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Peroxynitrite and Vascular Endothelial Dysfunction in Diabetes Mellitus

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Macro- and microvascular diseases are the principal causes of morbidity and mortality in patients with type I and II diabetes mellitus. Growing evidence implicates reactive nitrogen species (RNS), such as peroxynitrite ($ONOO^-$), derived from nitric oxide (NO) and superoxide anion ($O_2^{\cdot -}$), are important in diabetes. The mechanisms by which diabetes increases RNS, and those by which RNS modifies vascular function, are poorly understood. The authors recently discovered that physiologically relevant concentrations of $ONOO^-$ oxidize the zinc thiolate center in endothelial nitric oxide synthase (eNOS). In active eNOS dimers, a tetracoordinated zinc ion is held by four thiols, two from each 135-kDa monomer. Because it remains partially positively charged, the zinc thiolate center is subject to attack by the $ONOO^-$. This oxidant disrupts the zinc thiolate center, releasing zinc, and oxidizing the thiols. Upon thiol reduction, eNOS dimers dissociate into monomers. This modification of eNOS results in reduced NO bioactivity and enhanced endothelial $O_2^{\cdot -}$ production, which reacts with NO, further generating $ONOO^-$ (eNOS uncoupling). In addition, the authors' studies also demonstrate that low concentrations of $ONOO^-$ selectively nitrate and inactivate prostacyclin synthase (PGIS), which not only eliminates the vasodilatory, growth-inhibiting, antithrombotic, and antiadhesive effects of prostacyclin (PGI_2), but also increases release of the potent vasoconstrictor, prothrombotic, growth- and adhesion-promoting agents, prostaglandin H_2 (PGH_2) and thromboxane A_2 (TxA_2). In diabetic mice and rats, eNOS is uncoupled resulting in an increased tyrosine nitration of PGIS. The authors' studies indicate that in diabetes the synthetic enzymes of the two major endogenous vasodilators undergo oxidative inactivation by different mechanisms, which are, however, tightly interdependent.

Keywords 3-Nitrotyrosine, Diabetes, Endothelial Dysfunction, Nitric Oxide, Prostacyclin, Peroxynitrite

OXIDANT STRESS AND CARDIOVASCULAR DISEASES

The formation of free radicals and oxidants in the vasculature is both an essential part of its normal function and a potential route to vascular injury [1]. A key determinant of endothelial cell biology is the cell redox state, and a key molecule that mediates endothelial function is nitric oxide (NO) [2, 3]. Homeostatic balance between $NO^{\cdot}$ and reactive oxygen species (ROS), such as $O_2^{\cdot -}$ and hydrogen peroxide (H_2O_2), regulates cell redox and is essential for normal endothelial function [1–3]. Cardiovascular risk factors, such as dyslipidemia, elevated blood pressure, diabetes, and smoking, can initiate endothelial dysfunction by altering the cell redox state in the vessel wall [1–3]. ROS, which include NO, $O_2^{\cdot -}$, H_2O_2, and $ONOO^-$, play many important roles in vascular physiology and pathophysiology; but in this review, we will focus on the roles of $O_2^{\cdot -}$ and $ONOO^-$ in diabetes.

Nitric Oxide

NO is normally produced by endothelial nitric oxide synthase (eNOS) in the vasculature, but in inflammatory states, NO may be produced by inducible NOS, which can be expressed in macrophages and smooth muscle cells. NO derived from eNOS

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is a crucial mediator of endothelium-dependent vasodilation and may also play a role in platelet aggregation and in maintaining the balance between cell growth and differentiation of vascular cells.

Superoxide Anion

$O_2{\cdot}^-$ is generated by one-electron reduction of oxygen by NAD(P)H oxidases, the respiratory chain in mitochondria, xanthine oxidase, cyclooxygenases (COXs), as well as by NOS in the absence of optimal concentrations of its substrate, L-arginine, or its cofactor, tetrahydrobipterin [1, 2]. Both endothelial cells and smooth muscle cells produce $O_2{\cdot}^-$ under basal conditions, which increase during stimulation by agents such as bradykinin or the calcium ionophore, A23187, by cytokines such as interferon-γ and interleukin-1, or during ischemia-reperfusion. Xanthine oxidase and NAD(P)H oxidase have been identified as sources of $O_2{\cdot}^-$ in the endothelium [1, 2]. $O_2{\cdot}^-$ is water soluble and can act either as an oxidizing agent or as a reducing agent. In aqueous solution at neutral pH, its preferred reaction is the dismutation reaction, which yields H_2O_2. However, recent evidence suggests that $O_2{\cdot}^-$ may preferentially react with NO to form $ONOO^-$ in living tissues (see below).

Peroxynitrite

$ONOO^-$ is a potent oxidant formed by a direct bimolecular reaction of NO with $O_2{\cdot}^-$ at near diffusion-limited rates ($6.7 \times 10^9\ M^{-1}\ s^{-1}$) [4–7]. This rate constant is three times faster than the enzymatic dismutation of $O_2{\cdot}^-$ catalyzed by superoxide dismutase (SOD) at neutral pH ($k_{SOD} = 2 \times 10^9\ M^{-1}\ s^{-1}$). Thus, $ONOO^-$ formation represents a major potential pathway of NO reactivity that depends on local rates of production of both NO and $O_2{\cdot}^-$ [4–7]. $ONOO^-$ has a half-life of less than 1 s under physiological conditions, due to proton-catalyzed decomposition and targeted molecular reactions. Thus, the reaction of NO with $O_2{\cdot}^-$, which was initially viewed as a route for NO inactivation, instead yields the potent oxidizing species $ONOO^-$, which exhibits direct oxidative reactivity and will protonate to peroxynitrous acid (ONOOH) at physiological pH (pK_a, 6.8) [4–7]. $ONOO^-$ is a potent oxidant capable of directly oxidizing sulfhydryl groups and thioethers, as well as nitrating and hydroxylating aromatic groups, including tyrosine, tryptophan, and guanine [4–7]. This process is mediated by both one- and two-electron transfer reactions. $ONOO^-$ is also capable of reaction with metal centers to yield a species with the reactivity of nitronium (NO^{2+}), an oxidizing and nitrating intermediate [4–7]. These reactions, when occurring during the reaction of $ONOO^-$ with enzymes, macromolecules, and lipids, strongly influence cellular functions. For example, tyrosine nitration may lead to inactivation of prostacyclin synthase [PGIS] [8–10], SOD [11], cytoskeletal actin [5], and neuronal tyrosine hydroxylase [12]. Oxidation of critical sulfhydryl groups is responsible for the inhibition of mitochondrial and cytosolic aconitase and other critical enzymes in the mitochondrial respiratory chain, as well as disruption of zinc-thiolate centers at the active site of many enzymes [4–6].

OXIDANT STRESSES AND DIABETIC CARDIOVASCULAR COMPLICATIONS

Cardiovascular complications in diabetic patients represent by far the most common and devastating manifestation and are the major cause of hospital admissions [13–16]. In the United States, 77% of hospitalizations of diabetic patients are related to cardiovascular diseases and 10% to diabetic nephropathy [13–16]. One of the striking features of diabetic cardiovascular complications is accelerated atherosclerosis, which is associated with metabolic syndrome, insulin resistance, and oxidant stress [15–17]. The Diabetes Control and Complications Trial (DCCT), for type I diabetes, and the United Kingdom Prospective Diabetes Study (UKDPS), for type II diabetes, established the importance of intensive diabetes control in dramatically reducing the devastating complications that result from poorly controlled diabetes [13–16]. Both the DCCT and UKPDS demonstrated the efficacy of intensive glucose control in reducing the risk for the microvascular complications of diabetes, such as retinopathy, neuropathy, and nephropathy [13–17]. Recent findings from the long-term follow-up of participants in DCCT demonstrate a decreased incidence of atherosclerosis in the study participants who intensively controlled blood glucose [18], suggesting that hyperglycemia is important in the development of macrovascular diseases in diabetes. In addition, numerous studies have recently demonstrated that intensive blood pressure control is essential in preventing both micro- and macrovascular complications of diabetes [19]. Aggressive management of dyslipidemia has also been shown to decrease macrovascular complications [20–22]. However, how these risk factors increase diabetic complications remains a subject of intense investigation.

Increasing evidence implicates the generation of reactive oxygen species (oxidative stress) is playing an important role in the etiology of diabetic complications [23]. This hypothesis is supported by evidence that many biochemical pathways strictly associated with hyperglycemia (glucose autooxidation, polyol pathway, prostanoid synthesis, protein glycation) can increase the production of free radicals and oxidants. For example, glucose may combine directly with low-density lipoprotein (LDL) phospholipids or apo B lysine groups to form advanced glycosylation end products (AGEs) that facilitate lipid peroxidation [23]. In addition, the autooxidation of glucose and nonenzymatic glycation of proteins can result in the generation of $O_2{\cdot}^-$. Hyperglycemia and elevated levels of FFA also increase cellular production of $O_2{\cdot}^-$, particularly in endothelial cells [23]. Recent studies by Inoguchi et al. [24] suggest that the increase in oxidative stress is secondary to activation of NAD(P)H oxidase and is dependent upon protein kinase C (PKC)-β, whereas work by Brownlee and his coworkers [25] has suggested that hyperglycemia-induced mitochondrial oxidative stress may be the earliest event.

It is now accepted that inflammation plays a key role in cardiovascular diseases. Inflammatory factors, such as cytokines and adhesion molecules, are involved in the development and

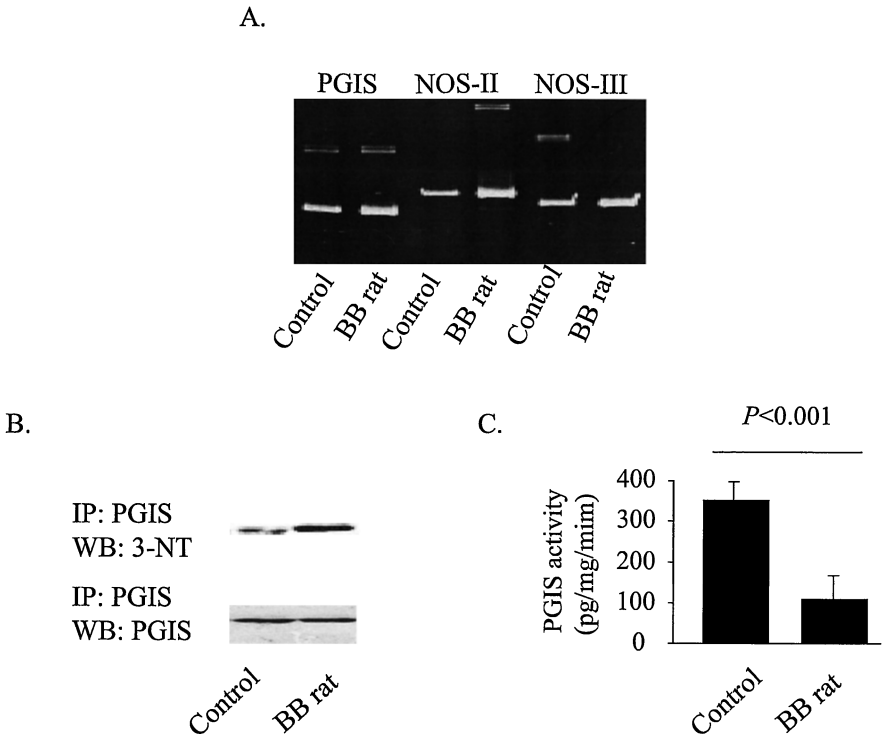

FIG. 1. Tyrosine nitration and PGIS expression in an aorta of the diabetic BB rats. (*A*) The expression of mRNA of PGIS, NOS-II, and NOS-III in aorta of control and BB rats, as assayed by RT-PCR. (*B*) Western blots of PGIS and 3-nitrotyrosine–positive protein in aortic homogenates from control and BB rats. The results represent four separate experiments. (*C*) The PGIS activity in the aorta of BB rats, as measured by the conversion of PGH_2 into PGI_2, was significantly decreased compared to those from control rats (5.7 ± 1.1 versus 11.7 ± 2.1 ng/min/mg protein, $n = 8$, $p < .01$). Of note is the expression of mRNA and protein of PGIS was unchanged by RT-PCR or Western blot with an antibody against PGIS.

propagation of atherosclerotic lesions [23]. Obese humans and patients with type II diabetes have elevated levels of proinflammatory cytokines, such as tumor necrosis factor (TNF) alpha, interleukin-6, and C-reactive protein (CRP) [26, 27]. CD40 ligand, a proinflammatory and atherogenic cytokine, and CRP are elevated in type I diabetic patients [26, 27], and these proinflammatory cytokines might also contribute to increased oxidant stress in diabetes.

TARGETS OF ONOO⁻ IN DIABETIC VESSELS

Although the production of $O_2^{\cdot -}$ has been previously recognized as an important cytotoxic factor contributing to tissue damage, neither NO nor $O_2^{\cdot -}$ alone can be considered as strong oxidants towards most types of biological molecules [5]. Their reaction product, $ONOO^-$, might however play a central role in the pathophysiology of various diseases including diabetes [5–7]. This possibility is therapeutically important because classical antioxidants such as vitamin C and E may not protect from reactive nitrogen species (RNS) such as $ONOO^-$. Recent clinical data demonstrate significantly more intense 3-nitrotyrosine (3-NT) staining in diabetic patients and animals when compared to normal controls [28–31]. The localization of the 3-NT staining indicates that endothelium-derived NO (in the presence of increased oxidant stress) is inactivated due to formation of $ONOO^-$ (Figure 1*A*). In addition, increased detection of 3-NT has been described in the human plasma when infused with glucose [30]. Of particular note is the finding of Frustaci et al. that apoptosis is increased 61- and 85-fold, respectively, in endothelial cells and cardiomyocytes in ventricular myocardial biopsies from diabetic humans [31]. Interestingly, they found that apoptosis in both cell types was strongly associated with positive staining for 3-NT, suggesting a correlation with oxidative stress and $ONOO^-$ generation [31]. It must be emphasized that these studies do not prove that 3-NT plays a key role in the pathogenesis of macrovascular disease in diabetes, but they do establish that it is at least a marker of endothelial dysfunction or cell damage in diabetes. The identities of nitrated proteins and functional implications in diabetes remain largely unknown.

PROSTACYCLIN SYNTHASE

Prostacyclin (PGI_2) is produced by the two rate-limiting cyclooxygenases, COX-1 and COX-2, which form the prostaglandin (PG) endoperoxide, PGH_2. PGH_2 is transformed enzymatically into PGI_2 by PGI_2 synthase (PGIS) in blood vessels or

into thromboxane A_2 (TxA_2) by TxA_2 synthase in platelets. In blood vessels, particularly larger arteries, the predominant PG is PGI_2. The capacity of blood vessels to generate PGI_2 is essential to the integrity of the endothelium. The actions of PGI_2 are opposed by activation of the thromboxane (TP) receptor by PGH_2 and/or TxA_2. Both TxA_2 and PGH_2 stimulate contraction of vascular smooth muscle and cause platelet aggregation via activation of the TP receptor [8–10]. Activation of the TP receptor in vascular cells also induces cell apoptosis and abnormal expression of adhesion molecules (intercellular adhesion molecule [ICAM]-1, vascular cell adhesion molecule [VCAM]-1, and endothelial-leukocyte adhesion molecule [ELAM]-1) and mitogenic or hypertrophic activities [32].

Using microsomal and purified PGIS and various cultured cells, we have established the selective inhibition of PGIS by $ONOO^-$. The inhibition was accompanied by the nitration of tyrosine residues in the active site of PGIS [8]. The mechanism of PGIS nitration is explained by a catalytic reaction of the iron-thiolate active center of PGIS with $ONOO^-$, which is 1000-fold faster than those of cellular antioxidants with the activated form of $ONOO^-$, peroxynitrous acid (HOONO) [33–35]. Tyrosine 430 of PGIS has been demonstrated by analytical methods, including mass spectrometry, to be nitrated by $ONOO^-$ [35]. In addition, we have utilized ischemia-reperfusion [37] and endotoxic shock [38], nitrate tolerance [39], endotoxin [40], or cytokine [41] as systemic conditions associated with overwhelming oxidative and nitrosative stresses to investigate the role of $ONOO^-$-PGIS pathways in the development of endothelial dysfunction. Short-term exposure of bovine coronary arteries to lipopolysaccharide (LPS) (endotoxin) or to hypoxia-reoxygenation caused a similar pattern of endothelial dysfunction as seen after treatment with $ONOO^-$ itself. A role for endogenous $ONOO^-$ in this process was indicated from finding that both NOS inhibitors and SOD efficiently prevented the inactivation and nitration of PGIS as well as PGH_2-mediated vasoconstriction, indicating that excessive formation of $O_2\cdot^-$ within vascular tissues not only neutralizes NO, but also forms $ONOO^-$ and triggers vasoconstriction by the increased accumulation of PGH_2 resulting from inactivation of PGIS [37–40].

Previous studies demonstrate that vascular PGI_2 synthesis and/or release are reduced in diabetes [41–48]. Blood vessels obtained from streptozotocin- or alloxan-induced diabetic animals show reduced production of PGI_2, whereas their platelets release more TxA_2 than normal [41–48]. PGI_2 production by blood vessels from patients with diabetes is depressed and urinary, as well as circulating levels of the PGI_2 metabolite, 6-keto-$PGF_{1\alpha}$, are reduced in diabetic patients with proliferative retinopathy and premature pregnancy. Decreased PGI_2 has been linked to platelet hyperaggregability, increased adhesiveness, and increased release of PGH_2/TxA_2 in diabetes. Moreover, a reduction in PGI_2 production has been proposed as a mechanism for accelerated atherosclerosis in patients with diabetes [41–48].

To establish if PGIS nitration occurs in diabetes, we first examined the effect of elevated glucose on the release of NO and $O_2\cdot^-$, the bioactivity of NO, and the activity of PGIS [32]. Human aortic endothelial cells (HAECs) were incubated in media containing 5 mM glucose, 30 mM glucose, or 24.5 mM mannitol plus 5.5 mM glucose for 10 days. Elevated glucose exposure was associated with significant increases in $O_2\cdot^-$ and decreased NO bioactivity as judged by levels of cyclic guanosine monophosphate (GMP), despite the fact that total NO breakdown products ($NO_2^-$and NO_3^-) were increased. There was also a 70% reduction in PGIS activity as judged by formation of 6-keto-$PGF_{1\alpha}$ produced from PGH_2. Associated with the reduced activity of PGIS was tyrosine nitration of the enzyme, as demonstrated by immunoblotting techniques and high-performance liquid chromatography (HPLC) measurement of 3-NT in immunoprecipitated PGIS. Because tyrosine nitration of PGIS was prevented by treating the cells for 10 days with either polyethylene glycolylated (PEG-SOD) or N^{ω}-nitro-L-arginine methyl ester (L-NAME), these studies indicated that the oxidant stress caused by elevated glucose was associated with reactive nitrogen species, likely $ONOO^-$ [32].

The results obtained by using immunohistochemistry, immunoprecipitation, and Western blots demonstrated an increased nitration of PGIS in high glucose–exposed vessels. Critical proof for the generation of nitrating species after high-glucose incubation came from a direct identification of 3-NT by HPLC. Interestingly, the amount of 3-NT contained in the PGIS immunoprecipitates represented a large proportion of the total content of 3-NT in the endothelial cells. Consistent with this, an immunoprecipitation obtained with antibody against 3-NT stained with 3-NT antibodies showed a major band at the molecular weight of PGIS, although other fainter bands were seen. Importantly, exposure of HAECs to high glucose increased $O_2\cdot^-$, inactivated NO, formed $ONOO^-$, tyrosine nitrated PGIS, and decreased the activity of PGIS, but did not alter the level of its expression, indicating its oxidative inactivation is the principal mechanism accounting for the decreased PGI_2 production.

$ONOO^-$ AND TP RECEPTOR ACTIVATION

Various studies demonstrate that the release by the aorta of PGH_2/TXA_2-like vasoconstrictors is increased in hyperglycemic alloxan- and streptozotocin (STZ)-induced diabetic animals [48–53]. Further, in the same models, the impaired relaxation is restored by COX inhibitors such as indomethacin or blockers of the PGH_2/TxA_2 receptor but not by the TxA_2 synthase inhibitors, suggesting the constrictor responses are mediated by PGH_2 rather than by TxA_2. Because PGH_2 is metabolized in blood vessels primarily by PGIS to yield PGI_2, the PGH_2-mediated response is minimal in the aorta of control animals, decreased activity of PGIS or increased COX activity is likely to be the cause of PGH_2-dependent vasoconstriction contributing to impaired relaxation in diabetes.

To elucidate if PGIS inhibition contributes to TP receptor stimulation in diabetes, we have performed additional studies in HAECs exposed to 30 mM glucose for 7 to 10 days in separate studies [32]. Exposure to glucose for 10 days increased both

apoptosis and ICAM-1 expression. Direct activation of the TP receptor with U46619 (1 μmol/L, 16 h) also significantly increased endothelial cell apoptosis and ICAM expression. Both the effect of U46619 and that of elevated glucose were significantly decreased by cotreatment with the TP receptor antagonist, SQ29548 (10 μM). These studies confirm that TP receptor stimulation and exposure to elevated glucose increase apoptosis of endothelial cells and adhesion molecule expression, and provide evidence that these effects of elevated glucose are mediated, to a major degree, by TP receptor stimulation. These results might provide a plausible potential mechanism for the involvement of TP receptor in the effect of hyperglycemia on endothelial dysfunction via its effects on endothelial cell apoptosis and adhesion molecular expression. Furthermore, we demonstrated that not only was the increase in ICAM expression prevented by TP receptor blockade, but also by PEG-SOD or L-NAME, consistent with $ONOO^-$ mediating the change. Importantly, we showed that the decrease in PGIS activity and PGIS nitration, which was prevented by PEG-SOD or L-NAME, was not prevented by TP receptor blockade, consistent with the requirement for the oxidative inactivation of PGIS for TP receptor stimulation [32].

Having shown that PGIS is tyrosine nitrated and inactivated in cultured HAECs we have performed a few preliminary studies to investigate if the changes observed in cultured cells also occur in vivo. We investigated if PGIS nitration occurs in diabetes. The diabetes-prone biobreeding (BB) diabetic rat is a genetic diabetes-prone model that more closely resembles type I diabetes. Aortas of 20- to 25-week-old diabetic BB rats, which had elevated plasma glucose of $>309 \pm 43$ mg/dL, were isolated and probed for PGIS nitration. As shown in Figure 1, diabetes caused increased nitration of PGIS in aortas of diabetic BB rats. Nitration of PGIS was further supported by the increased tyrosine nitration of PGIS in both endothelium and vascular smooth muscle layers (green fluroscence). Tyrosine nitration of PGIS was further confirmed from the increased 3-NT staining in PGIS immunoprecipitated from the aorta of diabetic BB rats, but not from control rats. In addition, PGIS activity, as judged by 6-keto-$PGF_{1\alpha}$ formation from exogenous PGH_2 substrate, was more than 75% lower than that from diabetic BB rats. These results indicate that diabetes increases tyrosine nitration and inhibition of PGIS in vivo. We have also made similar observations of tyrosine nitration and decreased PGIS activity in LDL receptor–deficient mice occurring in association with accelerated atherosclerotic lesion development caused by streptozotocin-induced diabetes (data not shown).

$ONOO^-$ AND eNOS UNCOUPLING

All NOS isoforms possess two catalytic domains consisting of a C-terminal reductase where NADPH, FMN, and FAD bind, and an N-terminal oxygenase domain where heme, tetrahydrobiopeterin (BH4), oxygen, and L-arginine bind [1]. The catalytic mechanisms of NOS involve flavin-mediated electron transport from the C-terminus–bound NADPH to the N-terminal heme center where oxygen is reduced and incorporated into the guanidine group of L-arginine, giving rise to NO and L-citrulline [1]. If the reaction is uncoupled, NOS will become a net producer of damaging $O_2^{\cdot -}$ or $ONOO^-$ instead of the protective NO (NOS uncoupling). Indeed, NOS can produce both $O_2^{\cdot -}$ and $ONOO^-$ in different disease states, and the resulting production of $ONOO^-$ can exert marked autocrine toxicity [1–3].

After having established that PGIS is tyrosine nitrated in cells exposed to high glucose, we next determined the source of $O_2^{\cdot -}$ and $ONOO^-$, which is responsible for PGIS nitration in HAECs exposed to elevated glucose. Figure 2*A* showed $O_2^{\cdot -}$ in HAECs, measured by the SOD-inhibitable cytochrome *c* reduction assay was significantly increased after 7 days exposure to 30 mM glucose. Neither oxypurinol (100 μM), a xanthine oxidase inhibitor, nor mitochondrial respiratory chain inhibitors, antimycin (100 μM), rotenone (100 μM), or the inducible NOS inhibitor, L-Nil (1 μM), blocked high glucose–up-regulated $O_2^{\cdot -}$ release. Notably, neither 25 mM mannitol (not shown) nor L-glucose had any effect, indicating that the metabolism of D-glucose, not hyperosmolarity, mediated the increased $O_2^{\cdot -}$ production. Both L-NAME, a nonselective NOS inhibitor (500 μM) and diphenyleneiodonium (DPI; 100 μM), a nonselective inhibitor of flavin-containing enzymes, significantly decreased high glucose–up-regulated $O_2^{\cdot -}$ release, suggesting eNOS as a source of $O_2^{\cdot -}$. The increase in $O_2^{\cdot -}$ caused by elevated glucose in HAECs was accompanied by an increase in eNOS expression, appearing primarily as an increase in eNOS monomers (see below; Figure 2*B*), and PGIS nitration (Figure 2*C*). Of note, inhibition of eNOS with L-NAME reduced high glucose–induced PGIS nitration, indicating that eNOS is a main source of oxidants triggering PGIS nitration (Figure 2*C*).

We next investigated how eNOS becomes uncoupled and releases oxidants. Because all three NOS have a zinc tetrathiolate (ZnS_4) cluster formed by a zinc ion coordinated in a tetrahedral conformation with pairs of symmetrically oriented and phylogenetically conserved cysteine residues at the dimer interface and the zinc-thiolate cluster is essential in regulating NOS activity, we next investigated if pathophysiologically relevant concentrations of $ONOO^-$ affect the zinc-thiolate structure of eNOS. Exposure of purified recombinant eNOS to $ONOO^-$ effectively disrupts and releases zinc from the zinc-thiolate cluster of eNOS and presumably forms disulfide bonds between the monomers [54]. As a result, disruption of sodium dodecyl sulfate (SDS)-resistant eNOS dimers occurs under reducing conditions [54]. The catalytic activities of eNOS are exquisitely and uniquely sensitive to $ONOO^-$, which decreases NO synthesis and increases $O_2^{\cdot -}$ production by the enzyme. Exposure of BAECs to elevated glucose resulted in loss of zinc, uncoupling of eNOS, and increased $O_2^{\cdot -}$ release similar to that seen with recombinant eNOS exposed to $ONOO^-$ [54]. The effects of elevated glucose were prevented by SOD-PEG or L-NAME, giving credible evidence that endogenous generation of $ONOO^-$ was responsible. Interestingly, high glucose significantly increased the expression of the zinc-depleted eNOS monomer in BAECs (Figure 2*B*). The increased zinc-depleted eNOS dimers might produce $O_2^{\cdot -}$ instead of NO [54].

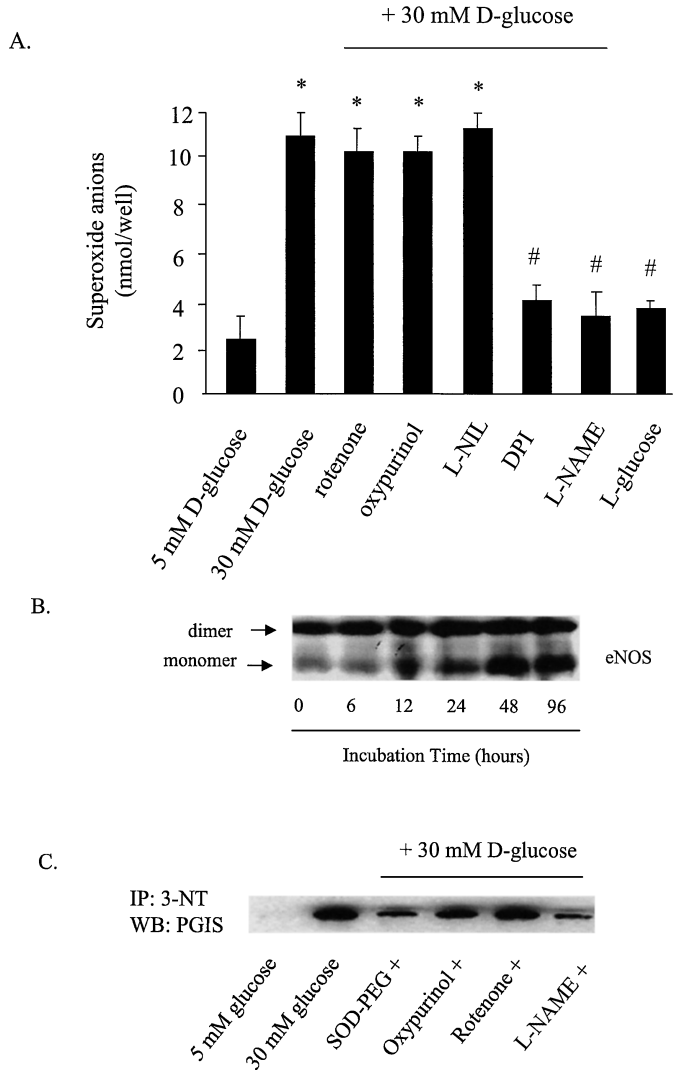

FIG. 2. The eNOS uncoupling in HAEC exposed to 30 mM D-glucose. (*A*) The eNOS-dependent $O_2{\cdot}^-$ release in HAEC cells exposed to elevated glucose. Human aortic endothelial cells (HAECs) were exposed to 30 mM D-glucose or L-glucose for 3 days. The production of $O_2{\cdot}^-$ was assayed by the SOD-inhibitable cytochrome *c* reduction assay as described previously [32, 52]. The pharmacologic inhibitors were added into cells 30 min before the assay. Glucose at 30 mM significantly increased $O_2{\cdot}^-$ release ($n = 7, p < .05$). L-NAME and DPI attenuated 30 mM glucose–induced $O_2{\cdot}^-$ ($n = 7, p < .001$ by two-way ANOVA)-treated HAECs ($n = 12$, $^*p < .01$). (*B*) Increased eNOS monomers in HAECs exposed to 30 mM glucose. The eNOS dimer and monomer were assayed by the low-temperature SDS-PAGE with Western blots as described previously. The blot is a representative of five blots obtained from five independent experiments. (*C*) Tyrosine-nitration of PGIS in HAECs exposed to 30 mM D-glucose. Tyrosine-nitrated proteins were immunoprecipitated with the antibodies against 3-nitrotyrosine. Nitrated PGIS was detected by using the antibody against PGIS in Western blot. The blot is a representative of three blots obtained from three independent experiments.

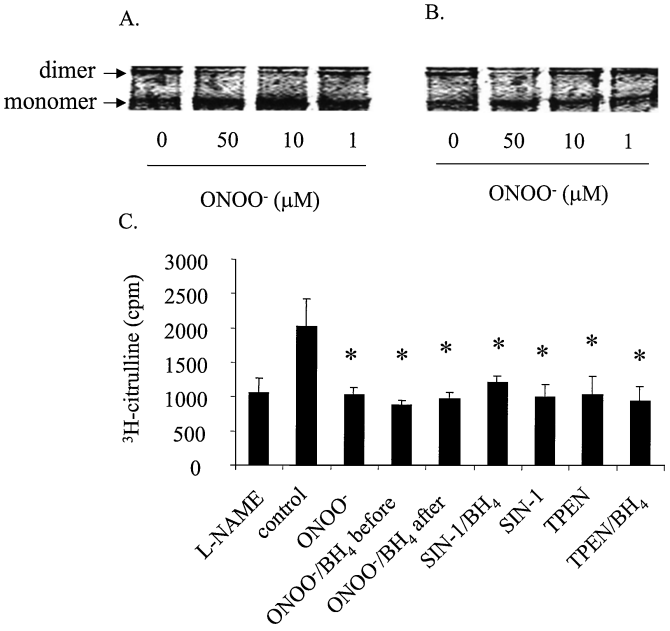

FIG. 3. Effects of BH_4 on $ONOO^-$-induced dissociation of eNOS and eNOS activity. Of note, supplementation of BH_4 did not reverse the effects of $ONOO^-$, SIN-1, or TPEN, a zinc chelator, on eNOS dimers and eNOS activity. (*A*) BH_4 (100 μmmol/L) was added into recombinant bovine eNOS 10 min before addition of $ONOO^-$. The blot represents those in three independent experiments. (*B*) BH_4 (100 μM) were added into recombinant bovine eNOS 30 min after $ONOO^-$ (50 μM). (*C*) Effects of BH_4 on $ONOO^-$-induced inhibition on eNOS activity. SIN-1 (0.5 mM) or TPEN (1 mM) were incubated with BAECs 2 h before assaying eNOS activity. The eNOS activity was assayed by the conversion of ^{3}H-arginine into ^{3}H-citrulline ($n = 6$, control versus treated, $^*p < .05$; $n = 6$, $ONOO^-$ versus treated, $^\#p < .05$).

BH_4 is an important factor regulating eNOS activity, and increased oxidation of BH_4 has been found in various diseases including diabetes [54]. Supplementation with BH_4 has been found to improve impaired endothelial dysfunction associated with diseases [54]. Recent studies indicate that $ONOO^-$ is able to deplete BH_4 resulting in eNOS uncoupling [55, 56]. We then compared the reactivity of $ONOO^-$ with both BH_4 and the zinc-thiolate center of eNOS. High concentrations of $ONOO^-$ (>100 μM) oxidized BH_4 (1 mM) with an EC_{50} of >100 μM [54]. Lower concentrations of $ONOO^-$ (50 μM), which did not cause detectable oxidation of BH_4 [54], effectively oxidized the zinc-thiolate center of BH_4 (Figures 3*A* and *B*). Supplementation with BH_4 neither prevented the oxidation of eNOS by $ONOO^-$, nor restored the enzymatically active dimers dissociated by $ONOO^-$ (Figures 3*A* and *B*). In addition, in intact BAECs, 0.1 mM BH_4 did not prevent $ONOO^-$-induced inhibition of eNOS activity (Figure 3*C*). These results indicate that $ONOO^-$ preferentially reacts with the zinc-thiolate center of eNOS. Kinetic studies demonstrate that the reaction of zinc-thiolate clusters with $ONOO^-$ is at least 100 times faster than that with BH_4 [55] and 1000 times faster than that with cysteine-thiols [54]. For example, the rate constant for the reaction with $ONOO^-$ with the zinc-thiolate center of alcohol dehydrogenase is $5.2 \times 10^5\ M^{-1}\ s^{-1}$ compared to that for BH_4 with $ONOO^-$ ($6 \times 10^3\ M^{-1}\ s^{-1}$) [54–56]. The faster reaction with $ONOO^-$ is due to the zinc atom, which has the highest charge-to-atomic radius ratio of any element and maintains partial cationic character

even in a tetracoordinate complex like the zinc-thiolate cluster. Thus, zinc will attract anionic oxidants, which results in the loss of zinc and disulfide formation within the metal binding site. Because the BH_4 binding site is close to the zinc binding site, disruption of the zinc-thiolate cluster of eNOS might distort the BH_4 binding site of eNOS. How oxidation of the zinc-thiolate cluster of eNOS leads to eNOS uncoupling will require further investigation.

We have also demonstrated dysfunction of eNOS in diabetic LDL receptor–deficient mice in vivo [54]. Increased amounts of eNOS protein were detected by Western blotting after boiling under reducing conditions in homogenates of the heart, kidney, and liver of the diabetic LDL receptor–deficient mice compared to normoglycemic littermates. In order to determine if dysfunction of eNOS occurs in the tissues of these diabetic atherosclerotic mice, tissue eNOS was purified and assayed for dimers and monomers, zinc content, and activity. After purification, it was apparent that the increased eNOS observed in the homogenate was primarily represented by an increase in eNOS monomers, because SDS-resistant eNOS dimers were significantly decreased. Despite the fact that increased amounts of eNOS protein was present in the tissues of the diabetic mice, the zinc content of eNOS purified from the heart and kidney was significantly decreased, whereas that of the liver was not affected [54]. The decrease in eNOS dimers and zinc content was paralleled by significantly decreased NO synthesis with an increase in atherosclerotic lesions in the diabetic LDL receptor–deficient mice [54]. Therefore, disruption of the zinc-thiolate cluster of eNOS by $ONOO^-$ might lead to eNOS uncoupling, which might be an important mechanism in the development of vascular complications in diabetes.

CONCLUSIONS

In summary, our studies indicate an important role for $ONOO^-$ in the pathogenesis of diabetic vascular dysfunction. Our data demonstrate that pathophysiologically relevant concentrations of $ONOO^-$ trigger eNOS uncoupling, tyrosine nitration, and inactivation of PGIS. Therefore, the formation of $ONOO^-$ is key in the initiation and progression of vascular complications in diabetes mellitus because of the down-regulation of protective actions of NO and PGI_2, and because the nonmetabolized PGH_2 via TP receptors tips the balance towards platelet aggregation, endothelial apoptosis, atheroma accumulation, and thrombus formation. The net vascular damage can initiate and propagate clinical events characterized by vasoconstriction, adherence of platelets and monocytes, atherosclerosis and/or micro-occlusive vascular disease, and, ultimately, vascular thrombosis and tissue damage (Figure 4).

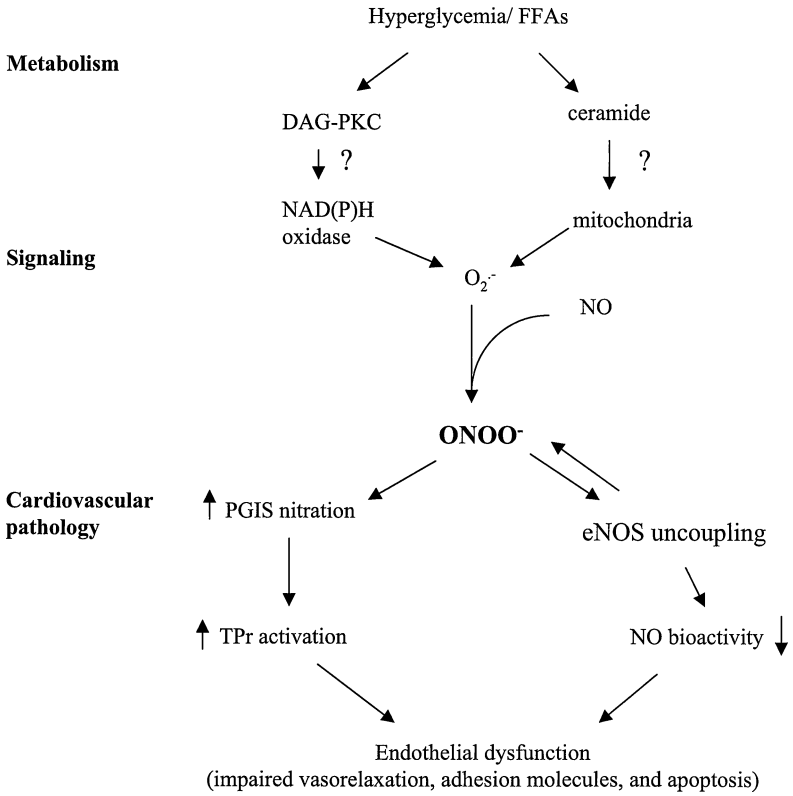

FIG. 4. Proposed mechanisms of the $ONOO^-$-mediated diabetic endothelial dysfunction. Hyperglycemia/free fatty acid increases increase intracellar oxidant stress through mechanism involving NADPH oxidase or the mitochondria. The generation of $O_2^{\cdot-}$ will react with NO to form $ONOO^-$ resulting in eNOS uncoupling, PGIS nitration, and TPr stimulation, and impaired insulin signaling in diabetes.

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