Nitric Oxide, Oxidative Excess, and Vascular Complications of Diabetes Mellitus

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The prevalence of diabetes mellitus is rising worldwide and has reached epidemic dimensions. Diabetes mellitus places patients at high cardiovascular risk. High blood glucose levels, altered insulin signaling, reactive oxygen species (ROS), inflammation, and protein kinase C activation might lead to a decrease in nitric oxide (NO) bioavailability. Diminished NO and enhanced oxidative stress play a central role in several pathophysiologic pathways, leading to vascular damage, such as endothelial dysfunction, vascular inflammation, atherosclerotic plaque formation and vulnerability, and promotion of a prothrombotic state. Possible sources of oxidative excess in diabetes are reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, uncoupled NO synthase, and the mitochondria. Advances in understanding the pathophysiologic mechanisms leading to vascular damage in diabetes will result in discovery of new therapeutic targets, which should help reduce cardiovascular risk in these patients.

Introduction

Diabetes mellitus represents an increasingly heavy health burden in our society and has acquired epidemic dimensions. The lifetime risk for developing diabetes for individuals born in the year 2000 in the United States is 32.8% for males and 38.5% for females [1]. If an individual is diagnosed with diabetes at the age of 40 years, men will lose 11.6 life-years, and women will lose 14.3 life-years; the loss of quality-adjusted life-years is even higher.

Most diabetic complications are related to the vasculature. Macrovascular complications of diabetes include atherosclerosis, leading to myocardial infarction, stroke, and peripheral artery disease, the latter being the leading cause of limb amputation in the United States. Microvascular complications include diabetic nephropathy and retinopathy, which represent the leading causes of end-stage renal disease and blindness. The cardiovascular risk in diabetic patients is further enhanced in the presence of other risk factors, such as hypertension. Diabetes and hypertension are often part of a clustering of risk factors. The mechanisms whereby diabetes affects the vasculature are complex, and new findings are enriching our pathophysiologic understanding of the disease. Two important risk factors, which are the focus of this review, have emerged: nitric oxide (NO) and oxidative excess. Because more than 90% of diabetic patients have the type 2 form of diabetes, this review is focused on the vascular pathology of type 2 diabetes.

Endothelial Dysfunction

Endothelial dysfunction is an almost inevitable finding associated with vascular damage in diabetes, and is linked in part to the balance between oxidative stress and the NO system. Rizzoni *et al.* [2] and Shofield *et al.* [3] have described vascular remodeling and endothelial dysfunction in small resistance arteries of diabetic patients, as well as elevated adhesion molecules, such as intercellular adhesion molecules (ICAM)-1 and vascular cell adhesion molecules (VCAM)-1 [2]. ICAM-1 and VCAM-1 are markers of endothelial dysfunction that correlate with blood glucose levels, as indicated by measurement of glycated hemoglobin [4]. In diabetic subjects, ICAM and VCAM are associated with markers of inflammation, such as C-reactive protein (CRP), interleukin (IL)-6, and tumor necrosis factor (TNF)-α [5]. Endothelial dysfunction is associated with inflammation and is a powerful promoter of progression of atherosclerosis [6]. Endothelial dysfunction has been shown to predict the risk of death in diabetic patients [7•].

There is impaired NO-dependent dilation of skeletal muscle arterioles in hypertensive diabetic obese Zucker rats. Oxidative excess promotes endothelial dysfunction in this model, as demonstrated by correction of the endothelial dysfunction by superoxide dismutase mimetics [8]. Similar results were seen by inhibition of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the OLETF diabetic rat model [9]. The endothelial

dysfunction occurs as a result of reduction of NO or tetrahydrobiopterin (BH_4) bioavailability. Diminished BH_4 results in uncoupling of endothelial NO synthase (eNOS), and oral administration of L-arginine [10], the substrate for eNOS, or BH_4 [11], reverses this endothelial dysfunction in the streptozotocin-induced rat model of diabetes.

Effects of Decreased Nitric Oxide Bioavailability in Diabetes

Nitric oxide bioavailability is decreased in diabetes [12]. Besides causing endothelial dysfunction, decreased NO bioavailability can also lead to several other pathophysiologic effects. There is a decrease in platelet-derived NO in diabetic patients with coronary heart disease, and the decreased NO affects arachidonic acid metabolism in the platelets, resulting in higher platelet sensitivity to aggregating stimuli [13]. A prothrombotic state increases the chance of coagulation on a vulnerable plaque, and, therefore, increases the risk of myocardial infarction.

In diabetes, inflammation is an important mechanism promoting vascular damage. Markers of vascular inflammation, such as chemokines and activated platelets, are increased in diabetic patients [14•]. NO and oxidative excess play a pivotal role in the vascular inflammatory state. In human umbilical vein endothelial cells (HUVEC), oxidized low-density lipoprotein (LDL) increases monocyte chemoattractant protein (MCP)-1 expression through an enhanced nuclear factor (NF)-κB activity; this process can be prevented by activation of the NO system [15]. Therefore, diminished NO bioavailability in diabetes can increase MCP-1 expression, promoting leukocyte chemotaxis and adhesion, two processes that are characteristic of the inflammatory response [14•].

Nitric oxide interferes with other mechanisms in diabetic vascular damage, such as the polyol pathway, in which reduced glucose results in sorbitol accumulation. This polypol pathway has been linked to the development of vascular complications in diabetes mellitus. In aortic tissue from diabetic rats, sorbitol accumulation can be increased by eNOS blockade and decreased by the addition of L-arginine, indicating a regulatory role for NO for the polyol pathway. The proposed target for NO on the polyol pathway might be the enzyme aldose reductase, a redox-sensitive protein [16•].

Causes of Diminished Nitric Oxide in Diabetes

Diminished NO in both human and animal models of diabetes might have several causes, such as variations in the expression of the enzyme NO synthase. In human aortic cells, glucose increases eNOS activity [17]; however, eNOS activity can be decreased by glycosylated and oxidized LDL [18]. In human endothelial cells, the addition of glucose increases oxidative stress and reduces NO bioavailability [19]. In HUVEC, there is a decrease in NO production, despite a threefold increase in eNOS expression, when grown on glycated collagen, accompanied by increased nitrotyrosine-modified proteins and premature cell senescence, which could be prevented by peroxynitrite scavenging [20]. NO is scavenged by superoxide anion forming peroxynitrite, which is responsible for the nitration of tyrosine residues on proteins. Nitrotyrosine in plasma proteins might be regarded as indirect evidence of peroxynitrite production. Indeed, nitrotyrosine plasma levels were increased in patients with diabetes and correlated with plasma glucose levels [21]. Additionally, peroxynitrite is a cytotoxic oxidant. By promoting generation of BH_2 rather than BH_4, eNOS dimer formation is decreased. Uncoupled eNOS loses its oxygenase function (NO production) and functions as a reductase, forming superoxide anion instead of NO [22]. Peroxynitrite is an important mediator of oxidation of LDL, contributing to its proatherogenic role [23••]. Using a novel peroxynitrite decomposition catalyst, FP15, target-organ damage in the form of endothelial and cardiac dysfunction can be prevented in diabetic mice [24•].

Another source of low NO in diabetes relates to the process of inflammation that has been shown to decrease NO levels. For example, the calcium-dependent protease calpain, which is increased in acute inflammatory conditions in the cardiovascular system, has been shown to mediate inflammation secondary to glucose excess. Inhibition of calpain activity decreases the interaction between leukocytes and the endothelium [25•]. The inflammatory marker CRP, which is elevated in diabetes, has also been shown to decrease eNOS activity [26]. Additionally, advanced glycation end products (AGEs) quench NO and impair endothelial function [27]. Aminoguanidine inhibits AGEs and improves NO function.

There is also a relationship between the insulin-signaling pathway and NO regulation. The actions of insulin are mediated through two major pathways. One pathway is through activation of insulin-receptor substrate-1 (IRS-1), phosphatidylinositol 3-kinase (PI3K), phosphoinositide-dependent kinase 1 (PDK-1), and Akt. This same pathway can lead to phosphorylation and activation of eNOS [28]. The other insulin-signaling pathway leads to mitogen-activated protein kinases (MAP-K), including mitogen-activated protein kinase kinase (MEK)-1, ERK-1, and ERK-2, which have major effects on growth and proliferation [29]. In states of insulin resistance, such as those seen in type 2 diabetes, the PI3K pathway is reduced, whereas the MAP-K pathway is unaffected [30]. High levels of glucose lead to inhibition of the PI3K pathway through the hexosamine pathway [31••]. Insulin excess also leads to the stimulation of inflammatory/atherogenic factors, such as MCP-1 and plasminogen activator inhibitor (PAI)-1 [32]. Therefore, in the insulin resistance and hyperinsulinemia of type 2 diabetes mellitus, the high insulin levels could lead to reduced eNOS expression coupled to increased growth

and mitogenic action, and the disparate effects of insulin on NO and growth in insulin-resistant states might favor the progression of atherosclerosis.

The protein kinase C (PKC) pathway that is activated by elevated glucose levels in diabetes presents another factor in the etiology of the complications of diabetes [14•]. PKC contributes to increased superoxide anion formation via inhibition of eNOS activity [33] and activation of NADPH oxidase [34], both of which play an important role in endothelial dysfunction. The specific PKC inhibitor ruboxistaurin improves diabetic nephropathy in animal models of diabetes [35,36]. This agent reduces PKC that, in turn, decreases oxidative stress, inflammation, and profibrotic mediators in diabetes.

Effects of Oxidant Excess in Diabetes

Reactive oxygen species (ROS) are downstream targets of PKC activation, but also appear to be upstream of PKC, as well as of NF-κB activation, the polyol, and the hexosamine pathways [37]. By activating the hexosamine pathway, ROS reduce NOS activity [38]. ROS can activate matrix metalloproteinases (MMPs) (namely MMP-2 and MMP-9), which degrade the extracellular matrix and contribute to the instability of the atherosclerotic plaque and plaque rupture [39]. In the vasculature, ROS upregulate adhesion (VCAM-1 and ICAM-1) and chemotactic (MCP-1) molecules, contributing to vascular inflammation [23••]. In resistance arteries from genetic hypertensive rats, which are known to be insulin-resistant, correction of oxidant excess with antioxidants results in improved endothelial function and vascular remodeling [40]. In diabetic pigs, oxidant excess induces an inflammatory response in the adventitia, with increased expression of IL-6, TNF-α, MCP-1, and VCAM-1 [41•].

In diabetes, ROS promotes the formation of AGEs acting on specific receptors (AGE-R1–3 [RAGE]), scavenging receptors (ScR-II), and the fatty acid receptor/transporter CD-36, which contributes to vascular and end-organ damage [42]. AGEs promote crosslinking of long-living proteins, such as vascular collagen, which is conducive to an age-associated increase of arterial and cardiac stiffness, and is enhanced in diabetes. Interestingly, new crosslink breakers, such as ALT-711, have improved arterial and ventricular function in elderly humans [43]. AGEs induce cell activation, and stimulate growth-related mediators, cell proliferation, and proinflammatory responses [41•,42]. In renal dysfunction, clearance of AGEs is delayed, which promotes vascular and renal injury in diabetic nephropathy [44]. AGEs themselves induce ROS [41•]. Therefore, AGEs are important promoters of vascular inflammation and accelerated atherosclerosis in diabetes. In fact, RAGE blockade decreases atherosclerosis in streptozotocin-induced diabetes [45•]. Finally ROS can induce insulin resistance, as demonstrated in angiotensin II–infused rats [46].

Sources of Reactive Oxygen Species in Diabetes

The main source of oxidant excess in the vasculature is NADPH oxidase [23••], and, seemingly, this is also the case in diabetes. In pigs with streptozotocin-induced diabetes, superoxide formation was augmented because of increased NADPH oxidase activity [41•]. PKC-induced activation of NADPH oxidase was a major source of ROS in the glomeruli of diabetic rats [47]. In vitro, glucose stimulates NADPH oxidase in endothelial cells, vascular smooth muscle cells, and renal mesangial cells, suggesting that it is the main source of ROS in diabetes [34]. In other studies, xanthine oxidase is the leading source of ROS in human and experimental diabetes [48]. In mice with streptozotocin-induced diabetes, plasma xanthine oxidase was increased, leading to enhanced superoxide formation that could be normalized by the xanthine oxidase inhibitor allopurinol [49].

Mitochondria have lately become the focus of research as a source of oxidant excess. Nishikawa *et al.* [37] showed that mitochondria-derived ROS can activate PKC, increase the formation of AGEs and sorbitol, and induce NF-κB activation. The age-related decline in mitochondrial function might contribute to the increasing insulin resistance seen in the elderly [50]. Recently, a unifying mechanism for these effects was proposed [51••]. Mitochondrial superoxide production might lead to DNA strand breaks, which activate poly (ADP-ribose) polymerase (PARP), inhibiting glyceraldehyde-phosphate dehydrogenase (GAPDH) activity. This leads to increased delivery of glycolytic intermediates to the mitochondria, increasing, in turn, superoxide production. Increases in glycolytic intermediates activate PKC, increase flux in the hexosamine pathway, with increased AGE formation, and activate the polyol pathway. The activation of PKC, hexosamine, AGE formation, and the polyol pathway can be prevented by competitive PARP inhibitors.

New Therapeutic Targets

Diabetic patients are at high cardiovascular risk. Intensive blood-pressure lowering is critical to lower their risk. Nevertheless, we recently showed that diabetic hypertensive patients with controlled blood pressure, most of them on ACE inhibitors and lipid-lowering agents, show marked remodeling of resistance arteries, even more pronounced than patients with untreated hypertension do [52]. Additionally, normotensive diabetic patients already display vascular abnormalities [2,3]. These newer studies of abnormal vascular biology in diabetes underline that the current treatment regimens for diabetic patients do not completely reduce the cardiovascular risk in diabetes to normal. In addition to strict control of glycemia, hypertension hypercholesterolemia, obesity, sedentary lifestyle, smoking, and high calorie diet, we need additional new treatment options with specific effects on the vascular pathology in diabetic patients. The newer mechanisms of

vascular damage in diabetes mellitus should provide new targets for intervention, of which blockade of oxidant excess might be the most important.

Peroxisome proliferator-activated receptors (PPAR)-γ activators are used in type 2 diabetes due to their insulin-sensitizing effects. PPAR-γ as well as PPAR-α agonists exhibit pleiotropic effects that include antioxidant and vascular-protective properties [53]. This class of agents has several potential benefits in diabetes in cardiovascular prevention but their effects must be proven in large outcome trials. The peroxynitrite decomposition catalysts, specific PKC inhibitors, and RAGE-blockade agents represent other new potential therapies for the complications of diabetes. The new crosslink breaker ALT-117 (Alteon, Ramsey, NJ) has already improved arterial and ventricular function in the elderly. Whether it will benefit diabetic patients needs to be investigated [43].

Conclusions

Diabetic subjects are at increased risk for cardiovascular disease, and the imbalance between the NO system and formation of ROS products are linked to advanced vascular damage. NO and ROS are at the center of feedback loops that lead to vascular injury. Such feedback loops include the uncoupling of eNOS due to ROS, leading to more ROS formation, ROS promotion of inflammation, superoxide anion stimulation of PKC with NADPH oxidase-mediated production of superoxide anion, or glycolytic intermediates overloading mitochondria leading to increased superoxide anion and PARP activation with inhibition of GAPDH, leading to further accumulation of glycolytic intermediates. New therapeutic approaches hopefully will be able to successfully interfere with these targets.

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