



Serial Review: The Role of Oxidative Stress in Diabetes mellitus  
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## Oxidative stress and diabetic cardiovascular complications<sup>☆</sup>

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### Abstract

Diabetes diagnoses are increasing at an alarming rate worldwide. The majority of diabetes-related deaths arise from cardiovascular complications such as myocardial infarction, stroke, and peripheral vascular disease. Oxidative stress has been demonstrated to be present in animal models as well as in patients with diabetes and has been suggested as a possible contributor to the accelerated atherosclerosis seen in diabetics. The generation of reactive oxygen species in diabetes occurs via several mechanisms and is initiated not only by glucose, but also by other substances that are found at elevated levels in diabetic patients. The resulting oxidative stress leads to a number of proatherogenic events. The elucidation of the mechanisms of oxidative stress in diabetes and their relationship with atherosclerosis could potentially identify molecular targets of therapy for this condition and its cardiovascular consequences.

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Diabetes mellitus is a serious and growing health problem. A recent study by the World Health Organization estimated that the worldwide prevalence of diabetes in 2002 was 170 million, with the number predicted to grow to 366 million by 2030 [1]. Both type 1 and type 2 diabetes exhibit hyperglycemia as their hallmark. Type 1 diabetes, accounting for 5–10% of diabetes diagnoses, is caused by pancreatic  $\beta$ -islet

cell failure with resulting insulin deficiency. Type 2 diabetes encompasses 90% of diabetics and is characterized by insulin resistance often accompanied by obesity and dyslipidemia. Gestational diabetes accounts for a small percentage of diabetes diagnoses and usually resolves after delivery. However, patients who have had gestational diabetes are 50% more likely to develop overt diabetes within 10 years of diagnosis [2,3].

The vascular complications of diabetes are conventionally divided into macrovascular and microvascular categories. Although the microvascular complications such as retinopathy, neuropathy, and nephropathy are important causes of morbidity and mortality in diabetes patients and have been shown to involve oxidative stress, this review will focus on oxidative stress and the development of macrovascular or cardiovascular complications. The role of oxidative stress in diabetic retinopathy will be reviewed in another installment of this series. Summaries of oxidative stress in diabetic neuropathy and nephropathy are beyond the scope of this discussion, but the reader is directed to recent, in-depth reviews [4–8].

Cardiovascular complications cause the majority of diabetes-related deaths [9]. Atherosclerosis in patients with diabetes tends to occur earlier and be more aggressive. The risk of diabetics for cardiovascular events is equivalent to that of nondiabetic patients who have established cardiovascular disease [10–12]. Diabetics are also two to four times more likely to suffer from stroke [13,14]. Furthermore, patients with diabetes who have had myocardial infarctions (MI) have poorer outcomes than nondiabetics who have had MI [15–18].

In this review we will summarize the current perspective on how diabetes induces oxidative stress, how diabetes-induced oxidative stress may lead to the development of accelerated atherosclerosis, and what potential avenues of therapy may be pursued in diabetic patients in order to prevent the onset and progression of cardiovascular complications.

## Diabetes and oxidative stress

There are several studies demonstrating that patients with diabetes not only have increased levels of circulating markers of free radical-induced damage, but also have reduced antioxidant defenses [19–22]. Hyperglycemia can induce oxidative stress via several mechanisms. These include glucose autooxidation, the formation of advanced glycation end-products (AGE), and activation of the polyol pathway. Other circulating factors that are elevated in diabetics, such as free fatty acids and leptin, also contribute to increased reactive oxygen species (ROS) generation. Fig. 1 summarizes the sources of ROS in diabetes and their associations with atherosclerosis.

### Glucose autooxidation

The increased metabolism of glucose due to intracellular hyperglycemia leads to the overproduction of

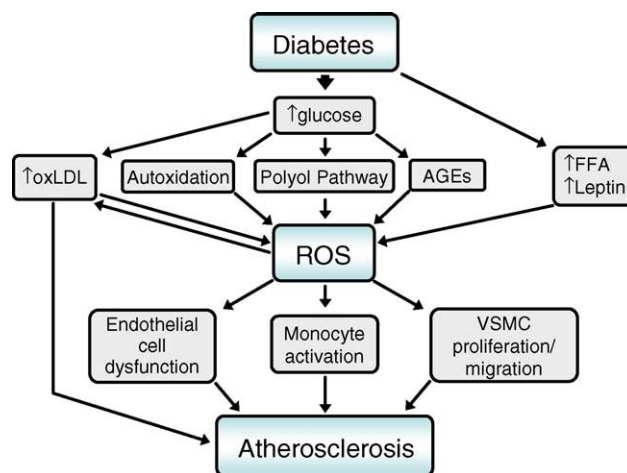


Fig. 1. Overview of the sources of ROS in diabetes and their links to atherosclerosis. oxLDL, oxidized LDL; FFA, free fatty acids; AGEs, advanced glycation end-products; VSMC, vascular smooth muscle cells; ROS, reactive oxygen species.

nicotinamide adenine dinucleotide (NADH) and flavin adenosine dinucleotide, which are used by the electron transport chain to generate adenosine triphosphate [23]. When NADH is in excess, an increase in the mitochondrial proton gradient is produced and electrons are transferred to oxygen, producing superoxide [24]. Production of superoxide by the electron transport chain occurs at two main sites: the NADH dehydrogenase of complex I and the interface between ubiquinone and complex III [25]. It is thought that mitochondrial-derived superoxide causes increased diacylglycerol (DAG) synthesis and subsequent protein kinase C (PKC) activation [23]. However, some investigators have shown that hyperglycemia induces de novo synthesis of DAG, independent of mitochondrial metabolism [26].

### Advanced glycation end-products

The formation of AGE begins with nonenzymatic covalent bonding of ketone or aldehyde groups of reducing sugars to the free amino groups of proteins and other molecules. A series of rearrangements and reactions occurs to irreversibly produce AGE [27]. AGE have been demonstrated in atherosclerotic lesions from patients with diabetes and their tissue concentration increases with disease severity [28]. They are proposed to contribute to atherosclerosis by modifying the extracellular matrix and circulating lipoproteins, as well as binding to and activating the receptor for AGE (RAGE), which is present on many vascular cells [27]. It is through their receptor-mediated effects that AGE have been shown to induce ROS production [29]. Stimulation of the RAGE causes the production of ROS, perhaps via an NAD(P)H oxidase [30], and subsequent activation of redox-sensitive transcription factors and expression of inflammatory mediators [31,32].

The polyol pathway

Two enzymes of the polyol pathway contribute to ROS generation. The first, aldose reductase, uses NADPH for the reduction of glucose to sorbitol. Under normal conditions, sorbitol production by aldose reductase is a minor reaction. However, under conditions of hyperglycemia, up to 30–35% of glucose is metabolized by this pathway [33]. When this occurs, the availability of NADPH is reduced, which in turn reduces glutathione regeneration and NO<sup>\*</sup> synthase activity, thus producing oxidative stress [23]. The second enzyme, sorbitol dehydrogenase, oxidizes sorbitol to fructose with concomitant NADH production. Increased NADH may be used by NAD(P)H oxidases to produce superoxide [34] and, as mentioned previously, can induce mitochondrial superoxide production as well.

Other sources of oxidative stress in diabetes

Free or nonesterified fatty acids (FFA) are elevated in diabetic patients [35]. Excess FFA enter the citric acid cycle and generate acetyl-CoA to produce excess NADH, which,

as noted, increases mitochondrial superoxide production. In humans, acute infusion of FFA has been shown to cause elevations in isoprostanes [36,37], which are markers of lipid peroxidation.

Leptin is a hormone cytokine secreted by adipocytes that acts on the central nervous system to decrease food intake. It also exerts effects on endothelial cells, vascular smooth muscle cells, monocytes, and macrophages [38]. Plasma levels of leptin are increased in type 2 diabetics [39,40] and are associated with cardiovascular disease [41]. Endothelial cells incubated with leptin produce increased levels of ROS [42,43], but the mechanisms by which this occurs have not been elucidated.

Mechanisms of diabetes-induced oxidative stress and their potential roles in atherosclerosis

Oxidative stress leads to many proatherogenic events such as LDL oxidation, endothelial dysfunction, and vascular smooth muscle proliferation and migration. In the following sections and in Fig. 2, we outline the role of

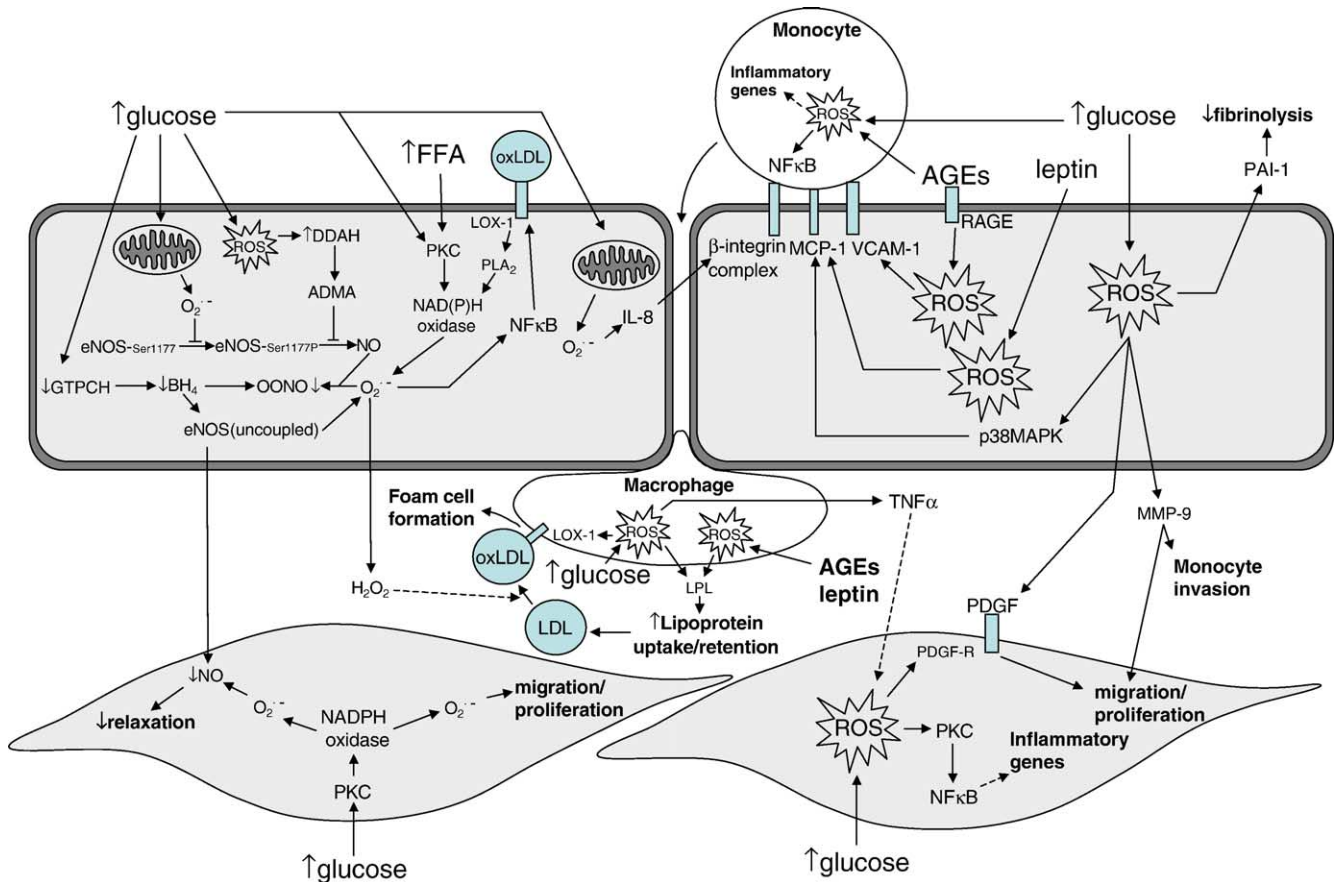


Fig. 2. Consequences of oxidative stress-induced signaling mechanisms in diabetes. ROS, reactive oxygen species; NO<sup>\*</sup>, nitric oxide; O<sub>2</sub><sup>•-</sup>, superoxide; eNOS, endothelial nitric oxide synthase; NFκB, nuclear factor κB; FFA, free fatty acids; AGEs, advanced glycation end-products; RAGE, receptor for AGE; PDGF, platelet-derived growth factor; PDGR-R, PDGF receptor; ADMA, asymmetric dimethyl arginine; DDAH, dimethylarginine dimethylaminohydrolase; PKC, protein kinase C; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; BH<sub>4</sub>, tetrahydrobiopterin; GTPCH, GTP-cyclohydrolase I; MMP-9, matrix metalloproteinase-9; p38MAPK, p38 mitogen-activated kinase; IL-8, interleukin-8; oxLDL, oxidized LDL; LOX-1, lectin-like oxLDL receptor; MCP-1, monocyte chemoattractant protein-1; VCAM-1, vascular cellular adhesion molecule-1; TNFα, tumor necrosis factor α.

diabetes-induced oxidative stress generation in the context of atherogenesis.

#### *Oxidized LDL*

The proposed role of oxidized low-density lipoprotein (oxLDL) in atherosclerosis has largely come from in vitro studies linking oxLDL with vascular cell injury. In vivo, the study of oxLDL is complex because of its heterogeneity [44]. Furthermore, it is not known where LDL undergoes its oxidative modification [44]. However, it is generally agreed that oxLDL is produced in vivo and that it contributes to atherogenesis [45]. There are several studies linking diabetes [46,47] and even postprandial hyperglycemia [48] with increased LDL oxidative susceptibility. Treatment of diabetics with insulin represses LDL oxidation [49]. In addition, the entry and retention of lipoproteins in the vascular wall is a recognized step in atherogenesis [45,50]. The vasculature in diabetics has been demonstrated to produce ROS [51], and thus LDL in these patients may be more easily oxidized simply because of its presence in an oxidative milieu. Moreover, the rate of LDL transvascular transport is higher in diabetics [52]. One of the factors that makes LDL in diabetics more prone to oxidation is glycation [27,53], and glycated LDL from diabetics has been shown to affect vascular tone when injected into mice [54]. Oxidized LDL has itself been shown to produce oxidative stress in endothelial cells via activation of a NADPH oxidase through a phospholipase A<sub>2</sub> signaling mechanism [55]. The effect of oxLDL signaling may also be increased through the upregulation of its receptor, the lectin-like oxLDL receptor (LOX-1), in human aortic endothelial cells [56]. This seems to occur through a PKC, ROS, and NF- $\kappa$ B-dependent mechanism [56]. Oxidative stress-induced LOX-1 upregulation also occurs in macrophages treated with hyperglycemia and may facilitate foam cell formation [57].

#### *Endothelial cell dysfunction*

Alterations in endothelial cell function are proposed to play an important role in atherogenesis. These perturbations include the loss of endothelial cell-directed vasodilatation, the increased expression of cellular adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1, increased permeability to circulating lipoproteins (notably LDL), and increased retention of these lipoproteins [45,50]. A number of studies have shown that patients with either type 1 [58,59] or type 2 [60,61] diabetes exhibit endothelial dysfunction. Furthermore, the endothelial function in diabetic patients can be improved with antioxidants, suggesting that oxidative stress plays an important role in the pathogenesis of endothelial dysfunction in diabetes [62,63].

The major contributor to endothelial oxidative stress is the increased production of superoxide. This seems to

occur via two principal sources: NAD(P)H oxidases and uncoupled eNOS. Hyperglycemia [26,64], AGE [30], FFA [26], and oxLDL [55] have been shown to increase endothelial NAD(P)H oxidase activity. The activation of NAD(P)H oxidases by hyperglycemia and FFA has been shown to be mediated by PKC [26]. Vessels isolated from diabetic patients exhibit increased superoxide production that is inhibited by diphenylene iodonium, and demonstrate increased expression of several NAD(P)H oxidase subunits (p22phox, p47phox, and p67phox) [51], suggesting that NAD(P)H oxidases are more active in diabetes. Not only does excess superoxide itself cause increased oxidative stress, but it can also react with nitric oxide (NO<sup>\*</sup>) to produce peroxynitrite [65], which, in turn, can oxidize tetrahydrobiopterin (BH<sub>4</sub>), thus reducing its availability to eNOS [66]. In the presence of reduced concentrations of BH<sub>4</sub>, eNOS becomes uncoupled and transfers electrons to molecular oxygen instead of L-arginine to produce superoxide rather than NO<sup>\*</sup> [67,68]. The presence of uncoupled eNOS in the diabetic vasculature is supported by a study in which diabetic vessels were found to produce less superoxide when incubated with the NO<sup>\*</sup> synthase inhibitor NG-nitro-L-arginine methyl ester [51]. BH<sub>4</sub> availability may also be decreased by a reduction of its synthesis. The expression of GTP-cyclohydrolase I (GTPCH), the rate-limiting enzyme for de novo BH<sub>4</sub> synthesis, is reduced in diabetic rats [69]. Furthermore, transgenic mice overexpressing GTPCH treated with streptozotocin (STZ) are able to maintain endothelial function [70]. Clinical studies have demonstrated that BH<sub>4</sub> supplementation given to diabetic patients improves their endothelium-dependent vasodilation, indicating that uncoupled eNOS plays a role in diabetic endothelial dysfunction [71].

The oxidative stress caused by diabetes leads to the decreased bioavailability of NO<sup>\*</sup> and subsequent impairment of endothelial-directed vasodilation. As noted above, superoxide can react with NO<sup>\*</sup>. There is also evidence for several mechanisms that lead to the inactivation of eNOS itself. Hyperglycemia causes O-linked *N*-acetylglucosamine modification of serine 1177 on eNOS, the Akt activation site [72]. This modification prevents its phosphorylation and is caused by hyperglycemia-induced mitochondrial superoxide production and activation of the hexosamine pathway [72]. The oxidative stress produced by diabetes may also inhibit Akt activity. It has been demonstrated that oxidative stress induces serine phosphorylation of insulin receptor substrate-1 (IRS-1) and targets it for degradation [73]. The decrease in IRS-1 leads to the impaired activation of the phosphatidylinositol 3-kinase/Akt pathway. Hyperglycemia also seems to result in the accumulation of asymmetric dimethylarginine (ADMA), an inhibitor of eNOS. In STZ-treated rats, the activity of dimethylarginine dimethylaminohydrolase (DDAH, an enzyme which catabolizes ADMA) is decreased, resulting in an increase in ADMA [74]. This decreased DDAH activity also seems to be caused by oxidative stress, as polyethylene glycol-

conjugated superoxide dismutase was able to reverse the effects of hyperglycemia-induced DDAH inactivation [74]. Together, these findings suggest that oxidative stress can inhibit eNOS activity in diabetic patients through multiple mechanisms.

Elevated concentrations of glucose, FFA, and leptin and the presence of AGE cause a multitude of proatherogenic consequences that are mediated by ROS in endothelial cells. Hyperglycemia can increase monocyte adhesion by the increased expression of MCP-1 via p38 mitogen-activated kinase [75] and through the activation of  $\beta$ 1-integrin by interleukin-8 and ROS from a mitochondrial source [76]. Monocyte invasion and vascular smooth muscle cell (VSMC) migration may be facilitated by the ROS-mediated expression of MMP-9, which has been shown to be induced by glucose [77]. Glucose-induced ROS also increases the secretion of platelet-derived growth factor (PDGF), a known smooth muscle cell mitogen [78], and plasminogen activator-1 [79]. ROS produced by leptin have been shown to activate the transcription factors NF- $\kappa$ B and activated protein 1 (AP-1) [43] as well as increasing MCP-1 expression [42]. FFA-induced ROS also increase NF- $\kappa$ B binding [80]. AGE cause increases in VCAM-1 expression [30] and vascular permeability [81] through ROS. In vivo studies have demonstrated that the increased levels of soluble adhesion molecules found in diabetics can be decreased through the administration of antioxidants [82,83]. Furthermore, FFA-induced endothelial dysfunction produced in healthy volunteers is improved by coadministration of vitamin C [84]. These findings demonstrate the central role of oxidative stress in many aspects of the endothelial contribution to atherosclerosis.

#### *Monocytes and macrophages*

The migration and accumulation of monocytes into atherosclerotic plaques have been well described [45]. Activation of monocytes causes them to elaborate various cytokines that contribute to plaque progression. Monocytes develop into macrophages within the vessel wall and scavenge modified lipoproteins to become foam cells, which secrete inflammatory mediators and produce ROS [45].

There is evidence suggesting that hyperglycemia causes oxidative stress in monocyte/macrophages, resulting in increased production of proatherogenic agents. A single oral dose of glucose has been shown to increase ROS generation in monocytes of healthy volunteers [85]. Furthermore, monocytes isolated from diabetic patients produce increased levels of superoxide through PKC-dependent activation of a p47phox-containing NAD(P)H oxidase [86]. Monocytes of patients with poorly controlled type 1 diabetes have increased NF- $\kappa$ B activation that can be suppressed with the antioxidant thiocetic acid [87]. When exposed to chronic hyperglycemia, monocytes secrete tumor necrosis factor- $\alpha$  via ROS-dependent activation of NF- $\kappa$ B

[88]. Tumor necrosis factor- $\alpha$  secretion also involves p38 MAPK and JNK-1, two members of the MAPK family that have been shown in other systems to be ROS sensitive [89].

AGE have been shown to cause oxidative stress in both monocytes and macrophages. In RAW macrophages, glycated albumin causes extracellular signal-regulated kinase phosphorylation and subsequent ROS production and NF- $\kappa$ B activation [90]. Hyperglycemia, leptin, and AGE via RAGE stimulate human macrophages to synthesize lipoprotein lipase (LPL), a proatherogenic ligand secreted by macrophages that associates with lipoproteins and promotes their uptake and retention into the vessel wall [91]. LPL induction seems to involve PKC, ROS, and the transcription factor AP-1 [91].

#### *Vascular smooth muscle*

Vascular smooth muscle proliferation and migration are proposed to play important roles in atherogenesis and restenosis. Diabetics seem to also have dysfunctional vascular smooth muscle, as they have a decreased vasodilatory response to direct application of NO $\cdot$  donors [92]. Hyperglycemia has been shown to increase superoxide production in VSMC via NAD(P)H oxidase activation [26,64,93]. This increased superoxide could potentially react with NO $\cdot$  from endothelial cells, thus limiting its effect on VSMC relaxation. The production of NO $\cdot$  in VSMC themselves may be affected as well, because high glucose concentrations can inhibit iNOS activity in these cells through a PKC- and calcium-dependent mechanism [94].

The oxidative stress produced in VSMC in diabetes may shift them from a contractile to a proliferative phenotype, thus further inhibiting vasodilation and enhancing lesion formation. Patients with diabetes have increased proliferation and migration of VSMC into atherosclerotic lesions [95]. There are a number of studies showing that exposure of VSMC to high glucose conditions results in oxidative stress and subsequent cell proliferation. Hyperglycemia causes PKC activation and subsequent ROS production via NAD(P)H oxidase in cultured aortic smooth muscle cells [64]. In STZ-treated rats, a p22phox-containing NAD(P)H oxidase was found to be a mediator of VSMC proliferation [96]. Additionally, the polyol pathway has been implicated in hyperglycemia-induced, PKC-directed NF- $\kappa$ B activation, as inhibition of aldose reductase is able to mitigate both PKC and NF- $\kappa$ B activation in cultured rat aortic smooth muscle cells (RASM) [97]. Aldose reductase also seems to mediate hyperglycemia-induced upregulation of the  $\beta$  subunit of the PDGF receptor protein in these cells [98]. Another stimulus for VSMC proliferation is a decrease in NO $\cdot$  concentration. NO $\cdot$  has been shown to be an antimitogenic factor in RASM [99] and, as mentioned above, high glucose concentrations inhibit iNOS activity [94].

Despite the increased proliferation and migration of VSMC in patients with diabetes [95], atherosclerotic lesions

from these patients have fewer VSMC, suggesting that VSMC death potentially plays a role in plaque instability and subsequent rupture [100]. A study in human aortic smooth muscle cells revealed that high glucose conditions cause cell necrosis via hydrogen peroxide [101]. Additionally, oxLDL is able to induce VSMC apoptosis via ROS and may be increased in diabetic patients [102].

### Implications for therapy

As the pathogenesis of both diabetes and cardiovascular disease involves oxidative stress, the use of antioxidants is an appealing therapy. However, the use of traditional antioxidants such as vitamin E or C in large clinical trials has failed to demonstrate any beneficial effect on cardiovascular disease or all-cause mortality, even when only diabetic patients were analyzed [103,104]. Furthermore, a recent meta-analysis of clinical trials studying vitamin E therapy suggests that the use of high-dose vitamin E (greater than 400 IU/day) may actually increase mortality [105]. Many explanations for these findings have been put forward. These trials did not select patients on the basis of measurable oxidative stress biomarkers. It is conceivable that the possible beneficial effect of antioxidant therapy was diluted by subjects who had no or very low levels of oxidant stress and who may not have shown a benefit [106–108]. Also, the use of antioxidant vitamins may have been initiated too late or for too short a time in the course of atherogenesis to demonstrate an effect [107]. Finally, there is evidence that both vitamins C and E not only are ineffective at lowering biological markers of oxidative stress [109,110], but also can act as pro-oxidants [111,112]. These findings have prompted a search for new antioxidants which may be able to impact atherosclerosis. One of these, AGI-1067 (the monosuccinic acid ester of probucol), has shown promising results and is currently undergoing phase III trials [113–115]. Importantly, pharmacologic agents currently in use that have been shown to be effective in reducing cardiovascular mortality are known to have antioxidant properties. Angiotensin converting enzyme inhibitors (ACEI) and HMG-CoA reductase inhibitors or statins have already demonstrated beneficial effects on diabetic patients in large randomized controlled-trials [15,116]. Interestingly, ACEI, which act partially to prevent the pro-oxidant effects of Ang II, were shown to prevent the onset of type 2 diabetes in the Heart Outcomes Prevention Evaluation Study [15]. Statins have been shown to exert vascular antioxidative properties [117,118]. The ability of thiazolidinediones or glitazones to improve endothelial dysfunction by decreasing oxidative stress via the peroxisome-proliferator-activated receptor  $\gamma$  has prompted the initiation of two clinical trials designed to investigate their effects on cardiovascular disease in diabetics [119,120].

Another strategy for therapy uses pharmacological agents that block AGE–RAGE interactions or interrupt

the formation of AGE themselves. Soluble RAGE or RAGE-specific IgG are possible approaches for the first strategy. Soluble RAGE has been shown to inhibit atherosclerosis in ApoE knockout mice [121]. Blockade of specific components of ROS-sensitive signaling cascades such as those initiated by PKC may attenuate the progression of diabetic cardiovascular complications. Ruboxistaurin (LY333531), a specific inhibitor of PKC $\beta$  has demonstrated encouraging effects on microvascular disease and is being evaluated in phase III clinical trials. Another PKC inhibitor, CGP53353, was able to abrogate hyperglycemia-induced NF- $\kappa$ B activation and VCAM-1 expression in human aortic endothelial cells [122]. Because of their potential unrivalled specificity, siRNAs directed at a component or components of a pathologic signaling pathway are an attractive concept. An important issue in the use of either antioxidants or specific inhibitors of ROS signaling is that of tissue- or cell-specific delivery, as oxidative molecules are also active in tissues and cells that are not participating in a pathologic process. An approach that is currently being tested is the use of nanoparticle or liposome-enclosed pharmacologic agents [123,124]. These ligand-conjugated vehicles can deliver a therapeutic agent to cells expressing a specific receptor.

### Conclusions

This review has presented evidence to support the role of oxidative stress in the development of diabetic cardiovascular complications. Hyperglycemia seems to cause proatherogenic events via oxidative stress in many of the cells involved in atherosclerosis. However, the metabolic derangements in diabetic patients also lead to the excess of other substances which induce oxidative stress and signaling pathways involved in atherosclerosis. The early initiation of therapy aimed at reducing oxidative stress and/or modulating ROS-sensitive signaling pathways may be of benefit for reducing cardiovascular disease in diabetes. Further insights into the molecular mechanisms of the metabolic basis of diabetes will prove invaluable in the treatment and prevention of this debilitating condition.

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