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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).

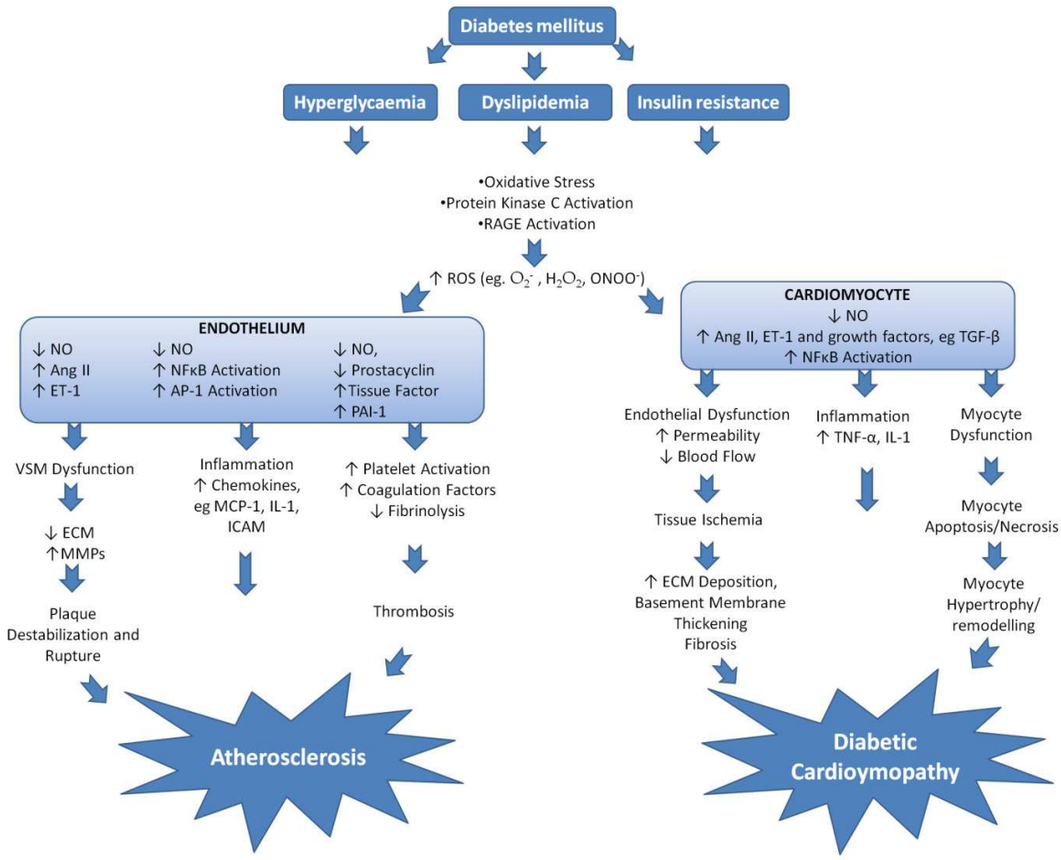


Fig. 1. Roles of reactive oxygen species (ROS) in the pathophysiology of diabetic macrovascular complications, namely atherosclerosis and diabetic cardiomyopathy.

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).

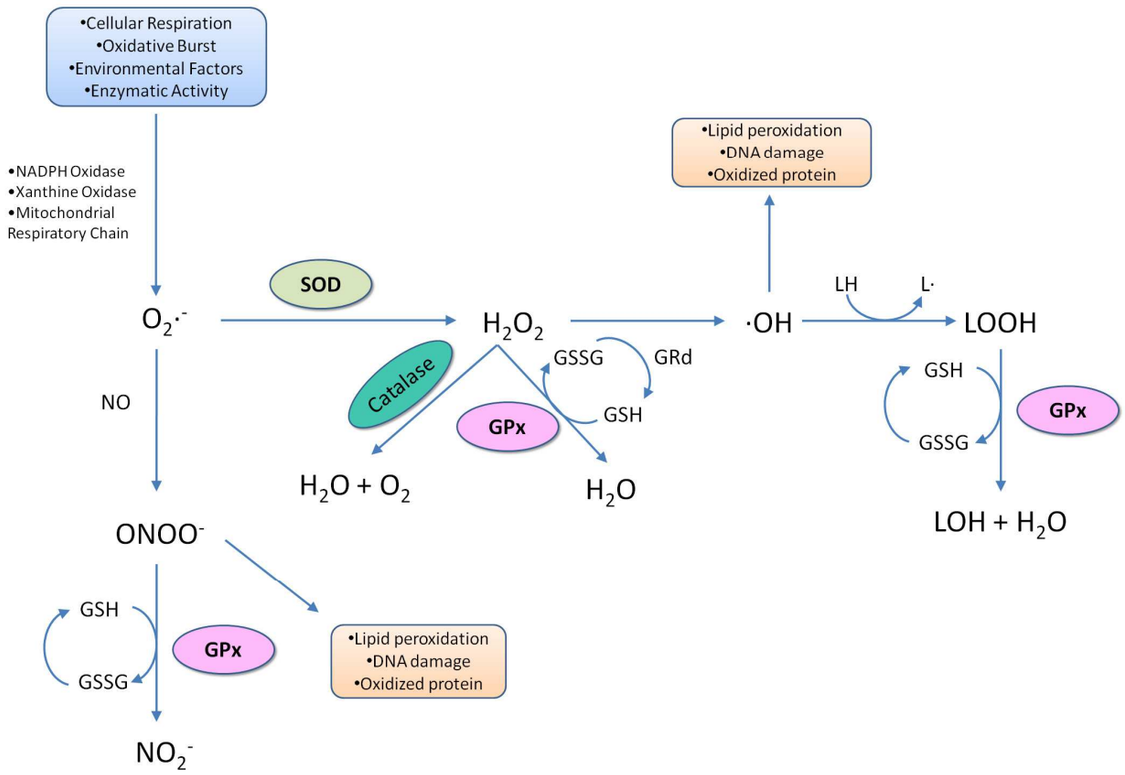


Fig. 2. Removal of reactive oxygen species (ROS) by antioxidant defence systems. Superoxide radical ($^{\bullet}O_2^-$) is generated in low levels under physiological states but its production is greatly enhanced under pathological situations via enzymes such as NADPH oxidase, xanthine oxidase and a 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.

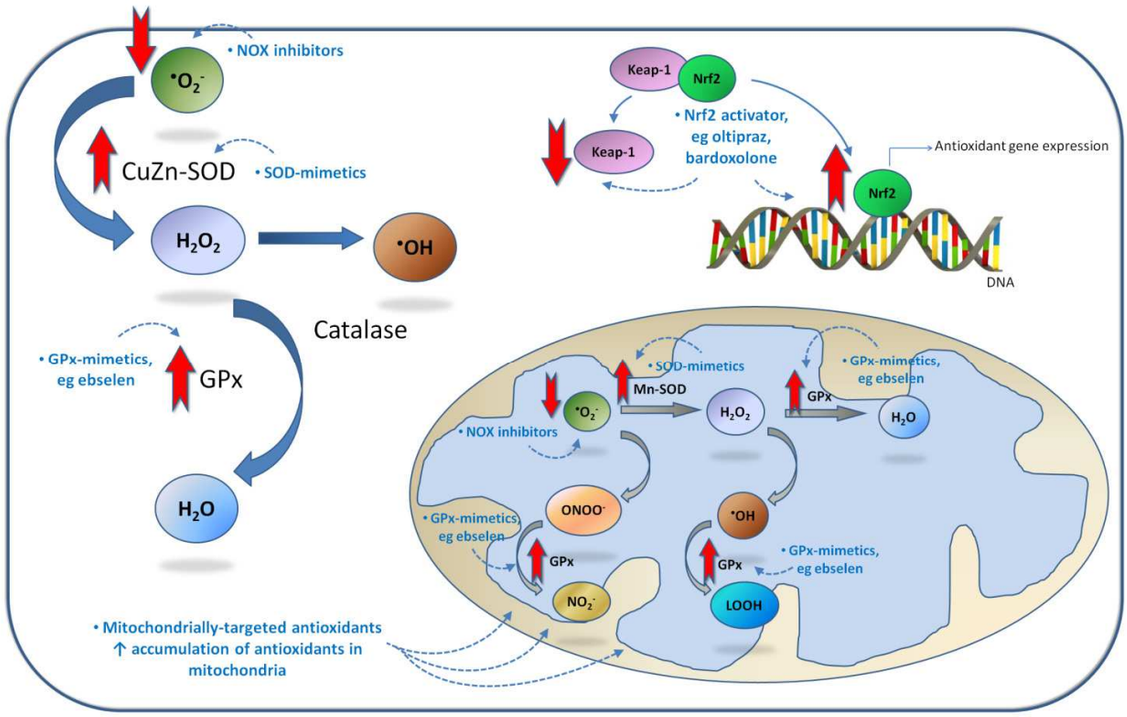


Fig. 3. Novel antioxidant strategies to attenuate increased cellular ROS production and/or increase the activity of endogenous antioxidant defence systems in diabetes-associated complications.

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.

6. References

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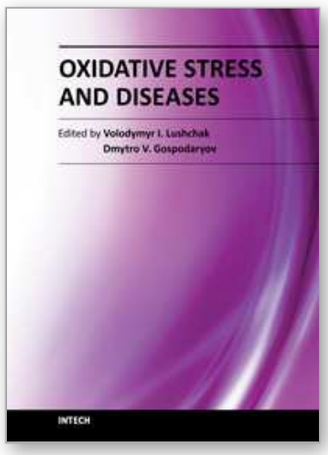
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The development of hypothesis of oxidative stress in the 1980s stimulated the interest of biological and biomedical sciences that extends to this day. The contributions in this book provide the reader with the knowledge accumulated to date on the involvement of reactive oxygen species in different pathologies in humans and animals. The chapters are organized into sections based on specific groups of pathologies such as cardiovascular diseases, diabetes, cancer, neuronal, hormonal, and systemic ones. A special section highlights potential of antioxidants to protect organisms against deleterious effects of reactive species. This book should appeal to many researchers, who should find its information useful for advancing their fields.

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