidative processes:
   - lipid compounds - conjugated dienes, hydroperoxides, aldehydes (malondialdehyde, hydroxynonenal)
   - ethane and pentane measurements in expired gases
   - DNA beta-hydroxydeoxyguanosine
   - protein derivatives (carbonyl or thiol groups)
   - amino acids - methionine sulfoxide, ortho-tyrosine, dityrosine, nitrotyrosine, chlorotyrosine.
6. Measurement of the antioxidants / oxidizing substances ratio (eg. ascorbic acid / dehydroascorbic acid and reduced glutathione / oxidized glutathione) [Prior & Cao, 1999].

2. Antioxidants

Determination of trace elements is also of interest because some are in the active center of antioxidant enzymes: for example Cu, Zn and Mn are found in the structure of SOD, and Se is present in the active center of GPX, but also possesses antioxidant effect independent of this enzyme [Olinescu, 1994].

The variety of antioxidant substances in the body, the difficulty of measuring their individual level and the interactions between them require methods for measuring total antioxidant capacity (TAOC) in different biological samples [Cadenas & Packer, 2002].

By measuring TAOC one can assess not only the interaction effects of antioxidants known, but also the antioxidant action of unidentified components present in human plasma. In most methods uric acid is the major contributor to the TAOC of plasma, so increasing plasma levels of uric acid can mask the depletion of ascorbate or other antioxidants in some pathological conditions if only TAOC measurement is carried out.

TAOC are methods used to measure indirect inhibition involving a prooxidant (typically a free radical) and an oxidizable substrate. The prooxidant induces oxidative deterioration of the substrate, which is inhibited in the presence of the antioxidant.

Antioxidant capacity can be defined as the ability of a compound to reduce prooxidants. In biological systems, prooxidants are usually defined as toxic substances causing oxidative damage to lipids, proteins, nucleic acids, leading to a variety of pathological events, but not

every prooxidant is necessary a toxic compound. Antioxidants are substances that, in low concentrations compared to an oxidizable substrate, prevent or delay oxidation initiated by a prooxidant [Dejica, 2001].

Several vitamins are well known for their antioxidant properties: vitamin C exhibits its effect in hydrophilic environment, while vitamin A and E protect especially the cell membranes, and act in hydrophobic phase. Many other, more complex antioxidant substances are found in plants.

3. Implication of oxidative stress and glycation process in diabetes mellitus

In type 2 diabetic patients, besides the relative insulin deficiency, there is a certain grade of insulin resistance. The relationship between reactive oxygen species and the effect of insulin has been studied, and the results showed that in elderly people, presenting intense exposure to oxidative stress, the ratio between GSH/GSSG is reduced, leading to intensified lipoperoxidation. This phenomenon might exhibit a negative influence on the integrity of plasma membranes, leading to their disfunction, regarding for instance the transmembrane glucose transport. There is a high probability that the periferal action of insulin is disturbed by the negative effect of reactive oxygen species on membrane ATP-ase activity [Dejica, 2000].

Peroxidation of lipids (especially LDL) plays an important role in inducing macrovascular lesions found in both diabetes and in atherosclerotic disease. Susceptibility to oxidation of lipoproteins seems to be a key element in the initiation and propagation of the atherogenic process [Dobreanu M., Módy, 1998]. In diabetic patients, lipid oxidation affects circulating lipids, and also those present in cell membranes or myelin layers. Hyperglycemia, excessive autooxidation and decreased antioxidant capacity registered in diabetic patients are responsible for intensified oxidative processes.

The speed of non-enzymatic glycation is proportional to the blood sugar level. Glucose (and fructose and galactose) are attached to the extreme N-terminal peptide chain, initially forming a Schiff base (aldimine), which is unstable, then by Amadori rearrangement a stable cetoimine is formed, and advanced glycation end products, leading to alterations in configuration, of the electronegative charge and molecular recognition of proteins.

Amadori-products can also be oxidized, by reactions catalyzed by transition metals, releasing eritronic acid and forming carboxymethylated lysine. Its level is double in the collagen of the skin of diabetic patients compared to non-diabetic subjects, and it is positively correlated with the presence of retino- and nephropathy in diabetic patients [Wolff, 1993].

All structural proteins and those circulating in the body can suffer non-enzymatic glycation processes. They can alter protein structure and function of vessels, nerves, liver, skin and other organs. Glycosylation of LDL lipoprotein particles decreases their catabolism and accelerates HDL catabolism, disorders that may explain in part the modifications present in macroangiopathy. Glycosylated proteins are more susceptible to attack by reactive oxygen species.

Enzymatic glycosylation of proteins is also important in development of chronic diabetic complications. The collagen molecules are thus glycosylated, glycoproteins and

proteoglycans suffer similar processes. At the level of nerves, lens, livers or other organs, glycosylation of proteins is involved in the occurrence of diabetic neuropathy, cataracts, dysmetabolic hepatopathy [Gherasim, 1998], [Kovács, 2001].

Based on these evidences, antioxidant treatment is a promising approach for complementary therapy in diabetes mellitus. Several phytotherapeutical products contain a complexity of free radical scavengers and exhibit no side effects on long term treatment.

4. Evaluation of oxidative stress and the efficacy of antioxidant treatment in diabetes mellitus

4.1. Studies on animals

Some of the plants known for their high antioxidant power are: Allium sativa, Ricinus communis, Securinega virosa, Viscum album, pomegranates, berries (including strawberries, blueberries, and raspberries), walnuts, sunflower seeds, ginger and several plants of Indian traditional medicine: Emblica officinalis L., Curcuma longa L., Mangifera indica L., Momordica charantia L., Santalum album L, Swertia chirata Buch-Ham, Withania somnifera and Cassia auriculata [Capasso et al, 2003], [Dejica, 2001], [Moshi & Mbwambo, 2003], [Pari & Latha, 2003], [Pietta, 1998], [Scartezzini & Speroni, 2000], [Varga et al., 2001].

Our studies carried out in streptozotocin diabetic rats demonstrated that treatment with the blueberry (Eridiarom®) or blueberry and sea buckthorn concentrate (Diavit®) for two months had regenerative effect on pancreatic beta cells [Crăciun et al., 2007], [Morar et al., 2004].

The therapeutic properties of blueberry are attributed to its anthocyanosides which belong to a class of substances known as plant bioflavonoids. Pharmacologically, anthocyanosides are thought to decrease vascular permeability and improve microcirculation. They are also thought to have antioxidant activity.

Diavit® is a dietary supplement with a more complex composition compared to Eridiarom®, it contains quinolizidine alkaloids, anthocyanosides, sugars, carotenoids, vitamins (C, E, PP, B_1, B_2, folic acid), minerals (K, Ca, P, S, Mg, Cl, Mn, Fe), organic acids, flavonoids, etc..

Carotenoids are best recognized for their antioxidant capacity, they are considered the most potent biological quenchers of singlet oxygen [Morar, 2003], [Paiva & Russell, 1999], [Pizzorno & Murray, 2003], [Rombi, 1998], [Slosse & Hootele, 1981], [Timberlake & Henry, 1988], [Verette, 1984], [Zeb, 2004].

The experiment was carried out on 5 groups of male Wistar rats, the first five group received Streptozotocin i.v. 4 mg/100 g body weight to induce diabetes.

The toxicity of Streptozotocin can be counteracted by desferioxamine, suggesting that oxidative reactions catalyzed by transition metals could be responsible for the toxicity of this substance. Streptozotocin, being one of the glico-nitrosureas, could exhibit its diabetes-inducing effect also by inadequate NO release [Giugliano et al., 1995].

The first group served as a diabetic witness, the second group was treated with Siofor® ½ tablet/day/group (equivalent to 12 mg/kg), the third group received Meguan® (2 tablets of

0.5 g/group/day), the 4th group was treated with Eridiarom® (1.2 g/group/day), and the fifth group was given Diavit® (1.2 g/group/day). Healthy, normally fed rats formed the 6th, non streptozotocin-treated witness group.

After 5 days, and than each month glycaemia was measured, and after 100 days histopathologic examination (pancreas, liver, kidney, heart, muscles, eyes) were performed on 2 animals of each group, and the rest of the rats were kept under observation.

During the experiment one animal in each treated group died, and in the group treated with Siofor two animals died.

The dynamics of glycaemia increases at 7 days after the induction of subclinical diabetes (176.8 – 185.5 mg/dl +/- 3.0 – 5.6 SD) in each of the streptozotocin-treated groups compared to the initial values (103.2 – 105.3 mg/dl +/- 1.6 – 2.2 SD).

After 30 days the glycaemia is close to normal (120.1 – 127.8 mg/dl +/- 2.0 – 2.1 SD) in groups 4 and treated with the phytotherapeutic products Eridiarom® and Diavit®5 (14.6-22.6% higher than the initial values), and it is high (151.6 mg/dl +/- 4.0 – 4.1 SD) in groups 2 and 3 treated with the antidiabetic sulphamides Siofor® and Meguan® (45.46% higher than the initial values), but lower compared to the first, witness group (164.6 mg/dl +/- 4.2 SD). After 90 days the glycaemia is practically normalized in groups 4 and 5 (102.8 – 114.3 mg/dl +/- 1.5 -2.3 SD) (the final value 1.37% lower, and 9.6% higher than the initial values) and it is slightly increased in the first 3 groups (135.0 – 133.3 mg/dl +/- 3.3 – 4.0 mg/dl) (29,1-27.9% higher than the initial values).

The histopathologic examination of the pancreas (Trichromic stain) revealed a strong destructive action of streptozotocin against the endocrine and exocrine pancreas.

In the first witness group we can observe pancreatic cytonecrosis, atrophy with hypofunction and disturbing of the ratio between α and β secretory cells after administering streptozotocin. 100 days later the Langerhans islets of the rats in the first, witness group present partial regeneration and recovery of pseudolobules with β cells, general interstitial oedema, atrophy of the acini's level with the significant reducing of the cellular secretory pole.

In group 2, treated with Siofor®, 100 days after the beginning of the experiment partial recovery can be observed in the Langerhans islets, with β cells during the pseudolobules' reconstruction, and partial recovery of the exocrine secretor function by acinary and lobular hyperfunction.

In group 3 treated with Meguan®, 100 days after the initiation of the experiment regenerated Langerhans islets can be seen with α and β cells' reorganisation and the formation of sinusoidal capillary, and acinus-lobulary hyperfunction with the hypertrophy of the secretor pole in pancreatic acini.

In group 4, treated with Eridiarom®, 100 days after the beginning of the experiment, Langerhans islets with regenerated α and β cells can be observed, with increased mitotic index and complete recovery, and generalised acinus-lobulary functional hypertrophy.

In group 5, treated with Diavit®, 100 days after the initiation of the experiment, Langerhans islets with complete recovery of the cell-architectonics can be observed, specific for normal

functioning Langerhans cells, and hyperthrophy with general acinus-lobulary moderate secretion.

Based on these experimental data we can conclude that the two phytotherapeutic products exhibited a powerful regenerative effect on the pancreatic cells, presenting a better efficacy compared to the widely used antidiabetic sulphamides [Crăciun et al., 2007], [Morar et al., 2004].

4.2 Studies on human subjects

In our research in humans we evaluated oxidative stress using several methods, measuring lipid peroxidation products in type 1 diabetic patients compared to non-diabetic subjects of the same age-group. We used the LPO 586 (R&D Systems) kit and two methods based on the reaction between malondialdehyde and thiobarbituric acid [Nemes-Nagy et al., 2004a], [Satoh, 1978].

After the incubation period, malondialdehyde concentration was determined by photometric dosage, in case of some methods comparing the results obtained for the samples with those for the reference series with known concentrations, previously prepared.

We revealed that oxidative stress was more intense in diabetic children (78 patients, aged 12.8 years +/- 4.2 SD) compared to healthy subjects of their age group (P=0.0022).

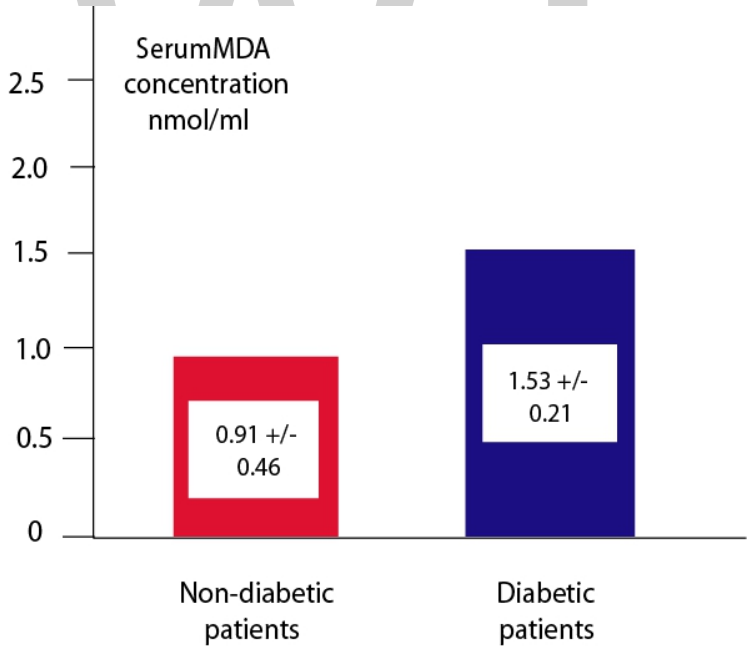

Fig. 1. Malondialdehyde concentration in diabetic and non-diabetic patients by Kei Satoh method

We obtained good correlation of the results provided by the three methods: r = 0.8087, P = 0.0151 comparing LPO-586 method with Fogelman procedure, r = 0.6580, P = 0.0007 comparing Fogelman method with the procedure described by Kei Satoh, and r = 0.9085, p = 0.0007 comparing the LPO-586 method with Kei Satoh procedure [Nemes-Nagy, 2004a].

In the diabetic children's group the average value of HbA1c was 10.0% +/- 1.9 (SD) (range 6.7 – 15.4 %), and the average malondialdehyde concentration was 3.7 nmol/ml +/- 1.04 (SD) (range 1.7 – 8.1 nmol/ml), and the MDA concentration was significantly higher ($P<0.001$) compared to the average value of children presenting glucose intolerance (2.6 nmol/ml +/- 1.1 SD).

HbA1c level highly correlated with malondialdehyde concentration ($r = 0.814$, $P<0.05$) in the diabetic children's group (60 patients).

HbA1c was determined by chromatographic method using venous blood samples collected on EDTA-K_2 as anticoagulant.

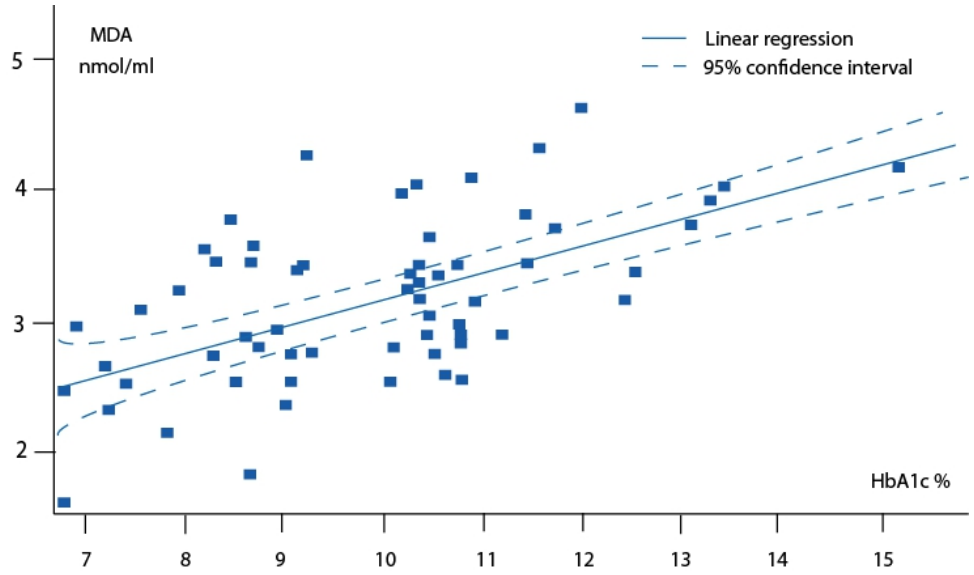

Fig. 2. Correlation between glycated hemoglobin level and serum malondialdehyde concentration in type 1 diabetic children

We also determined serum malondialdehyde concentration in three different groups of type 2 diabetic patients: subjects presenting cardiovascular (CV) diseases, patients having a high risk to develop such diseases, and those who had no other cardiovascular risk factor besides suffering from diabetes mellitus.

We obtained significant differences between the first and third (P = 0.0015), and the second and the third group (P = 0.0018) concerning malondialdehyde concentration, the patients in the third group having lower levels [Moldován, 2003] compared to those from the first two groups.

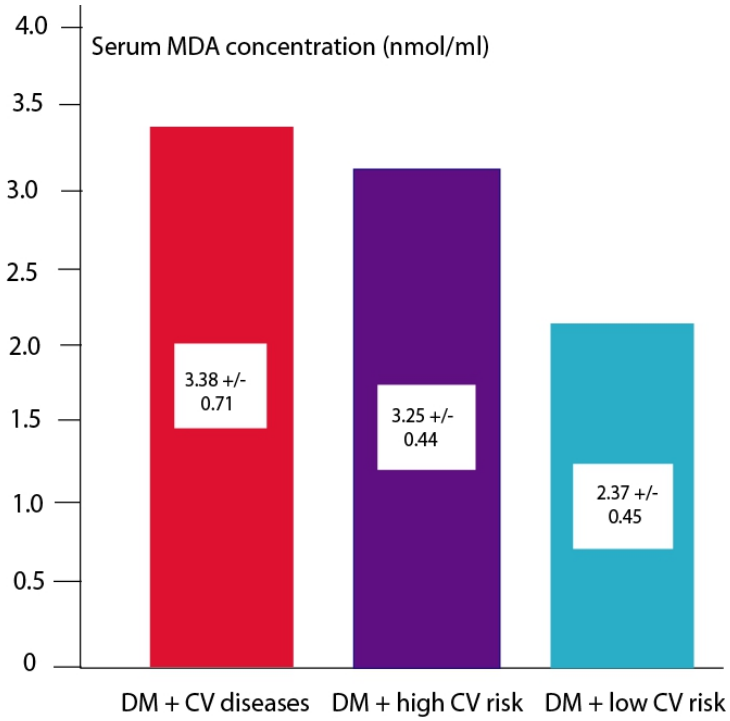

Fig. 3. Average values of serum malondialdehyde concentration in different cardiovascular risk groups of type 2 diabetic patients

We compared Cu/Zn SOD and GPX activities in diabetic children and a non-diabetic infant group of similar age. In diabetic infants we found significantly lower SOD activities (1200.8 U/gHb ± 101.4 SD) compared to the control group (1404.9 U/gHb +/- 125.4 SD), the difference is significant (P<0.005). No notable differences could be observed regarding GPX activity in the 2 studied groups of patients (P>0.05).

According to the literature normal values of erythrocyte SOD are considered between 1102-1601 U/gHb. In our diabetic group only 16,7% of the patients presented values lower than normal, the minimum SOD activity value was 1014.2 U/gHb and the maximum was 1386 U/gHb.

Physiological values of GPX activity are between 27.5-73.6 U/gHb, only one of the studied diabetic children exhibited a value slightly lower than normal [Jákó et al, 2009].

Our research team demonstrated the powerful antioxidant and hypoglycemic effect of a blueberry (*Vaccinium myrtillus*) concentrate (Eridiarom®) in diabetic children (initially this product was used for treatment of diarrhoea in humans). We selected 29 infants presenting poor carbohydrate metabolic balance to participate to the study.

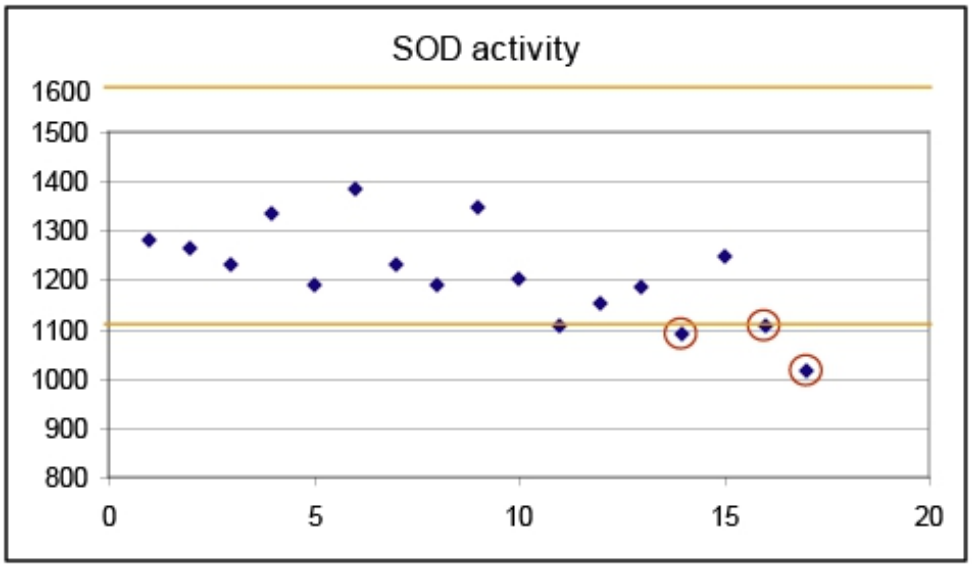

Fig. 4. Erythrocyte Cu-Zn superoxide dismutase activity in type 1 diabetic children

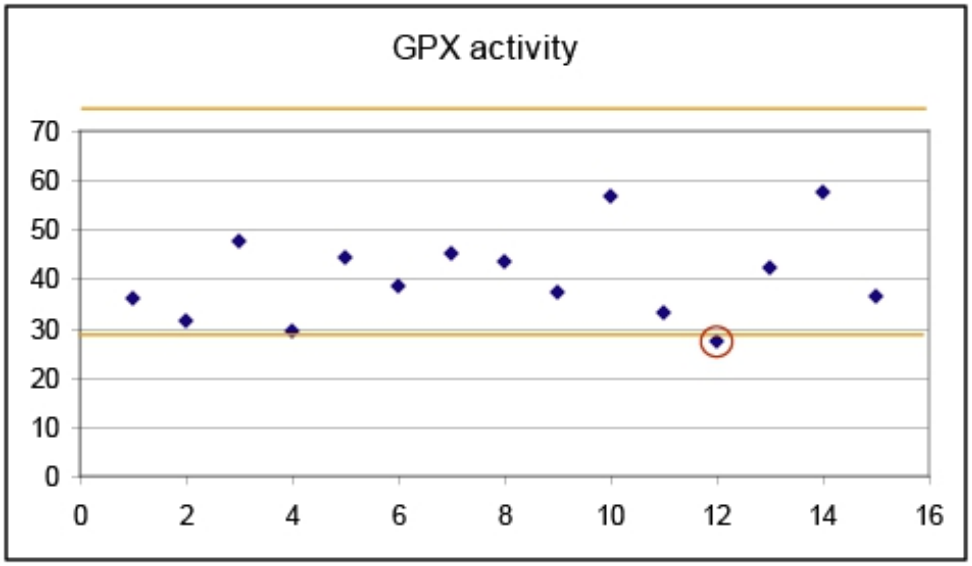

Fig. 5. Whole blood glutathione peroxidase dismutase activity in type 1 diabetic children

The average glycemic level at the beginning of the study was 179.4 mg/dl ± 39.2 (SD), after 3 months of Eridiarom® treatment the value decreased to 159.1 mg/dl +/- 40.7 (SD), the difference is significant (P<0.005).

Insulin doses (UI/kg) could be lowered in 78.6% of the patients. Initially the average dose was 0.98 UI/kg ± 0.2 (SD) and after 3 months of treatment 0.91 UI/kg ± 0.2 (SD), the difference is significant (P<0.05). This result can be explained by the regenerative effect of this phytotherapeutic product on the pancreatic beta cells, improving their insulin secretion.

We observed that the longer the treatment with this dietary supplement, better the results are.

After 3 months of Eridiarom® treatment HbA1c values presented decrease in 71.43% of the patients. Using the Student paired t test, we obtained significant differences (P<0.05) regarding the HbA1c values at the beginning of the study (9.6% ± 1.6 SD) and 8.5% ± 1.5 (SD) after 3 months of Eridiarom® treatment.

After 3 months of Eridiarom® treatment MDA concentration decreased in 92.9% of the patients, 7.1% showed practically no modification of the MDA level.

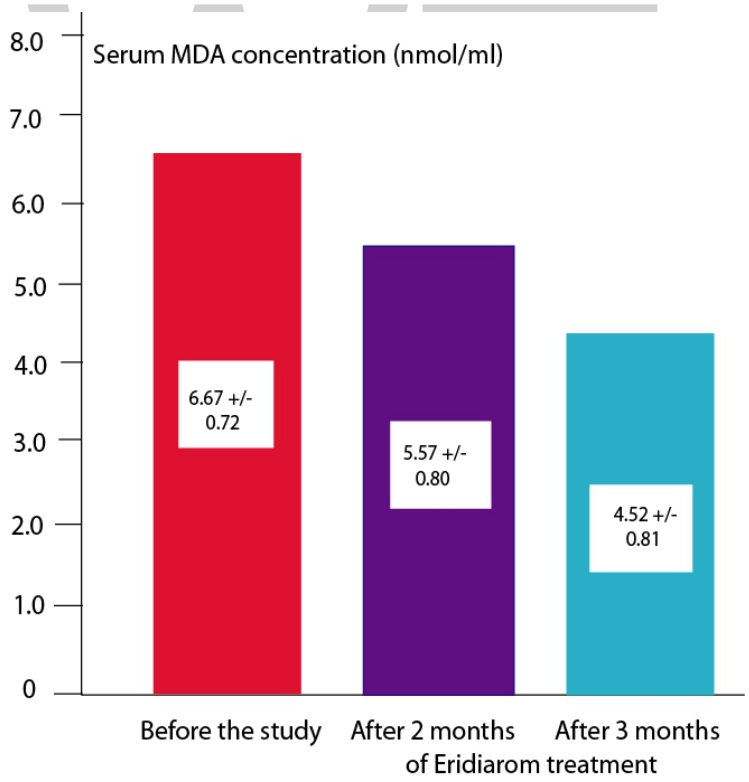

Fig. 6. Serum MDA concentration before and after Eridiarom® treatment

The average MDA concentration before the study was 6.7 nmol/ml ± 0.7 (SD), after 3 months of treatment the average value was 4.5 nmol/ml ± 0.8 (SD), the difference is significant (P<0.0001).

Prior to the study the serum magnesium and calcium concentrations were under the normal range in 43% of the patients. The average magnesium level at the beginning of the study was 1.7 mg/dl, while after 3 months of Eridiarom® treatment in case of all these patients the magnesium level turned to normal, the average value being 2.2 mg/dl; the difference is significant (P<0.0001) [Balogh-Sămărghițan et al., 2004], [Nemes-Nagy et al., 2006].

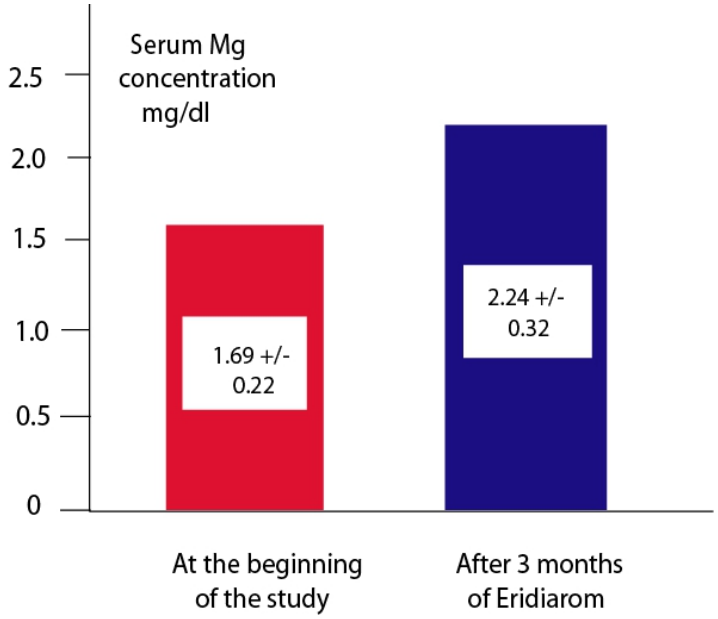

Fig. 7. Dynamics of serum magnesium concentration under blueberry containing Eridiarom® treatment

Several studies were made on the implications of magnesium in diabetes mellitus. Certain key enzymes of carbohydrate metabolism (glucokinase, hexokinase, glucose-6-phosphate dehydrogenase) and lipid metabolism (mevalonate kinase, lecithin-cholesterol acyl-transferase) are magnesium-dependent.

Insulin, along with catecholamines, has major effects on intracellular homeostasis of magnesium, being, besides vitamin D and taurine, an important magnesium-linking substance. Insulin receptors exhibit a magnesium-dependent kinase activity [Vereşiu, 2000].

Studies evaluating magnesium levels in diabetic patients compared to healthy controls showed decreases in case of diabetic subjects, and other studies have shown an increased risk for this disease in patients with magnesium deficiency. Possible correlation of Mg with chronic diabetes complications have been the subject of other studies, that have found lower values in patients with retinopathy [De Valk, 1999] and neuropathy [DeLourdes, 1998].

Effects of magnesium administration in patients with diabetes to improve glycemic control and prevention of chronic complications are conflicting and awaiting further confirmation.

A few years later we studied the effect of another dietary supplement (Diavit®), containing blueberry (*Vaccinum myrtillus*) and sea buckthorn (*Hippophae rhamnoides*) concentrate with a complex composition previously presented.

We compared glycaemic profile, glycated hemoglobin ($HbA1_C$) after 2 and 3 months of treatment, C peptide level and changes in antioxidant enzyme activity (Cu/Zn superoxide dismutase and glutation peroxidase) after two months of treatment with the Diavit® dietary supplement versus after placebo treatment.

Values for activity of erythrocyte Cu/Zn SOD, a scavenger of superoxide radicals, were significantly higher ($P<0.05$) in diabetic patients after two months of treatment with the concentrate (1260.9 ± 66.9 U/g Hb) compared to those obtained before treatment (1201.6 ± 105.6 U/g Hb).

There was no significant difference in GPX activity before (40.8 ± 9.2 U/g Hb) and after the study (43.9 ± 13.9 U/g Hb), only a slight, not significant increase could be observed ($P>0.05$) [Capasso et al, 2003], [Nemes-Nagy et al., 2007, 2008, 2010], [Paglia & Valentine, 1967], [Szőcs-Molnár et al., 2006].

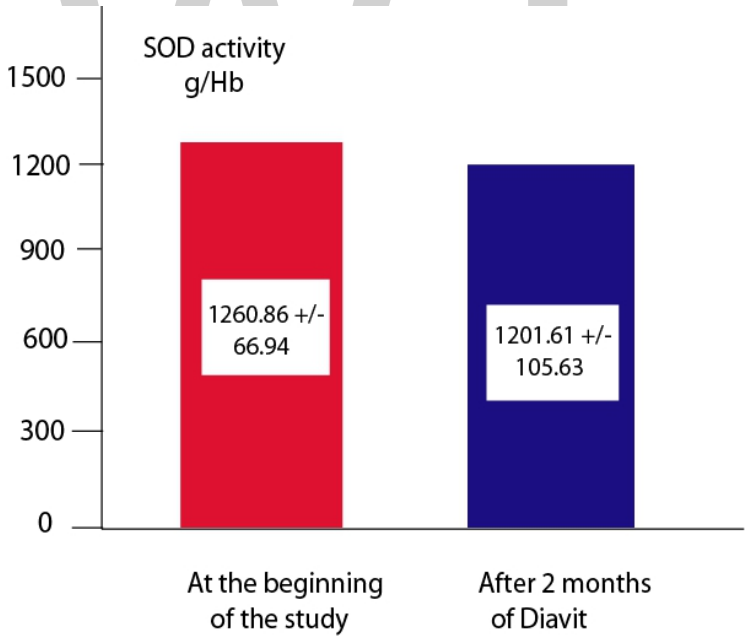

Fig. 8. Dynamics of blood SOD activity under blueberry and sea buckthorn containing Diavit® treatment

Hydrogen peroxide, formed by the reaction catalyzed by SOD, is decomposed by two enzymes: GPX and CAT. Dosage of blood catalase activity could have helped to have a better picture on the enzymatic antioxidant equipment of these diabetic patients under the treatment with this dietary supplement.

$HbA1_C$ levels were significantly lower after treatment with the dietary supplement (9.2 ±1.6 % versus 4the initial 10.2 ± 2.3 %; $P<0.05$) and C-peptide average value increased significantly ($P<0.05$) after 2 month of treatment with this dietary supplement (0.2 ng/ml ± 0.1 SD) compared to the initial average value (0.04 ng/ml ± 0.02 SD). Insulin requirement reduced significantly from the average of 0.96 ± 0.27 IU/kg bodyweight to 0.89 ± 0.28 IU/kg after 2 months of treatment with the product ($P<0.05$), insulin doses were reduced in 66.7% of the patients.

Lower blood glucose and HbA1c values may be due to a regenerative effect that the product has on pancreatic beta cells. Significantly higher C-peptide levels after 2 months of treatment with the extract support this hypothesis [Nemes-Nagy et al., 2008].

A scientific team in Harvard University, Howard Hughes Medical Institute, under the leadership of Prof. Douglas Melton, published their findings about the capacity of pancreatic beta cells to regenerate by self-duplication due to some latent embrional cell remains or adult stem cells [Zhou et al., 2008], and this regenerative process might explain our results with the product studied. We suppose that better results could be obtained if the treatment with the concentrate begins soon after the diabetic disease is diagnosed, maybe because long term insulin treatment causes the atrophy of pancreatic beta-cells, similar to corticosteroid-caused hypofunction of corticosuprarenals. This hypothesis should be verified in latter studies. Based on our experimental data it seems that the longer the treatment with these dietary supplements, the better the results are.

Hyperglycemia in diabetes mellitus produces increased oxidative stress via non-enzymatic glycation, glucose autooxidation, and alteration in polyol pathway activity. This is characterized by increased lipid peroxide production and decreased antioxidative defence (e.g. inactivation of SOD by glycation) which affects the entire body. Two months using Diavit® lead to a significant increase in SOD which may have occurred as a result of its antioxidant and hypoglycemic effects. The antioxidant effect of this product might be partially due to anthocyanosides, known as scavengers of superoxide anions, inhibitors of lipid peroxidation. Lower glycaemic levels during the study might cause lower superoxide radical production and decrease the inactivation rate of this antioxidant enzyme, leading to higher SOD levels than before.

Protection of free radical scavengers might help to maintain higher levels of antioxidants under treatment with this concentrate, and several components of the product (carotenoids, vitamin E, C) show important protective role against oxidative stress. According to recent studies, it might be a link between GPX and ascorbate: intracellular vitamin C cooperates in enhancing glutathione recovery after oxidative challenge thus providing cells with enhanced survival potential, while extracellular vitamin C is recycled through a mechanism involving the simultaneous neutralization of oxidant species [Montecinos et al., 2007].

Several data suggest that oxygen metabolits are involved in the pathogenesis of autoimmune destruction of pancreatic beta cells, involving inflammatory process, and especially superoxide

radical is required for the expression of the disease. Pancreatic beta cells are particularly sensitive to superoxide mediated radical damage, having a poor antioxidant defence system. Superoxide itself or derivative radicals may be the direct cause of cell damage. Radical generation leads to breakage of cell DNA, which initiates the repair process resulting in depletion of cellular NADH + H^+ levels, leading to an inhibition of pro-insulin synthesis and renders the cell more sensitive to radical damage because NAD is involved in the electron-transport process required for radical scavenging by the cell. Oxygen radicals are also involved in the production of the cytokines (IL-1, TNF) by the cells of the inflammatory focus that could be involved in the cell damage [Nemes-Nagy et al., 2008].

Another important component of Diavit® is the phytoestrogen called resveratrol, which is a natural polyphenolic compound found largely in the skin of red grapes, but also in blueberries.

Growing evidence suggests that resveratrol may play an important role in the prevention of many human diseases. Many of the biological actions of this polyphenol have been attributed to its antioxidant properties, it exhibits anticoagulant, vasodilator, antiinflammatory effects, inhibits oxidation of LDL-cholesterol particles, thus preventing atherosclerosis, and also increases sensitivity to insulin.

Certain studies evaluated the effect of resveratrol on intracellular reduced glutathione (GSH) and membrane sulphydryl groups in erythrocytes subjected to oxidative stress in vitro to test the efficacy of the antioxidant effect of resveratrol on human erythrocytes. In one of these studies subjecting erythrocytes to oxidative stress (in vitro) by incubating them with t-BHP (10 micromolar) caused a significant decrease in the intracellular GSH level and membrane -SH content compared with basal values.

Incubation of erythrocytes/membranes with resveratrol (1-100 micromolar final concentration) resulted in significant protection against the t-BHP-induced oxidative stress as evidenced by the increase in GSH level and membrane -SH content. It was observed that the effect of resveratrol is dose/concentration and time-dependent, it protects erythrocytes experimentally exposed to oxidative stress. Since resveratrol is naturally present in many fruits and vegetables, a diet rich in resveratrol, or dietary supplements containing this substance may provide protection against degenerative diseases and prevent diabetes complications [Pandey & Rizvi, 2010].

A new promising study on humans demonstrated the effect of regular consumption of a resveratrol supplement and the health of patients with impaired glucose tolerance. Resveratrol has been tested in relation to diabetes before, but only in animal subjects or on cell lines. Those studies have repeatedly shown promising effects on insulin secretion, insulin sensitivity and glucose tolerance, leading to the initiation of this first-of-its-kind pilot clinical study on humans [Crandall, 2010].

5. Studies on antioxidant components of medicinal teas, fruit and vegetable juices

We also determined the flavonoid content and the antioxidant capacity of several medicinal teas used by diabetic patients. We found the highest amount of flavonoids in Juglandis

folium, and the smallest amount of flavonoids was found in Phaseoli tegumen. The results were calculated in hyperoside units.

The highest concentration of malondialdehyde was found in Centauri herba (149 mmol/100g), smaller amounts were found in Myrtilli folium (146 mmol/100g), Mori folium (115 mmol/100g), Crataegi summitas (100 mmol/100g), Urticae herba (97 mmol/100g), Menthae piperitae (97 mmol/100g), Visci albae stipes (94 mmol/100g), Juglandis folium (89 mmol/100g), Millefolii flos (79 mmol/100g) and Phaseoli tegumen (73 mmol/100g). The antioxidant capacity of these plants is inversely proportional to their MDA contents, a measure of ROS-induced damage to lipids [Nemes-Nagy et al., 2004], [Lushchak Vl., 2011].

Medicinal teas	**Flavonoid content (g% hyperoside)**
Juglandis folium	1.79
Crataegi summitas	0.75
Menthae piperitae	0.70
Urticae herba	0.68
Mori folium	0.59
Myrtilli folium	0.31
Millefolii flos	0.24
Centauri herba	0.24
Visci albae stipes	0.18
Phaseoli tegumen	0.03

Table 1. Flavonoid content of medicinal teas

We measured, by photometric diclorphenol-indophenol method the vitamin C concentration in freshly squeezed juices and in those preserved, available in stores. As a result we found that lemon, orange and grapefruit contain the biggest ascorbate quantity, especially freshly squeezed (22.0 – 22.2 mg/dl) the same volume of juice contains higher ascorbate concentration compared to those sold in boxes (15.0 mg/dl – 21.9 mg/dl).

In case of orange juices, we compared the vitamin C content of 3 samples from juices sold in boxes from different firms (the results obtained were 20.5 mg/dl, 18.2 mg/dl and 15.0 mg/dl), so we concluded that the concentration of ascorbic acid was 7.7%, 18.0% and 32.0% lower compared to the result obtained from the freshly squeezed juices.

Freshly squeezed juices	**Vitamin C content (mg/dl)**
Orange	22.2
Lemon	22.2
Grapefruit	22.0
Grapes	17.2
Tomato	17.0

Table 2. Vitamin C contents of freshly squeezed juices

6. Vitamin C dynamics in human milk

We also followed the dynamics of ascorbate in human milk after juice and vitamin C tablet ingestion. The highest ascorbate level in milk was 1 hour after juice ingestion and half an hour after taking 1000 mg vitamin C containing tablets, the assimilation from natural sources being better. It is a close relationship between mothers' diet and the quality of their milk. We can recommend to consume juices one hour before brestfeeding, to offer the infant the highest amount of ascorbate [Jákó et al., 2008].

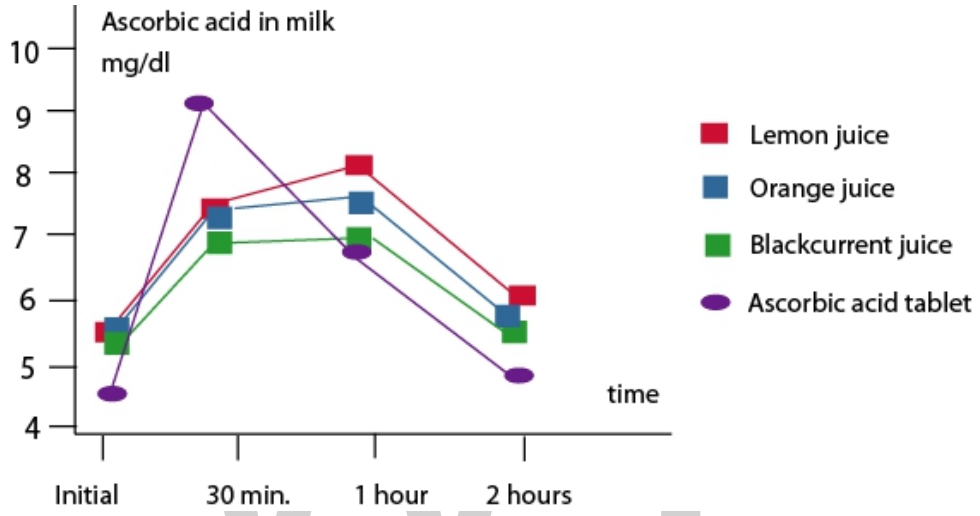

Fig. 9. The dynamics of ascorbic acid in human milk after ingestion of freshly squeezed juices and C vitamin tablets

7. Determination of carotenoid score in diabetic patients

Another study was made on adult subjects, we used Raman spectroscopy (a method that can identify carotene molecules in the skin) to determine the carotenoide score, which gives information on the antioxidant capacity of the body. Significantly lower average value was obtained in adult diabetic compared to the control non-diabetic group of similar age; the difference is significant ($P<0.05$) [Jákó et al, 2009].

Food carotenoids are transported into the skin, protecting the tegument against ultraviolet radiation. The skin carotenoid concentration undergoes smaller variation compared to that in the blood depending on the dietary intake. Skin carotenoid determination by HPLC method using bioptic material is an invasive procedure and cannot be introduced in the every day practice. A very good correlation can be found between the serum carotenoid concentration and that in the palm skin ($r = 0.78$, $P<0.001$) determined by Raman spectroscopy, and the biophotonic scanner operates based on this observation [Jákó et al, 2009], [Peng et al., 1995], [Smidt & Shieh, 2003]. Thus, skin carotenoid measurement appears to be a valuable biomarker of carotenoid nutritional status.

Raman scattering spectroscopy is a highly specific method for skin carotenoid determination. This method is able to discern carotenoids from other potentially interfering compounds present in the skin due to its ability to identify molecules with long conjugated double-bond structures. Raman spectroscopy involves a blue, low-energy laser light source of 470 – 490 nm, directed onto the surface of the skin, where Raman resonance light scattering events cause the carotenoids to emit a green signal at 510 – 530 nm, which is detected and quantified.

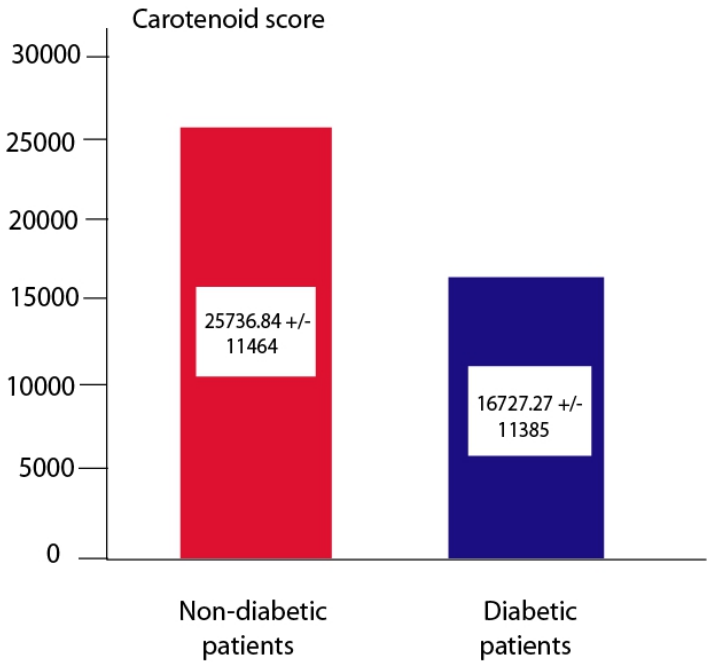

Fig. 10. Comparision of skin carotenoid score in diabetic and non-diabetic patients

Studies showed that the Raman spectroscopic method accurately reflects the presence of carotenoids in the human skin with high reproducibility. Significant differences in carotenoid concentrations were found between five different skin sites, the highest concentration being found in the palm.

An epidemiological study performed on a large number of healthy volunteers determined that the Raman spectroscopic method of measuring skin carotenoids is not really affected by gender, age or skin pigmentation.

Raman intensity measurements were positively related to the amount of fruit and vegetable intake and use of carotenoid-containing dietary supplements, and inversely related to body fat content and smoking. This study confirmed that skin carotenoids follow similar dietary and demographic patterns as in case of serum or plasma carotenoid measurements.

Before the measurement, every patient has to fill in a special questionnaire containing questions regarding its lifestyle, dietary habits and other aspects that could influence the result.

Fig. 11. Biophotonic scanner showing the carotenoid score of a non-diabetic patient

Fig. 12. Low carotenoid score of an old diabetic patient

8. Conclusion, perspectives

Regarding proper evaluation of antioxidant status in the human body, measuring lipoperoxidation products is a valuable tool because its high value positively correlates with the intensified oxidative stress. Interpretation of antioxidant enzyme activities could be sometimes difficult because it can decrease in case of intense consumption when free radical production is intensified, and in other situations even higher values can be observed due to compensatory mechanisms, being a way of adaptation to intensified oxidative stress.

We can conclude that proper nutrition including adequate fresh fruit and vegetable intake are important sources of natural antioxidants. Patients suffering from diseases like diabetes mellitus which involves high oxidative stress should take dietary supplements containing antioxidant vitamins, phytoterapeutical products and oligoelements, these could help diabetic patients to achieve a better metabolic balance and to prevent several complications of this disease.

It would be interesting to perform a placebo-controlled double-blind study on the effect of the dietary supplement Diavit®, containing blueberry and sea buckthorn concentrate, on a large group of type 2 diabetic patients followed for several years, to observe the long term effect of this phytotherapeutic product, or other, more complex dietary supplements could be used in similar studies. This could be a possibility to reduce the incidence of complications in diabetic patients, and to help them to achieve a proper metabolic balance without taking antidiabetic drugs, or at least lower the doses of their usual medication, decreasing the risk of developing side effects.

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The contributors of this book come from diverse backgrounds, making this book a truly international effort. This book will bring forth new frontiers with its revolutionizing research information and detailed analysis of the nascent developments around the world.

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This book was conceptualized with the vision of imparting up-to-date information and advanced data in this field. To ensure the same, a matchless editorial board was set up. Every individual on the board went through rigorous rounds of assessment to prove their worth. After which they invested a large part of their time researching and compiling the most relevant data for our readers. Conferences and sessions were held from time to time between the editorial board and the contributing authors to present the data in the most comprehensible form. The editorial team has worked tirelessly to provide valuable and valid information to help people across the globe.

Every chapter published in this book has been scrutinized by our experts. Their significance has been extensively debated. The topics covered herein carry significant findings which will fuel the growth of the discipline. They may even be implemented as practical applications or may be referred to as a beginning point for another development. Chapters in this book were first published by InTech; hereby published with permission under the Creative Commons Attribution License or equivalent.

The editorial board has been involved in producing this book since its inception. They have spent rigorous hours researching and exploring the diverse topics which have resulted in the successful publishing of this book. They have passed on their knowledge of decades through this book. To expedite this challenging task, the publisher supported the team at every step. A small team of assistant editors was also appointed to further simplify the editing procedure and attain best results for the readers.

Our editorial team has been hand-picked from every corner of the world. Their multi-ethnicity adds dynamic inputs to the discussions which result in innovative outcomes. These outcomes are then further discussed with the researchers and contributors who give their valuable feedback and opinion regarding the same. The feedback is then collaborated with the researches and they are edited in a comprehensive manner to aid the understanding of the subject.

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The publisher and the editorial board hope that this book will prove to be a valuable piece of knowledge for researchers, students, practitioners and scholars across the globe.

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