THE SOURCES AND THE TARGETS OF OXIDATIVE STRESS IN THE ETIOLOGY OF DIABETIC COMPLICATIONS

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*Abstract***.** Oxidative stress, defined as an imbalance between reactive oxygen species production and breakdown by endogenous antioxidants, is closely associated with aging and a number of diseases including inflammation, carcinogenesis, and atherosclerosis. Also, it has been shown that oxidative stress plays a role in the progression of diabetes. Hyperglycemia, which occurs during diabetes (both type 1 and type 2) and, to a lesser extent, during insulin resistance, causes oxidative stress. Oxidative stress may be important in diabetes, not just because of its role in the development of complications, but because persistent hyperglycemia, secondary to insulin resistance, may induce oxidative stress and contribute to beta cell destruction in type 2 diabetes. Glucose control plays an important role in the prooxidant/antioxidant balance. A supplementation with antioxidants has been proposed as a complementary treatment, and some antidiabetic agents may by themselves have antioxidant properties independently of their role on glucose control. The aim of this paper was to review the sources and the targets of oxidative stress in the etiology of diabetic complications.

Key words: diabetes, oxidative stress, glycation, lipid peroxidation, antioxidants.

INTRODUCTION

Diabetes mellitus (DM) is a syndrome characterized by abnormal insulin secretion, derangement in carbohydrate and lipid metabolism, and is diagnosed by the presence of hyperglycemia.

During diabetes or insulin resistance, failure of insulin-stimulated glucose uptake by fat and muscle causes glucose concentrations in blood to remain high. Consequently, glucose uptake by insulin-independent tissues increases. Increased glucose flux both enhances oxidant production and impairs antioxidant defenses by multiple interacting non-enzymatic, enzymatic and mitochondrial pathways [45, 56]. These include activation of protein kinase C isoforms [37], increased hexosamine pathway [41], glucose autooxidation [12], increased methyl-glyoxal and advanced glycation end-product (AGEs) formation [84], increased polyol

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pathway flux [49]. These seemingly different mechanisms are the result of a single process – that is, overproduction of superoxide by the mitochondrial electron transport chain [92]. This hyperglycaemia-induced oxidative stress ultimately results in modification of intracellular proteins resulting in an altered function, DNA damage, activation of the nuclear transcription NF-kB, causing abnormal changes in gene expression, decreased production of nitric oxide, and increased expression of cytokines, growth factors and procoagulant and proinflammatory molecules [20, 27, 46, 66, 83].

Since numerous studies demonstrated that oxidative stress, mediated mainly by hyperglycemia-induced generation of free radicals, contributes to the development and progression of diabetes and related contributions, it became clear that ameliorating oxidative stress through treatment with antioxidants might be an effective strategy for reducing diabetic complications.

In this article, sources of free radicals contributing to oxidative stress and the natural defense mechanisms in diabetes are briefly reviewed.

REACTIVE SPECIES AND OXIDATIVE STRESS

Oxidative stress is defined by Sies [76] as a disturbance in the prooxidant-antioxidant balance in favour of the former, leading to potential damage, and by Dean [22] as a disruption of redox signaling and control.

Cellular metabolism generates reactive oxygen species (ROS). Molecular ground-state oxygen can be activated to a ROS by means of energy transfer (e.g., under the influence of ultraviolet radiation), forming singlet oxygen ($^1O_2$), or by electron transfer, forming "incomplete" reduction products, i.e., the superoxide anion radical ($^{\bullet}O_2^-$). Small amounts of oxygen (between 0.4 and 4% of all oxygen consumed) are reduced to $^{\bullet}O_2^-$ by the mitochondrial electron transport chain during the course of normal oxidative phosphorylation, which is essential for generating ATP [11, 16].

Subsequently, $^{\bullet}O_2^-$ can be converted into other ROS and reactive nitrogen species (RNS) (Fig. 1.). Under normal conditions, $^{\bullet}O_2^-$ molecules are quickly converted to H_2O_2 by the key mitochondrial enzyme, manganese superoxide dismutase (Mn-SOD) within the mitochondria and by copper and zinc (CuZn-SOD) in the cytosol [28, 57].

H_2O_2 is then either detoxified to H_2O and O_2 by glutathione peroxidase (in the mitochondria) in conjunction with glutathione reductase, or diffuses into the cytosol and is detoxified by catalase in peroxisomes. H_2O_2 can also be converted to the highly reactive hydroxyl radical (HO•) in the presence of reduced transition metals such as Cu or Fe (Fenton reaction). Further reactive oxygen species may be

derived from H_2O_2, such as the hypochlorite (OCl^-), peroxyl radicals (ROO•) and alkoxyl radicals (RO•) or from peroxidation of polyunsaturated fatty acids (PUFA) such as conjugate dienes, lipid hydroperoxides and malonyldialdehyde (MDA) [83].

Production of one ROS may lead to the production of others through radical chain reactions. As summarized in Fig. 1, $^{\bullet}O_2^-$ is produced by one electron reduction of oxygen by several different oxidases including NAD(P)H oxidase, xanthine oxidase, cyclooxygenase and even endothelial nitric oxide synthase (eNOS) under certain conditions [32, 56].

RNS include free radicals like nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($^{\bullet}NO_2^-$), as well as nonradicals such as peroxynitrite ($ONOO^-$). $NO^{\bullet}$, also known as endothelium-derived relaxing factor (EDRF), produced from L-arginine by eNOS in the vasculature is considered a vasculoprotective molecule [91]. However, $NO^{\bullet}$ easily reacts with $^{\bullet}O_2^-$, generating the highly reactive molecule $ONOO^-$. Thus, variation in the production of $NO^{\bullet}$ and $^{\bullet}O_2^-$ by endothelium might provide one mechanism for the regulation of vascular tone and hence of blood pressure.

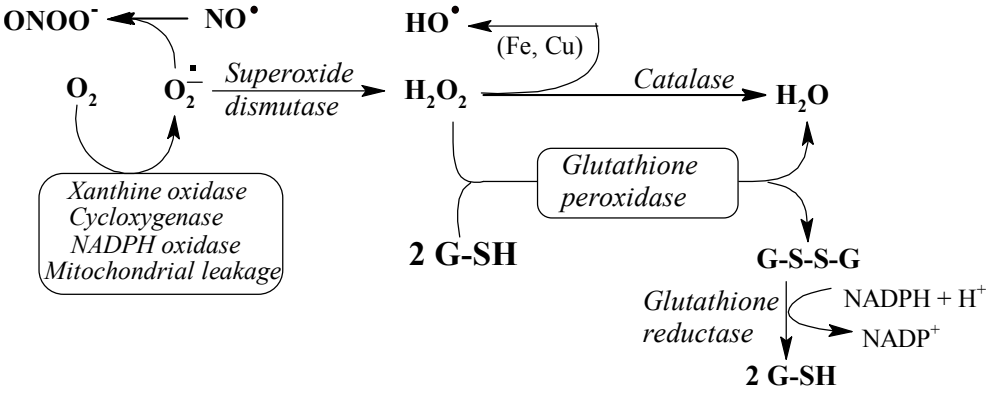

Fig. 1. Endogenous stimuli leading to ROS generation. The endogenous antioxidant enzymes: superoxide dismutase, glutathione peroxidase, and catalase function to maintain redox equilibrium.

Although these ROS and RNS differ with regard to their stability, reactivity and molecular targets, a common denominator is that their uncontrolled formation in cells, i.e., the generation of a ROS load exceeding the antioxidant capacity of the cell, results in damage and oxidation of lipids, proteins, and nucleic acids, as well as of several other biomolecules.

ANTIOXIDANTS

The reactive oxygen intermediates, produced in mitochondria, peroxisomes, and the cytosol, are scavenged by cellular defending systems, including enzymatic (ex. superoxide dismutase, glutathione peroxidase GPx, glutathione reductase and catalase) and nonenzymatic antioxidants (ex. glutathione G-SH, thioredoxin, lipoic acid, ubiquinol, albumin, uric acid, flavonoids, vitamins A, C and E, etc.). Some are located in cell membranes, others in the cytosol, others in the blood plasma [54].

One observation concerning antioxidants is their extraordinary chemical efficiency. These compounds should act rapidly, as soon as a source of increased amount of ROS appears. As ROS are very reactive with rate constants between 10^4–10^9 $M^{-1}s^{-1}$, antioxidants must possess a similar efficiency. A second common observation is their ability to function in cooperation. Effective protection from the formation and action of ROS requires antioxidant activity in both aqueous and lipid environments and in various part of the cell structure. When one AO reacts with a ROS, another antioxidant should be present to regenerate the first (Fig. 2). In addition, these AO may act by coupling to protect both the membrane and the cytoplasm. AO in cells and blood possess high capacity and redundancy to always be available to protect cellular structures and vital molecules (nucleic acids). A third characteristic has only become evident in the last few years. The role of antioxidants seems to be to sacrifice themselves to protect the essential cellular structures and molecules against ROS.

A major cellular thiol antioxidant and redox buffer of the cell is reduced glutathione (GSH), which is regenerated most efficiently from oxidised form GSSG by glutathione reductase and reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Fig. 1). Glutathione is highly abundant in the cytosol (1–11 mM), nuclei (3–15 mM), and mitochondria (5–11 mM) and is the major soluble antioxidant in these cell compartments [55, 93]. Because GSH is synthesized in the cytosol, its mitochondrial presence requires inner membrane transport. Two mitochondrial electroneutral antiport carrier proteins have been shown to have the capacity to transport GSH, the dicarboxylate carrier protein and the 2-oxoglutarate carrier protein. GSH in the nucleus maintains the redox state of critical protein sulphydryls that are necessary for DNA repair and expression. Oxidized glutathione is accumulated inside the cells and the ratio of GSH/GSSG is a good measure of oxidative stress of an organism [93]. Of course, there are many other redox couples in the cell: examples include NAD^+/NADH, ascorbate/dehydroascorbate, $NADP^+$/NADPH and α-lipoic acid (LA)/Dihydrolipoic acid (DHLA).

The main protective roles of glutathione against oxidative stress are [55]: (a) glutathione is a cofactor of several detoxifying enzymes against oxidative stress, e.g. glutathione peroxidase (GPx), glutathionereductase, glyoxalases and enzymes involved in leucotriene synthesis; (b) GSH can react with $ONOO^-$ leading to

formation of some nitrosothiol (GSNO), which can decompose to regenerate $NO^{\bullet}$; hence GSH can, to some extent, "recycle" $ONOO^{-}$ to $NO^{\bullet}$ [96] (c) GSH scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathionperoxidase; (d) glutathione is able to regenerate the most important antioxidants lipoic acid, vitamins C and E, back to their active forms; glutathione can reduce the tocopherol radical of vitamin E directly, or indirectly, via reduction of semidehydroascorbate to ascorbate (Fig. 2.). The capacity of glutathione to regenerate the most important antioxidants is linked with the redox state of the glutathione disulphide-glutathione couple (GSSG/2GSH) [67]. At high concentrations, ROS can be important mediators of damage to cell structures, nucleic acids, lipids (LH) and proteins [54].

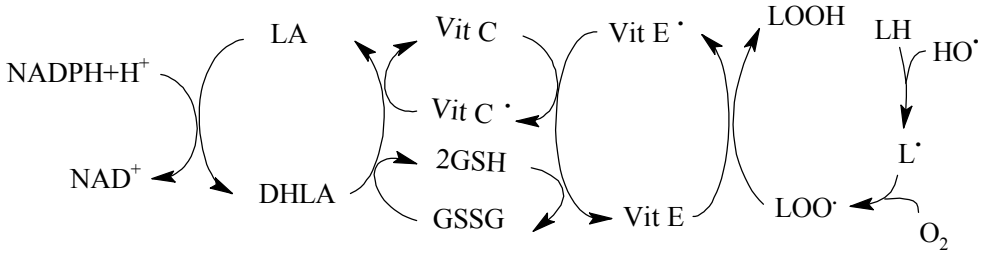

Fig. 2. Interaction between endogenous antioxidants in the process of detoxifying lipid peroxides. Regeneration of endogenous antioxidants occurs through a cooperative set of reactions. The hydroxyl radical can abstract an electron from polyunsaturated fatty acid (LH) to give rise to a carbon-centered lipid radical (L•). The lipid radical (L•) can further interact with molecular oxygen to give a lipid peroxyl radical (LOO•). The lipid peroxyl radical (LOO•) is reduced within the membrane by the reduced form of Vitamin E resulting in the formation of a lipid hydroperoxide and a radical of Vitamin E. The Vitamin E radical is reduced back to Vitamin E by Vitamin C leaving behind the Vitamin C radical. The oxidized Vitamin E radical is reduced by GSH. The oxidized glutathione (GSSG) and the Vitamin C radical are reduced back to GSH and Vit C, respectively, by the dihydrolipoic acid (DHLA) which is itself converted to α-lipoic acid (LA). α-Lipoic acid (LA) after reduction by nicotinamide adenine dinucleotide phosphate (NADPH) to dihydrolipoic acid (DHLA) is able to facilitate the nonenzymatic regeneration of vitamin C and GSH, both of which are able to regenerate vitamin E.

MECHANISMS FOR INCREASED OXIDATIVE STRESS IN DIABETES

In diabetes, an altered oxidative metabolism is a consequence either of the chronic exposure to hyperglycaemia or of the absolute or relative insulin deficit; insulin regulates several reactions involved in oxido-reductive metabolism [30].

Despite strong experimental evidence indicating that oxidative stress may determine the onset and progression of late-diabetes complications [70] controversy exists about whether the increased oxidative stress is merely associative rather than causal in diabetes. This is partly because measurement of

oxidative stress is usually based on indirect and nonspecific measurement of products of reactive oxygen species. Enhanced oxidative stress in hyperglycaemia is indicated by urinary excretion of 8-iso-PGF2α (8-iso-prostaglandin F2α) [21].

Oxidative stress as measured by indices of lipid peroxidation and protein oxidation has been shown to be increased in both insulin dependent diabetes, and non-insulin dependent diabetes, in obese diabetic patients and even in diabetic patients without complications [15, 60, 64, 78].

Measurement of thiobarbituric acid reactive substances (TBARS) concentration, although non-specific, is widely used as an indicator of ROS production lipid peroxidation process and indirectly, of oxidative stress. Increased TBARS together with alterations of antioxidant mechanisms reported in our previous study indicates that oxidative stress is associated with obesity in diabetic type 2 patients. Thus, in our obese diabetic patients, TBARS concentrations are significantly increased, and antioxidant molecules GSH concentrations are significantly decreased. Hyperglycemia, plasma lipid peroxidation and decreased level of glutathione may contribute to the significantly augmented plasma level of the dicarbonyls in obese diabetic patients versus healthy subjects [60]

During diabetes or insulin resistance, increased oxidative glucose metabolism itself increases mitochondrial production of $^{\bullet}O_2^-$, which will then be converted to HO•, and H_2O_2 [64]. Beyond glucose, ROS formation is also increased by FFAs (free fatty acids), through direct effects on mitochondria [27]. It has been proposed that overexpression and activity of a mitochondrial inner membrane uncoupling proteins (UCPs) contribute to an increase in superoxide formation under diabetic conditions [71].

The overproduction of superoxide, in particular by mitochondria, causes inhibition of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and of cytochrome enzymes of the electron transport chain responsible for oxidative phosphorylation associated with the Krebs cycle [64]. Hyperglycaemia-induced GAPDH inhibition was found to be a consequence of poly(ADP-ribosylation) of GAPDH by PARP [poly(ADP-ribose) polymerase], which was activated by DNA strand breaks produced by mitochondrial superoxide overproduction [24].

As a result, glycolytic intermediates upstream of GAPDH accumulate, leading to increased substrate-directed activity of the de novo DAG (diacylglycerol) synthetic pathway, which further activates PKC (protein kinase C isoforms) and NADPH oxidase, as well as the hexosamine and polyol biosynthetic pathways (Fig. 3).

In addition, glucose and glucose-derived dicarbonyl compounds react non-enzymatically with the basic amino acids lysine and arginine in proteins to form AGEs (advanced glycosylation end-products) both extra- and intra-cellularly. These different pathways are interrelated and potentiate each other.

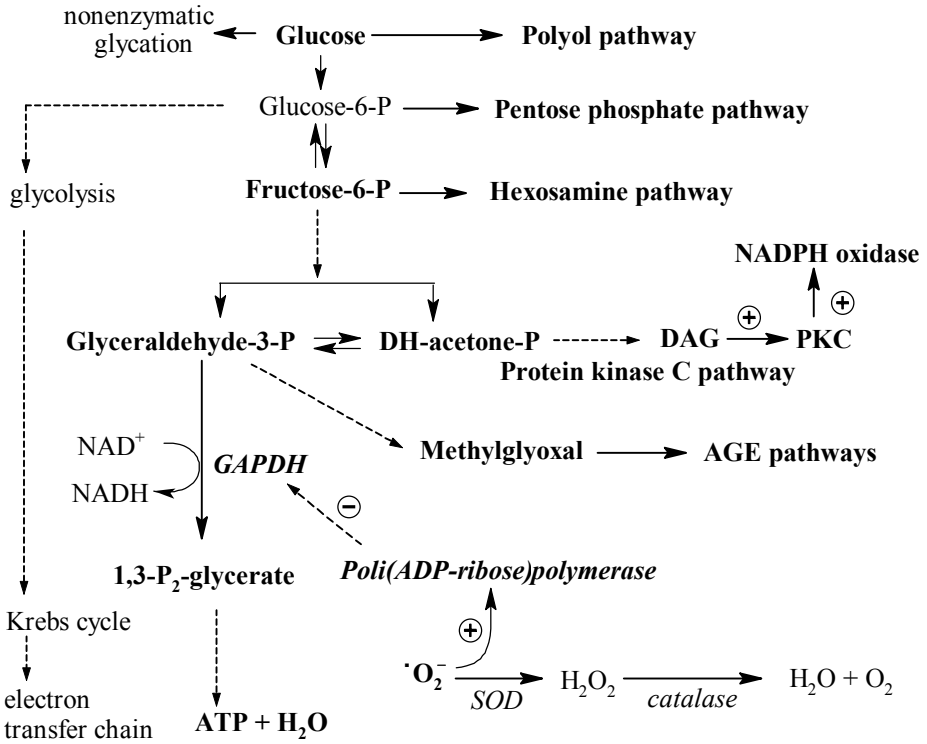

Fig. 3. Diagram of pathways that contribute to oxidative stress in response to increased glucose flux (GAPDH = glyceraldehyde-3-phosphate dehydrogenase, PARP = poly(ADP-ribose) polymerase, SOD = superoxide dismutase).

INCREASED GLUCOSE AUTOOXIDATION AND ADVANCED GLYCATION END PRODUCTS (AGES) FORMATION

Glucose in solution is a ring structure, in equilibrium with a small amount of an open-chain aldehyde form [61]. Glucose spontaneously reacts with free amino groups of proteins to form labile Schiff bases (early Maillard reaction) (Fig. 4). These Schiff bases are not stable and may either dissociate or undergo an Amadori rearrangement to become more stable, fructose-like compounds known as fructosamines [7]. Formation of fructosamines is followed by their slow conversion to a series of compounds known as advanced glycation end-products (AGEs) which are thought to participate in the development of diabetic complications [6, 13].

Glycation products can be oxidized by several ROS, including $HO^{\bullet}$ and $ONOO^{-}$, to give AGEs (Fig. 4) [1, 104]. Indeed, ROS have been described as "fixatives of glycation" [34]. When oxidation is involved in their formation,

so-called glycoxidation products such as pentosidine and N^{ε}(carboxymethyl)lysine result. These are the best chemically characterized AGEs compounds found in human. The non-enzymic glycation reaction proceeds slowly through different stages, leading to alterations of protein structure and molecular surface topology that profoundly change the affected molecule`s biochemical properties. The major biological effects of excessive glycation include: inhibition of regulatory molecule binding, crosslinking of glycated proteins, trapping of soluble proteins by glycated extracellular matrix, decreased susceptibility to proteolysis, inactivation of enzymes and transcription factors, abnormalities of nucleic acid function, and increased immunogenicity in relation to immune complex formation [1, 34, 90, 104].

Glycation *in vivo* is slow and reversible at physiological glucose levels, tending mostly to affect proteins with a very slow turnover, for example collagen in some connective tissues, and crystalline lens. Glycation is faster at elevated glucose, occurring in these and many other proteins in diabetic patients. Some tissues, such as the liver, kidneys, and erythrocytes are more susceptible to AGE formation than others [10].

Glycated haemoglobin (HbA_{1C}) contains a glucose Amadori product attached to the N-terminal valine of the β-chain. Whereas haptoglobin can prevent pro-oxidant effects of normal haemoglobin, it is less good (especially the Hp2-2 form) at doing so for glycated haemoglobin [81]. Indeed, diabetics with Hp2-2 haemoglobin were reported to suffer more cardiovascular disease [81].

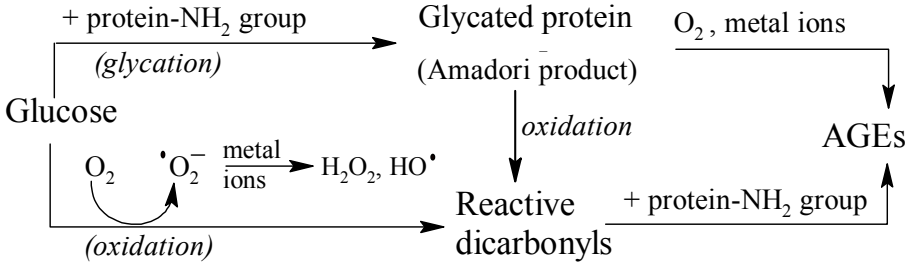

Fig. 4. Formation of advanced glycation end-products (AGEs) by combination of glycation and oxidation.

Glucose also glycates CuZnSOD in the erythrocyte, decreasing its activity; this may account for the lower SOD activity reported in the blood of some diabetics [5]. Significantly decreased SOD activity in obese and obese diabetic patients blood has been shown versus healthy subjects, none of the investigated patients or controls being anemic [60]. Both CuZnSOD and caeruloplasmin can fragment after glycation to release pro-oxidant copper ions [39].

Another way of making AGEs is to first oxidize the glucose and then allow the oxidation products to react with protein (Fig. 4). In the presence of the transition metals, glucose can oxidize, to produce $^{\bullet}O_2^-$, H_2O_2, $HO^{\bullet}$ and toxic

dicarbonyls which can damage proteins [34]. Oxidative stress thus contributes to AGE formation and the word glycoxidation is often used to describe the pathways involved. Once formed, AGE-modified proteins cause more oxidative stress. Glycation of proteins in the electron transport chain can impair normal electron flow and promote "leakage" to form $^{\bullet}O_2^-$.

Glucose is not the only contributor to AGEs formation. Recent data show that, in spite of the fact that sugars are the main precursors of AGE compounds, numerous intermediary metabolites, i.e. α-oxoaldehydes, also creatively participate in nonenzymatic glycation reactions. Such intermediary products are generated during glycolysis (methylglyoxal) or along the polyolic pathway, and can also be formed by autooxidation of carbohydrates (glyoxal) and by lipid peroxidation [86]. Alpha-oxoaldehydes modify AGEs surprisingly fast, in contrast to classical Maillard reactions, which are very slow (Fig. 5).

Levels of methylglyoxal formed from intermediates of glycolysis are increased in diabetes, and recently it has been reported that the higher level of methylglyoxal is associated with a poor level of glutathione in diabetes mellitus [63]. Short periods of hyperglycemia (as observed in impaired glucose tolerance) may be sufficient to increase methylglyoxal concentration *in vivo* [63]. Methylglyoxal can react with collagen, thereby interfering with crucial cell-matrix interactions, especially *via* the loss of specific arginine residues involved in integrin-mediated attachment [65, 68]. Methylglyoxal is physiologically detoxified by the cytosolic glutathione-dependent glyoxalase system [88], although its action could be compromised by falls in G-SH levels in diabetes.

In diabetic patients, AGEs are present in many tissues (some tissues, such as the liver, kidneys, and erythrocytes are more susceptible to AGEs formation than others), on circulating low density lipoproteins (LDLs) [53] and in atherosclerotic lesions. Binding of glucose to the amino groups on both apo B and on lipids in LDL facilitates LDL oxidation. Thus AGEs formation is probably a significant contributor to the onset of diabetic complications, mainly atherosclerosis [56].

Once AGEs are formed they bind to their cell-surface receptors termed RAGE to endothelial cells, mesangial cells and macrophages, resulting in the activation of postreceptor signaling, generation of intracellular ROS and the activation of gene expression [103]. Exposure of endothelial cells to AGEs activates NF-kB, increases adhesion molecule (including vascular cell adhesion molecule-1 (VCAM-1)) production and NOS activity, and may decrease GSH levels. This occurs in addition to the direct deleterious effects of high glucose on vascular endothelium.

The generation of intracellular ROS results in activation of the transcription factor NF-kB which translocates into nucleus and induces the expression of genes regulated by NF-kB. This receptor ligation increases the production of the transcription factor NF-kB, also causing increased oxidative stress [9].

It has been shown that blockage of RAGE inhibited the evolution of diabetic nephropathy and enhanced wound repair (known to be a problem in diabetics) in

murine models. The mechanism involved in RAGE-associated development of diabetic complications appears to be related to an increased production of ROS [84].

Normally, RAGE may enable macrophages to recognize and engulf glycosylated cells, for example AGE-modified erythrocytes. RAGE receptors bind several other ligands, including β-amyloid and HMGB1, a promoter of inflammation released from necrotic cells [1].

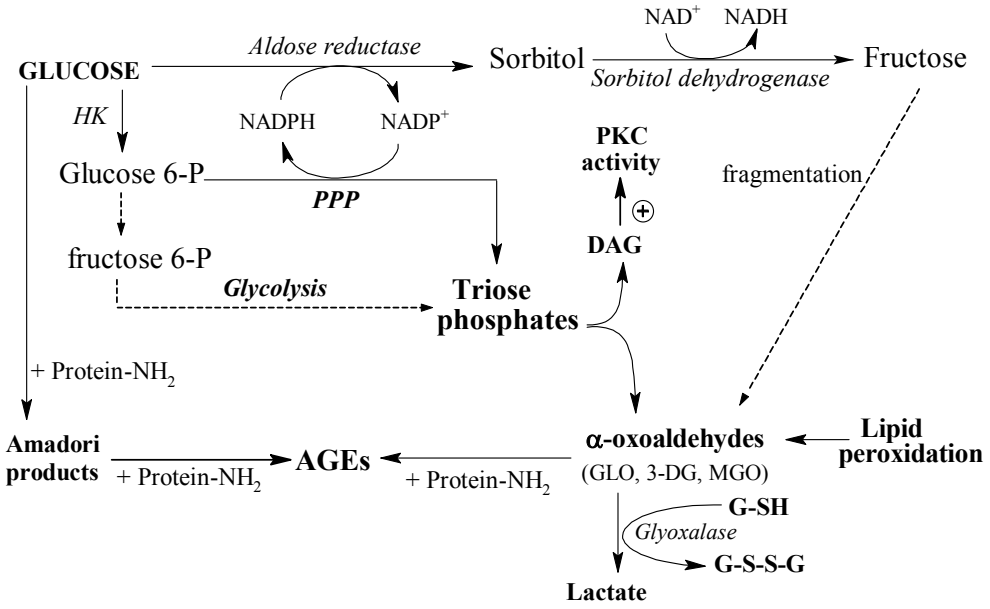

Fig. 5. Potential pathway leading to AGE formation. AGE arise from: decomposition of Amadori products, fragmentation products of polyol pathway, lipid peroxidation products which react with amino groups of protein. (HK=hexokinase; GLO=glyoxal; MGO=methylglyoxal; 3-DG=3-deoxyglucosone; PPP=pentose phosphate pathway).

Many aspects of diabetic complications are thus potentially related to the effect of Amadori-adducts and AGEs [1, 18]. Clinical trials with aminoguanidine, an AGE formation inhibitor that had shown promise in animal experiments, have unfortunately been halted because of unforeseen side effects [85], but trials with other AGE formation inhibitors and with AGE crosslink breakers are underway [58].

THE POLYOL PATHWAY

Under normoglycemia, most of the cellular glucose is phosphorylated into glucose 6-phosphate by hexokinase. A minor part of nonphosphorylated glucose (approximately 3 %) enters the so-called polyol pathway, the alternate route of

glucose metabolism [62], implicating the enzyme aldose reductase. Aldose reductase normally has the function of reducing toxic aldehydes in the cell to inactive alcohols, but when the glucose concentration in the cell becomes too high, aldose reductase also reduces, in the presence of NADPH, glucose to sorbitol, which is later oxidized to fructose by the sorbitol dehydrogenase at the cost of NAD^+ (Fig. 5).

Under hyperglycemia, there is an increase in the use of glucose through the pentose phosphate pathway together with increased conversion of glucose *via* the polyol pathway (more than 30 % of glucose) [31].

The sorbitol pathway increases in activity in diabetes in those tissues that do not require insulin for cellular glucose uptake, such as the retina, kidney, peripheral nerves and blood vessels [79]. This pathway may impair endothelial function through some mechanisms. First, sorbitol does not diffuse through cell membranes easily and accumulates, causing osmotic damages. Sorbitol accumulation decreases other osmolytes such as myo-inositol and taurine [79]. However, the relatively low expression of aldose reductase in endothelial cells may not be sufficient to cause significant sorbitol accumulation.

Secondly, hyperglycemia leads to overflow of the products of the polyol pathway along with depletion in the reduced nicotinamide adenine dinucleotide phosphate (NADPH). NADPH is an essential reducing equivalent for the regeneration of reduced glutathione (GSH) by glutathione reductase (GR) (Fig. 1), and for the activity of the NADPH-dependent thioredoxin system, two important cell antioxidants against oxidative damage. Cells have several sources of NADPH, including the two dehydrogenases of the pentose-phosphate pathway (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase; both insulin-induced enzymes), the malic enzyme and the NADPH-dependent isocitrate dehydrogenase. The impairment of the hexose monophosphate shunt leads to a reduced NADPH availability, and negatively influences other enzymes and systems involved in defensive processes against oxidative agents, such as the catalase and the glutathione system. Several papers have been published that underline the role of glucose-6-phosphate dehydrogenase deficiency [100] in the pathogenesis of diabetes.

NADPH is also a cofactor of important enzymes of the reactive nitrogen species (RNS) and reactive oxygen species (ROS) metabolism, NOS [64] and NADPH-oxidase [23], respectively. Intracellular depletion of NADPH leads to a decreased $NO^{\bullet}$ synthesis, since NADPH is cofactor of the NO-synthase, which synthetizes $NO^{\bullet}$ from L-arginine. All isoforms of NOS contain a reductase domain and an oxygenase domain separated by a calmodulin binding region. NOS requires five cofactors/prosthetic groups such as flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, tetrahydrobiopterin (BH_4) and Ca^{2+} – calmodulin (Fig. 6).

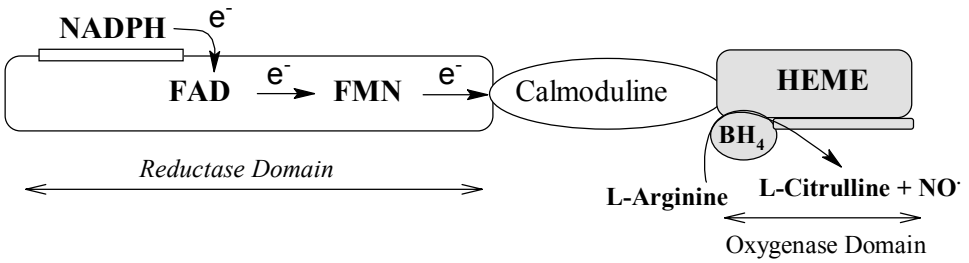

Fig. 6. Schematic illustration of electron transfer in NO synthase. NADPH binds to a specific region in the reductase domain and transfers electrons to the flavines (FAD and FMN). Calmoduline facilitates electron transfer from the reductase domain to the heme-iron contained in the oxygenase domain. The arginine substrate binding site is in the oxygenase domain adjacent to the hem-iron. Heme-bound oxygen serves as a cosubstrate and is incorporated into both products (NO· and citrulline) of the catalytic reaction.

If eNOS lacks its substrate L-arginine or one of its cofactors, NOS may produce $^{\bullet}O_2^-$ instead of $NO^{\bullet}$ and this is referred to as the "uncoupled state of NOS" [94] (Fig. 7).

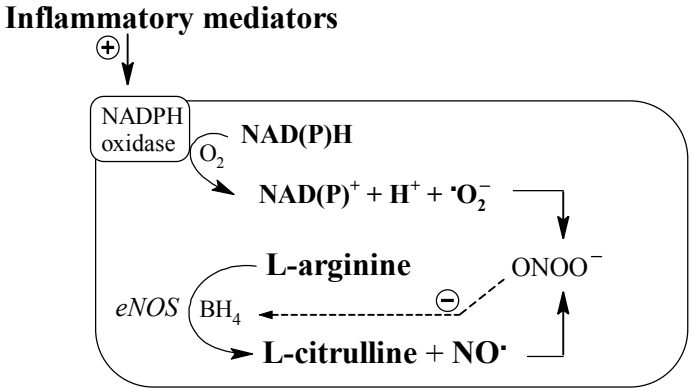

Fig. 7. Generation of reactive oxygen species (ROS) in vascular endothelial cells. The enzyme complex of NAD(P)H oxidase is activated in response to a variety of inflammatory mediators: Angiotensin II (Ang II), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and transforming growth factor-beta (TGF-β) and produces $^{\bullet}O_2^-$. The endothelial nitric oxide synthase (eNOS) generates nitric oxide, NO·. Superoxide can further react with pre-formed NO· and generate oxidising agent peroxynitrite $ONOO^-$ which can oxidize active eNOS cofactor BH_4 to inactive molecules such as BH_2. eNOS with the deficiency of cofactors L-arginine and BH_4 ((6*R*)-5,6,7,8-tetrahydrobiopterin) switches from a coupled state (generating nitric oxide, NO·) to an uncoupled state (generating superoxide, $^{\bullet}O_2^-$).

The elevated levels of inflammatory mediators, such as tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β), interleukin-6 (IL-6) and angiotensin II may lead to enhanced superoxide radical generation by NADPH oxidase pathway and oxidation of NO· to peroxynitrite [66]. Peroxynitrite, in turn,

can oxidize active eNOS cofactor BH_4 to inactive molecules such as BH_2, thus uncoupling $NO^{\cdot}$ formation [3]. The loss of $NO^{\bullet}$ can affect vascular relaxation [56].

The abundant pro-oxidant activity from peroxynitrite and NADPH oxidase system also facilitates oxidation of LDL to oxidized LDL particles. It has also been shown that oxidized low-density lipoprotein (oxLDL) can limit L-arginine availability in endothelial cells, and given the abundance of oxidized LDL in diabetes, L-arginine depletion may also contribute to eNOS uncoupling and precipitate a vicious cycle [68].

Thirdly, the increase in the cytosolic NADH/NAD^+ ratio results in a redox imbalance that resembles that which occurs in tissue hypoxia and therefore is termed hyperglycaemic pseudohypoxia [29].

Regeneration of NAD^+ in the mitochondria can lead to the formation of $^{\bullet}O_2^-$ as can the processes of autooxidation and glycation. Antioxidant enzymes including superoxide dismutase remove $^{\bullet}O_2^-$ (Fig. 1) [27].

The full impact of the sorbitol pathway in vascular dysfunction is not completely understood and the role of inhibition of aldose reductase in the prevention and treatment of diabetic complications remains unclear. Aldose reductase activity in endothelial cells of different origin is low and it thus appears unlikely that the improved nerve conduction in diabetic neuropathy observed with aldose reductase inhibitors or *myo*-inositol supplementation is related to improved endothelial function. In contrast, an excess aldose reductase activity in human retinal endothelial cells can be a mechanism for human diabetic retinopathy [19].

ACTIVATION OF PROTEIN KINASE C ISOFORMS

Glucose excess may activate protein kinase C (PKC) directly by several mechanisms, including through de novo synthesis of diacylglycerol (DAG), by activation of phospholipase C, and by inhibition of DAG kinase [44, 102] or indirectly (via ligation of AGE receptors [64] or increased activity of the polyol pathway [64]).

Increased activity of protein kinase C results in functional changes to vascular cells via activation of phospholipase A_2 (the enzyme supplying the substrate arachidonic acid for prostaglandin production), the expression of growth factors (e.g., transforming growth factor-β, endothelin, and vascular endothelial growth factor), and alterations in the expression of certain basement membrane proteins (e.g., fibronectin) [47, 80, 98, 102]. There is plausible evidence that PKC, which is stimulated in diabetes via multiple mechanisms activates NAD(P)H oxidase [56, 64].

Activation of the PKC pathway can synergize with other kinase pathways, that is, the MAPK pathway. The p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) (also known as stress-activated protein kinase (SAPK)) pathways are known to be activated by oxidative stress, and/or high glucose in several cell types. Recently it was shown that the JNK pathway is activated by oxidative stress in pancreatic β-cells and that activation of the JNK pathway is involved in reduction of insulin gene expression by oxidative stress and that suppression of the JNK pathway can protect β-cells from oxidative stress [4, 42]. Interactions between these pathways are likely to play a role in determining the long-term effects of hyperglycemia.

INCREASED HEXOSAMINE FLUX

Hyperglycemia increases the flux through the hexosamine pathway by providing more fructose-6-phosphate for glutamine**:** fructose-6-phosphate amidotransferase (GFAT), the rate-limiting enzyme of the pathway [4]. GFAT converts the fructose-6-phosphate to glucosamine-6 phosphate and finally to UDP (uridine diphosphate) – *N*-acetyl glucosamine. After that, by the addition of *N*-acetyl glucosamine to serine and threonine residues, increase O-linked glycosylation of the transcription factors. For example, increased modification of the transcription factor Sp1 results in increased expression of transforming growth factor-ß1 and plasminogen activator inhibitor-1, both of which are bad for diabetic blood vessels [25].

Glucosamine-6-phosphate, produced by the hexosamine biosynthetic pathway, inhibits activity of glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme in the pentose shunt pathway [43]. Since G6PD activity is coupled to reduction of $NADP^+$ to NADPH, activation of the hexosamine biosynthetic pathway would further decrease $NADPH/NADP^+$ ratios. Decreased $NADPH/NADP^+$ ratios, resulting from inhibition of G6PD or stimulation of NADPH oxidase, can increase oxidative stress by two mechanisms, first, by decreasing the regeneration of the important cellular antioxidant, reduced glutathione (GSH) from oxidized glutathione (GSSG), and second, by decreasing availability of NADPH, thereby decreasing activity of catalase, the enzyme responsible for converting the H_2O_2, to H_2O. Indeed, glutathione scavenging activity and NADPH content are decreased in vascular endothelial tissues by high glucose conditions [43].

The hexosamine pathway also functions as a cellular "sensor" of energy availability and mediates the effects of glucose on the expression of several gene products including leptin [72].

Recent data have implicated the activation of the hexosamine pathway by hyperglycemia-induced increase in ROS formation. In bovine endothelial cells,

hyperglycemia induced a significant increase in the hexosamine pathway [36], which was blocked by an inhibitor of electron transport chain complex II, by an uncoupler of oxidative phosphorylation, by uncoupling protein-1 and by manganese superoxide dismutase [14]. Normalizing levels of mitochondrial reactive oxygen species with each of these agents prevents glucose-induced activation of protein kinase C, formation of advanced glycation end-products, sorbitol accumulation and NF-kB activation.

DO ANTIOXIDANT SUPPLEMENTS HELP DIABETIC PATIENTS?

Given the involvement of oxidative stress in diabetic complications, supplementation with antioxidants could be of interest, by allowing a delay in the appearance or in the development of vascular complications [36]. Some information is available on the effects of treatments with classical antioxidants such as vitamin E, vitamin C or lipoic acid. Specifically, vitamin E normalizes retinal blood flow and PKC activity in vascular tissue of diabetic rats [48]. Diabetic embryopathy of rat or mouse embryos is prevented by vitamin C, vitamin E, superoxide dismutase, N-acetyl-cysteine, or glutathione ethyl ester [17, 95, 99]. Also, it was reported that treatment with antioxidants (*N*-acetyl-l-cysteine and taurine) prevented hyperglycemia-induced insulin resistance *in vivo* [33].

Although in short-term experiments, high doses of vitamin C can improve some aspects of endothelial dysfunction in diabetes [89], randomized clinical trials with antioxidants have failed to show a decrease in cardiovascular disease [51, 74]. Vitamin C has antioxidant properties, but it can also glycate proteins. One epidemiological study suggested that high ascorbate intake in subjects who had been diabetic for many years was associated with increased risk of complications [50].

In diabetic patients, long-term treatment with high doses of vitamin E has no beneficial effects on endothelial or left ventricular function [26]. Because vitamin E-treated patients had a worsening in some vascular reactivity measurements when compared with control subjects, the use of high dosages of vitamin E cannot be recommended.

Lipoic acid has been suggested, but not proven, to decrease the severity of diabetic neuropathy by maintaining G-SH levels and/or by its direct antioxidant properties [87, 97]. However, lipoic acid administration, improved endothelial function in subjects with metabolic syndrome, but did not decrease their plasma F_2-isoprostane levels, suggestive of benefit by non-antioxidant mechanisms [77]

By contrast, irbesartan (an agent that blocks the angiotensin receptor; angiotensin II is associated with increased ROS production) did decrease isoprostane levels improving vascular effects as well; the effects of lipoic acid and

irbesartan were additive, again suggesting that benefits of lipoate are not antioxidant-related [77].

In diabetic foot patients with retinopathy we observed high plasma values for uric acid and ceruloplasmin [59]. These plasma compounds could be important in the pathogenesis of retinal disease. Two aspects should be considered when these high values are analyzed. First, these antioxidant compounds may become prooxidant in diabetic vascular environment. Secondly, it is not known whether these modified plasma oxidative stress parameters are cause or effect in diabetic complication development.

Studies in experimental models provide a foundation for the clinical studies but results should be interpreted cautiously since the experimental models of diabetes, duration and type of antioxidant treatment and markers of oxidative stress investigated in these studies exhibit a wide range. In summary, there are differences in response to antioxidants in experimental diabetes in the prevention of complications. Antioxidants may provide short-term relief of oxidative stress and restore normal function, but it may be difficult to provide sufficient antioxidants like vitamin E or vitamin C that are not enzymatically regenerated (unlike GSH) at sufficient concentrations to scavenge free radicals on a long-term basis. Also, in tissues of patients with long-standing diabetes and preexisting structural and functional pathology, it may be impossible to eliminate or reverse tissue damage; if patterns of gene expression have been altered by oxidative stress, it may not be possible to reverse this process and restore normal patterns of gene expression [45].

THERAPEUTIC POTENTIAL FOR REDUCING OXIDATIVE STRESS

Thus, metformin treatment can prevent diabetic complications not only by lowering plasma glucose, but also by inhibiting AGE formation [82]. Similarly, sulfonylureas can exhibit antioxidant activity. Thus, gliclazide decreases LDL oxidation and monocyte adhesion to endothelial cells, suggesting a beneficial effect of this drug in the prevention of atherosclerosis associated with type 2 diabetes [69]. Treatment with gliclazide also induces a decrease in plasma lipid peroxides and an enhancement of erythrocyte Cu, Zn-SOD activity, this effect resulting from a free radical scavenging activity independent of glycemic control [40]. In our previous study treatment with antidiabetic drugs (sulfonylureas and metformin) of obese diabetic patients induces a decrease in plasma lipid peroxides and an enhancement of erythrocyte Cu, Zn-SOD activity compared to obese untreated patients. This makes us think that antidiabetic drugs (sulfonylureas and metformin), known to have a potential antioxidant activity, have a real impact on the improvement of these parameters [60].

CONCLUSION

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of normal cellular metabolism. ROS/RNS are known to act as secondary messengers controlling various normal physiological functions of the organism and therefore the production of NO• by NOS and superoxide by NAD(P)H is tightly regulated by hormones, cytokines, and other mechanisms. In diabetes, in a variety of tissues, hyperglycemia results in the generation of ROS and RNS, leading to increased oxidative stress. In the absence of a compensatory response from the endogenous antioxidant network, the stress-sensitive signaling pathways, such as sorbitol, PKC, NF-kB, AGE/RAGE, hexosamine and others are enabled. The consequence is the production of compounds which cause cellular damage and are responsible for the long-term complications of diabetes. Thus the use of antioxidants may be important in preventing activation of these pathways. To date, no recommendations can be made for the use of any particular antioxidant compound. It would seem justified, however, to maximize antioxidant defense, both nutritionally and through the use of antidiabetic and other drugs with antioxidant properties.

REFERENCES

1. AHMED, N., Advanced glycation end products-role in pathology of diabetic complications. *Diabetes Research and Clinical Practice*, 2005, **67**, 3–21.
2. ALDERTON, W.K., C.E. COOPER, R. G. KNOWLES, Nitric oxide synthases: structure, function and inhibition, *Biochem. J*., 2001, **357**, 593–615.
3. ALP, N.J., K.M. CHANNON, Regulation of endothelial nitric oxide synthase by tetrahydrobiopterin in vascular disease. *Arteriosclerosis, Thrombosis & Vascular Biology*, 2004, **24**, 413–420.
4. AMIRI, F., S. SHAW, X. WANG, J. TANG, J.L. WALLER, D.C. EATON, M.B. MARRERO, Angiotensin II activation of the JAK/STAT pathway in mesangial cells is altered by high glucose, *Kidney Int*., 2002, **61**, 1605–1616.
5. ARAL, K., S. IIZUKA, Y. TADA, K. OIKAWA, N. TANIGUCHI, Glycation and inactivation of human Cu-Zn-superoxide dismutase, *J. Biol. Chem*., 1987, **262**, 16969–16972.
6. BAGEACU, V., I. MIHĂILESCU, M. RIȚCO, M. MOHORA, I. BRUCKNER, Autoxidative glycosylation of proteins and oxidative stress in diabetes mellitus, *Rom. J. Biophys*., 1994, **4**, 203–209.
7. BAYNES, J.W., N.G. WATKINS, C.I. FISHER, C.J. HULL, J.S. PATRICK, M.U. AHMED, J.A. DUNN, S.R. THORPE, The Amadori product on protein: structure and reactions, *Prog. Clin. Biol. Res*., 1989, **304**, 43–67.
8. BEISSWENGER, P.J., S.K. HOWELL, A.D. TOUCHETTE, S. LAL, B.S. SZWERGOLD, Metformin reduces systemic methylglyoxal levels in type 2 diabetes, *Diabetes,* 1999, **48***,* 198–202.
9. BIERHAUS, A., S. CHEVION, M. CHEVION, M. HOFMANN, P.QUENHONBERGER, T. ILMER, Advanced glycation end product-induced activation of NFkB is suppressed by alpha-lipoic acid in cultured endothelial cells, *Diabetes,* 1997, **46**, 1481–1490.
10. BOHLENDER, J.M., S. FRANKE, G. STEIN, G. WOLF, Advanced glycation end products and the kidney, *Am. J. Physiol. Renal Physiol*., 2005, **289**, 645–659.

11. BOVERIS, A., Determination of the production of superoxide radicals and hydrogen peroxide in mitochondria, *Methods Enzymol*., 1984, **105**, 429–435.
12. BROWNLEE, M., Biochemistry and molecular cell biology of diabetic complications. *Nature*, 2001, **414**, 813–820.
13. BROWNLEE, M., Glycation and diabetic complications. *Diabetes*, 1994, **43**, 836–841.
14. BROWNLEE, M., The pathobiology of diabetic complications. A unifying mechanism, *Diabetes*, 2005, **54**, 1615–1625.
15. CERIELLO, A., F. MERCURI, L. QUAGLIARO, R. ASSALONI, E. MOTZ, L. TONUTTI, C. TABOGA, Detection of nitrotyrosine in the diabetic plasma: evidence of oxidative stress. *Diabetologia,* 2001, **44**, 834–838.
16. CHANCE, B., H. SIES, A. BOVERIS, Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.,* 1979, **59**, 527–605.
17. CHANG, T.I., M. HORAL, S. JAIN, F. WANG, R. PATEL, M.R. LOEKEN, Oxidant regulation of gene expression and neural tube development: insights gained from diabetic pregnancy on molecular causes of neural tube defects, *Diabetologia,* 2003, **46**, 538–545.
18. CHATURVEDI, N., C.G. SCHALKWIJK, H. ABRAHAMIAN, J.H. FULLER, C.D. STEHOUWER, Circulating and urinary transforming growth factor β1, Amadori albumin, and complications of type 1 diabetes: the EURODIAB prospective complications study, *Diabetes Care,* 2002, **25**, 2320–2327.
19. DAGHER, Z., Y.S. PARK, V. ASNAGHI, T. HOEHN, C. GERHARDINGER, M. LORENZI, Studies of rat and human retinas predict a role for the polyol pathway in human diabetic retinopathy, *Diabetes*, 2004, **53**, 2404–2411.
20. DANDONA, P., K. THUSU, S. COOK, B. SNYDER, J. MAKOWSKI, D. ARMSTRONG, T. NICOTERA, Oxidative damage to DNA in diabetes mellitus, *Lancet*, 1996, **347**, 444–445.
21. DAVI, G., G. CIABATTONI, A. CONSOLI**,** A. MEZZETTI, A. FALCO, S. SANTARONE, E. PENNESE, E. VITACOLONNA, T. BUCCIARELLI, F. CONSTANTINI, C. PATRONO, *In vivo* formation of 8-iso prostaglandin F2α and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation, *Circulation,* 1999, **99**, 224–229.
22. DEAN, P.J., Redefining Oxidative stress, *Antioxidants & Redox Signaling*, 2006, **8**, 1865–1879.
23. DECOURSEY, T.E., E. LIGETI, Regulation and determination of NADPH oxidase activity, *Cell Mol. Life Sci*., 2005, **62**, 2173–2195.
24. DU, X., T. MATSUMURA, D. EDELSTEIN, L. ROSSETTI, Z. ZSENGELLER, C. SZABO, M. BROWNLEE, Inhibition of GAPDH activity by poly(ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells, *J. Clin. Invest.,* 2003, **112**, 1049–1057.
25. DU, X.L., D. EDELSTEIN, L. ROSSETTI, I.G. FANTUS, H. GOLDBERG, F. ZIYADEH, J. WU, M. BROWNLEE, Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation, *Proc. Natl. Acad. Sci. USA,* 2000, **97**, 12222–12226.
26. ECONOMIDES, P.A., L. KHAODHIAR, A. CASELLI, E. CABALLERO, H. KEENAN, S. BURSEL, G. KING, The effect of vitamin E on endothelial function of micro and macrocirculation and left ventricular function in type 1 and type 2 diabetic patients, *Diabetes*, 2005, **54**, 204–211.
27. EVANS, J.L., I.D. GOLDFINE, B.A. MADDUX, G.M. GRODSKY, Oxidative stress and stress-activated signalling pathways: a unifying hypothesis of type 2 diabetes, *Endocrine Reviews*, 2002, **23**, 599–622.
28. FARACI, F.M., S.P. DIDION, Superoxide dismutase isoforms in the vessel wall, *Arteriosclerosis, Thrombosis, and Vascular Biology,* 2004, **24**, 1367.
29. FERON, O., R.A. KELLY, The caveolar paradox: Suppressing, inducing, and terminating eNOS signaling, *Circulation Research*, 2001, **88**, 129–131.

30. GOLDSTEIN, B.J., K. MAHADEV, W.X. XIANGDONG, Redox paradox. Insulin action is facilitated by insulin-stimulated reactive oxygen species with multiple potential signaling targets, *Diabetes*, 2005, **54**, 311–321.
31. GONZÁLEZ, R.G., P. BARNETT, J. AGUAYO, H.M. CHENG, L.T.J. CHYLACK, Direct measurement of polyol pathway activity in the ocular lens, *Diabetes*, 1984, **33**, 196–199.
32. GUZIK T.J., S. MUSSA, D. GASTALDI, J. SADOWSKI, C. RATNATUNGA, R. PILLAI, K.M. CHANNON, Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase, *Circulation*, 2002, **105**, 1656–1662.
33. HABER, C.A., T.K. LAM, Z. YU, N. GUPTA, T. GOH, E. BOGDANOVIC, A. GIACCA, I.G. FANTUS, *N*-acetylcysteine and taurine prevent hyperglycemia-induced insulin resistance in vivo: Possible role of oxidative stress, *Am. J. Physiol. Endocrinol. Metab.*, 2003, **285**, 744–753.
34. HALLIWELL, B., J.M.C. GUTTERIDGE, Non-enzymatic glycation and glycoxidation, in: *Free radicals in biology and medicine*, Oxford University Press, 2007, pp. 510–514.
35. HAMMES, H.P., X. DU, D. EDELSTEIN, T. TAGUCHI, T. MATSUMURA, Q. JU, J. LIN, A. BIERHAUS, P. NAWROTH, D. HANNAK, M. NEUMAIER, R. BERGFELD, I. GIARDINO, M. BROWNLEE, Benfotiamine blocks three major pathways of hyperglycemic damage and prevents experimental diabetic retinopathy, *Nat. Med.*, 2003, **9**, 294–299.
36. HAYOZ, D., T. ZIEGLER, H.R. BRUNNER, J. RUIZ, Diabetes mellitus and vascular lesions, *Metabolism*, 1998, **47**, 16–19.
37. INOGUCHI, T., P. LI, F. UMEDA, H.Y. YU, M. KAKIMOTO, M. IMAMURA, T. AOKI, T. ETOH, T. HASHIMOTO, M. NARUSE, H. SANO, H. UTSUMI, H. NAWATA, High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells, *Diabetes*, 2000, **49**, 1939–1945.
38. ISHII, H., M.R. JIROUSEK, D. KOYA, C. TAKAGI, P. XIA, A. CLERMONT, S.E. BURSELL, T.S. KERN, L.M. BALLAS, W.F. HEATH, L.E. STRAMM, E.P. FEENER, G.L. KING, Amelioration of vascular dysfunctions in diabetic rats by an oral PKC beta inhibitor, *Science*, 1996, **272**, 728–731.
39. ISLAM K.N., TAKAHASHI M, HIGASHIYAMA S, MYINT T, UOZUMIN N, KOSAKA H, TANIGUCHI N, Fragmentation of ceruloplasmin following non-enzymatic glycation reaction. J. Biochem. 1995, **118**, 1054-1059.
40. JENNINGS, P.E., J.J. BELCH, Free radical scavenging activity of sulfonylureas: a clinical assessment of the effect of gliclazide, *Metabolism,* 2000, **49**, 23–26.
41. KANETO, H., G. XU, K.H. SONG, K. SUZUMA, S. BONNER-WEIR, A. SHARMA, G.C. WEIR, Activation of the hexosamine pathway leads to deterioration of pancreatic β-cell function by provoking oxidative stress, *J. Biol. Chem.*, 2001, **276**, 31099–31104.
42. KANETO, H., G. XU, N. FUJII, S. KIM, S. BONNER-WEIR, G.C. WEIR, Involvement of c Jun N-terminal kinase in oxidative stress-mediated suppression of insulin gene expression, *J. Biol. Chem.*, 2002, **277**, 30010–30018.
43. KANJI, M.I., M.L. TOEWS, W.R. CARPER, A kinetic study of glucose-6-phosphate dehydrogenase, *J. Biol. Chem.*, 1976, **251**, 2258–2262.
44. KEOGH, R.J., M.E. DUNLOP, R.G. LARKINS, Effect of inhibition of aldose reductase on glucose flux, diacylglycerol formation, protein kinase C, and phospholipase A2 activation, *Metabolism*, 1997, **46**, 41–47.
45. KING, G.L., M.R. LOEKEN, Hyperglycemia-induced oxidative stress in diabetic complication, *Histochem. Cell Biol.*, 2004, **122**, 333–338.
46. KLOTZ, L.O., Oxidant-induced signaling: effects of peroxynitrite and singlet oxygen, *Biol. Chem.*, 2002, **383**, 443–456.
47. KOYA, D., M.R. JIROUSEK, Y.W. LIN, H. ISHII, K. KUBOKI, G.L. KING, Characterization of protein kinase C beta isoform activation on the gene expression of transforming growth factor-beta, extracellular matrix components, and prostanoids in the glomeruli of diabetic rats, *J. Clin. Invest.,* 1997, **100**, 115–126.

48. KUNISAKI, M., S.E. BURSELL, A.C. CLERMONT, H. ISHII, L.M. BALLAS, M.R. JIROUSEK, F. UMEDA, H. NAATA, G.L. KING, Vitamin E prevents diabetes-induced abnormal retinal blood flow via the diacylglycerol-protein kinase C pathway, *Am. J. Physiol. Endocrinol. Metab*., 1995, **269**, 239–246.
49. LEE, A.Y., S.S. CHUNG, Contributions of polyol pathway to oxidative stress in diabetic cataract, *FASEB J.,* 1999, **13***,* 23–30.
50. LEE, D.H., A. FOLSOM, L. HARNACK, B. HALLIWELL, D. JACOBS, Does supplemental vitamin C increase cardiovascular disease risk in women with diabetes?, *Am. J. Clin. Nutr*., 2004, **80**, 1194.
51. LONN, E., S. YUSUF**,** B. HOOGWERF, Effects of vitamin E on cardiovascular and microvascular outcomes in high-risk patients with diabetes: results of the HOPE study and MICRO-HOPE substudy, *Diabetes Care*, 2002, **25**, 1919–1927.
52. LOVE, D.C., J.A. HANOVER, The hexosamine signaling pathway: Deciphering the "O-GlcNAc Code", *Sci. STKE*, 2005, **312**, 13–21.
53. LYONS, T.J., A.J. JENKINS, Lipoprotein glycation and its metabolic consequences, *Curr. Opin. Lipidol*., 1997, **8**, 174–180.
54. MARITIM, A.C., R.A. SANDERS, J.B. WATKINS, Diabetes, oxidative stress, and antioxidants: A review, *J. Biochem. Mol. Toxicol*., 2003, **17**, 24–38.
55. MASELLA R., R. DI BENEDETTO, R. VARI, C. FILESI, C. GIOVANNINI, Novel mechanisms of natural antioxidant compounds in biological systems: Involvement of glutathione and glutathione related enzymes, *J. Nutr. Biochem.*, 2005, **16**, 577–586.
56. MEHTA, J. L., N. RASOULI, A. K. SINHA, B. MOLAVI, Oxidative stress in diabetes: A mechanistic overview of its effects on atherogenesis and myocardial dysfunction. *Int.J.Biochem.Cell Biology*, 2006, **38**, 794–803.
57. MENDEZ, J.I, W.J. NICHOLSON, W.R. TAYLOR, SOD isoforms and signaling in blood vessels: Evidence for the importance of ROS compartmentalization, *Arterioscler. Thromb. Vasc. Biol.,* 2005, **25**, 887–888.
58. MENE, P., F. FESTUCCIA, F. PUGLIESE, Clinical potential of advanced glycation end-product inhibitors in diabetes mellitus, *Am. J. Cardiovasc. Drugs*, 2003, **3**, 315–320.
59. MOHORA, M., B. VÎRGOLICI, A. COMAN, C. MUSCUREL, L. GAMAN , V. GRUIA, M. GREABU, Diabetic foot patients with and without retinopathy and plasma oxidative stress, *Rom. J. Intern. Med*., 2007, **1**, 45–51.
60. MOHORA, M., B. VÎRGOLICI, F. PAVELIU, D. LIXANDRU, C. MUSCUREL, M. GREABU, Free radical activity in obese patients with type 2 diabetes mellitus, *Rom. J. Intern. Med*., 2006, **1**, 69–78.
61. MONNIER, V.M., Nonenzymatic glycosylation, the Maillard reaction and the aging process. *J. Gerontol.*, 1990, **45**, 105–111.
62. MORRISON, A.D., R.S. CLEMENTS, S.B. TRAVIS, F. OSKI, A.I. WINEGRAD, Glucose utilization by the polyol pathway in human erythrocytes, *Biochem. Biophys. Res. Commun.,* 1970, **40**, 199–205.
63. MUCHOPADHYAY, S., R. GACHHUI, M. KAR, The role of methyl glyoxal in relation to patho-physiological complications in diabetes mellitus. *Biomed. Res*., 2006, **17**, 111–116.
64. Nishikawa, T., D. Edelstein, X.L. Du, S. Yamagishi, T. Matsumura, Y. Kaneda, M.A. Yorek, D. Beebe, P.J. Oates, H.P. Hammes, I. Giardino, M. Brownlee, Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage, *Nature*, 2000, **404**, 787–790.
65. OYA, T., N. HATTORI, Y. MIZUNO, S. MIYATA, S. MAEDO, T. OSAWA, K. UCHIDA, Methylglyoxal modification of protein. Chemical and immunochemical characterization of methylglyoxal-arginine adducts, *J. Biol. Chem.*, 1999, **274**, 18492–18502.
66. PALMER, R.M.J., D.S. ASHTON, S. MONCADA, Vascular endothelium cell synthesizes nitric oxide from L-arginine, *Nature*, 1988, **333**, 664–666.

67. PASTORE, A., G. FEDERICI, E. BERTINI, F. PIEMONTE, Analysis of glutathione: Implication in redox and detoxification, *Clin. Chim. Acta.*, 2003, **333**, 19–39.
68. PAUL, R.G., A.J. BAILEY, The effect of advanced glycation end-product formation upon cell-matrix interactions, *Int. J. Biochem. Cell Biol.*, 1999, **31***,* 653–660.
69. RENIER, G., A.C. DESFAITS, O. SERRI, Gliclazide decreases low-density lipoprotein oxidation and monocyte adhesion to the endothelium, *Metabolism,* 2000, **49***,* 17–22.
70. REUSCH, J.E.B., Diabetes, microvascular complications, and cardiovascular complications: what is it about glucose?, *J. Clin. Invest*., 2003, **112**, 986–988.
71. ROLO, A.P., C.M. PALMEIRA, Diabetes and mitochondrial function: Role of hyperglycemia and oxidative stress, *Toxicol. Appl. Pharmacol*., 2006, **212**, 167–178.
72. ROSSETTI, L., Perspective: hexosamines and nutrient sensing, *Endocrinology*, 2000, **141**, 1922–1925.
73. RUGGIERO-LOPEZ, D., M. LECOMTE, G. MOINET, G. PATEREAU, M. LAGARDE, N. WIERNSPERGER, Reaction of metformin with dicarbonyl compounds. Possible implication in the inhibition of advanced glycation end product formation, *Biochem. Pharmacol.,* 1999, **58**, 1765–1773.
74. SACCO, M., F. PELLEGRINI, M.C. RONCAGLIONI, F. AVANZINI, G. TOGNONI, A. NICOLUCCI, Primary prevention of cardiovascular events with low-dose aspirin and vitamin E in type 2 diabetic patients: results of the Primary Prevention Project (PPP) trial, *Diabetes Care*, 2003, **26**, 3264–3272.
75. SALVEMINI, D., Z.Q. WANG, J.L. ZWEIER, A. SAMOUILOV, H. MACARTHUR, T.P. MISKO, M.G. CURRIE, S. CUZZOCREA, J.A. SIKORSKI, D.P. RILEY, A nonpeptidyl mimic of superoxide dismutase with therapeutic activity in rats, *Science,* 1999, **286**, 304–306.
76. SIES, H., Oxidative Stress II: Oxidants and Antioxidants. Academic Press, London, 1991.
77. SOLA, S., Irbesartan and lipoic acid improve endothelial function and reduce markers of inflammation in the metabolic syndrome, *Circulation*, 2005, **111**, 343–353.
78. STEPHENS, J.W., D.R. GABLE, S.J. HUREL, G.J. MILLER, J.A. COOPER, S.E. HUMPHRIES, Increased plasma markers of oxidative stress are associated with coronary heart disease in males with diabetes mellitus and with 10-year risk in a prospective sample of males, *Clinical Chemistry*, 2006, **52**, 446–452.
79. STEVENS, M.J., I. OBROSOVA, E.L. FELDMAN, D.A. GREENE, The sorbitol osmotic and sorbitol-redox hypothesis. In: D. LeRoith, S.I. Taylor, J.M. Olefsky (eds.), *Diabetes mellitus: a fundamental and clinical text*, Lippincott Williams & Wilkins, Philadelphia, 2000, pp. 972–983.
80. STUDER, R.K, P.A. CRAVEN, F.R. DERUBERTIS, Role for protein kinase C in the mediation of increased fibronectin accumulation by mesangial cells grown in high-glucose medium, *Diabetes,* 1993, **42**, 118–126.
81. SULEIMAN, M., D. ARONSON , R. ASLEH , R. KAPELIOVICH , A. ROGUIN, R. MEISEL , M. SHOCHAT, A. SULIEMAN, R. LOTAN, N. LEVY, A. LEVY, Haptoglobin polymorphism predicts 30-day mortality and heart failure in patients with diabetes and acute myocardial infarction, *Diabetes*, 2005, **54**, 2802–2810.
82. TANAKA, Y, H. UCHINO, T. SHIMIZU**,** H. YOSHII, M. NIWA, C. OHMURA, N. MITSUHOSHI, T. ONUMA, R. KAWAMORI*,* Effect of metformin on advanced glycation endproduct formation and peripheral nerve function in streptozotocin-induced diabetic rats, *Eur. J. Pharmacol.,* 1999, **376***,* 17–22.
83. TANIYAMA, Y., K. K. GRIENDLING, Reactive oxygen species in the vasculature: Molecular and cellular mechanisms, *Hypertension*, 2003, **42**, 1075–1081.
84. THORNALLEY, P.J., Cell activation by glycated proteins. AGE receptors, receptor recognition factors and functional classification of AGEs, *Cell. Mol. Biol.,* 1998, **44***,* 1013–1023.
85. THORNALLEY, P.J., Use of aminoguanidine (Pimagedine) to prevent the formation of advanced glycation endproducts, *Arch. Biochem. Biophys*., 2003, **419**, 31–40.
86. THORNALLEY, P.J., A. LANGBORG, H.S. MINHAS, Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose, *Biochem. J*,. 1999, **344**, 109–116.

87. THORNALLEY, P.J., A.C. MCLELLAN, T.W. Lo, J. BENN, P.H. SÖNKSEN, Negative association between erythrocyte G-SH concentration and diabetic complications, *Clin. Sci.,* 1996, **91**, 575–582.
88. THORNALLEY, P.J., Glutathione-dependent detoxification of alphaoxoaldehydes by the glyoxalase system: involvement in disease mechanisms and antiproliferative activity of glyoxalase I inhibitors, *Chem. Biol. Interact.,* 1998, **111**, 137–151.
89. TING, H.H., F.K. TIMIMI, K.S. BOLES, S.J. CREAGER, P. GANZ, M.A. CREAGER, Vitamin C improves endothelium-dependent vasodilation in patients with non-insulin-dependent diabetes mellitus, *J. Clin. Invest*., 1996, **97**, 22–28.
90. TURK, Z., Glycation and complications of diabetes, *Diabet Croat*, 2001, **30**, 49–54.
91. TURKO, I.V., S. MARCONDES, F. MURAD, Diabetes-associated nitration of tyrosine and inactivation of succinyl-CoA:3-oxoacid CoA-transferase, *Am. J. Physiol. Heart Circ. Physiol.*, 2001, **281**, 2289–2294.
92. TUSHUIZEN, M.E., M. DIAMANT, R.J. HEINE, Postprandial dysmetabolism and cardiovascular disease in type 2 diabetes, *Postgrad. Med. J*., 2005, **81**, 1–6.
93. VALKO, M., D. LEIBFRITZ, J. MONCOLA, T.D. MARK, M.T.D. CRONIN, M. MAZURA, J. TELSER, Free radicals and antioxidants in normal physiological functions and human disease, *Int. J. Biochem. & Cell Biology*, 2007, **39**, 44–84.
94. VASQUEZ-VIVAR, J., B. KALYANARAMAN, P. MARTASEK, N. HOGG, B.S. MASTERS, H. KAROUI, P. TORDO, K. PRITCHARD, Superoxide generation by endothelial nitric oxide synthase: The influence of cofactors, *Proc. Natl. Acad. Sci. USA*, 1998, **95**, 9220–9225.
95. VIANA, M., E. HERRERA, B. BONET, Terotogenic effects of diabetes mellitus in the rat. Prevention by vitamin E, *Diabetologia*, 1996, **39**, 1041–1046.
96. VILLA, L.M., E. SALAS, V.M. DARLEY-USMAR, M.W. RADOMSKI, S. MONCADA, Peroxynitrite induces both vasodilation and impaired vascular relaxation in the isolated perfused rat heart, *Proc. Natl. Acad. Sci. USA*, 1994, **91**, 12383–12387.
97. VINCENT, A**.**M., T. JOHNSTONE, E. HORTON, A. VEVES, Oxidative stress in the pathogenesis of diabetic neuropathy, *Endocr. Rev*., 2004, **23**, 612-616.
98. WAY, K.J., N. KATAI, G.L. KING, Protein kinase C and the development of diabetic vascular complications, *Diabetic Med*., 2001, **18**, 945–959.
99. WENTZEL, P., L. THUNBERG, U. J. ERIKSSON, The teratogenic effect of diabetic serum is prevented by supplementation of superoxide dismutase and N acetylcysteine in rat embryo culture, *Diabetologia*, 1997, **40**, 7–14.
100. WEST, I.C, Glucose-6-phosphate dehydrogenase: A candidate for diabetes, *Diabet. Med*., 2002, **19**, 172–174.
101. WILLIAMSON, J.R., K. CHANG, M. FRANGOS, Hyperglycemic pseudohypoxia and diabetic complications, *Diabetes,* 1993, **42**, 801–813.
102. XIA, P., T. INOGUCHI, T.S. KERN, R.L. ENGERMAN, P.J. OATES, G.L. KING, Characterization of the mechanism for the chronic activation of diacylglycerol-protein kinase C pathway in diabetes and hypergalactosemia, *Diabetes*, 1994, **43**, 1122–1129.
103. YAN, S.D., D. STERN, A.M. SCHMIDT, What`s the RAGE? The receptor for advanced glycation end products (RAGE) and the dark side of glucose, *Eur. J. Clin. Invest*., 1997, **27**, 179–181.
104. YIM, M.B., H.S. YIM, C. LEE, S.O. KANG, P.B. CHOCK, Protein glycation. Creation of catalytic sites for free radical generation, *Ann. N.Y. Acad. Sci*., 2002, **928**, 48–53.