Original Article

Endothelial Nitric Oxide Synthase–Dependent Tyrosine Nitration of Prostacyclin Synthase in Diabetes In Vivo

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There is evidence that reactive nitrogen species are implicated in diabetic vascular complications, but their sources and targets remain largely unidentified. In the present study, we aimed to study the roles of endothelial nitric oxide synthase (eNOS) in diabetes. Exposure of isolated bovine coronary arteries to high glucose (30 mmol/l D-glucose) but not to osmotic control mannitol (30 mmol/l) switched angiotensin II–stimulated prostacyclin (PGI_2)-dependent relaxation into a persistent vasoconstriction that was sensitive to either indomethacin, a cyclooxygenase inhibitor, or SQ29548, a selective thromboxane receptor antagonist. In parallel, high glucose, but not mannitol, significantly increased superoxide and 3-nitrotyrosine in PGI_2 synthase (PGIS). Concurrent administration of polyethylene-glycolated superoxide dismutase (SOD), L-nitroarginine methyl ester, or sepiapterin not only reversed the effects of high glucose on both angiotensin II–induced relaxation and PGI_2 release but also abolished high-glucose–enhanced PGIS nitration, as well as its association with eNOS. Furthermore, diabetes significantly suppressed PGIS activity in parallel with increased superoxide and PGIS nitration in the aortas of diabetic C57BL6 mice but had less effect in diabetic mice either lacking eNOS or overexpressing human SOD ($hSOD^{+/+}$), suggesting an eNOS-dependent PGIS nitration in vivo. We conclude that diabetes increases PGIS nitration in vivo, likely via dysfunctional eNOS. *Diabetes* 55:3133–3141, 2006

Endothelial dysfunction is a hallmark of vascular injury in diabetes and is preceded by the development of overt cardiovascular diseases (1–3). There is an overwhelming mass of evidence demonstrating the development of endothelial dysfunction in animal models of diabetes and in human blood vessels from diabetic patients, as evidenced by increased release of reactive oxygen species, decreased nitric oxide (NO) bioactivity, decreased release of prostacyclin (PGI_2), and enhanced endothelial production of vasoconstrictor thromboxane (Tx)A_2/prostaglandin (PG)H_2 in early stages of diabetes (1–3). The net effect of endothelial dysfunction is vascular damage, which is responsible for complications in both types of diabetes.

Evidence has accumulated indicating that the generation of reactive oxygen and nitrogen species plays an important role in the etiology of diabetes complications. Our previous studies (4,5) and others (6,7) have shown that exposure of human aortic endothelial cells to high glucose leads to augmented production of superoxide anion ($O_2^{\cdot -}$), which may quench NO, thereby reducing the efficacy of this potent endothelium-derived vasodilator system and generating toxic oxidant species, such as peroxynitrite ($ONOO^-$) (4,5). $ONOO^-$ is a highly reactive species and can initiate both nitrosative and oxidative reactions in vitro and in vivo (8–10). A characteristic reaction of $ONOO^-$ is the nitration of protein-bound tyrosine residues to generate 3-nitrotyrosine–positive proteins (8–10).

PGI_2 synthase (PGIS; EC5.3.99.4) catalyzes the conversion of PGH_2 into PGI_2 (11,12). The limiting step of PGI_2 is the formation of the PG endoperoxide, PGH_2, by the two rate-limiting cyclooxygenases (COXs), COX-1 and -2. In blood vessels, particularly larger arteries, the predominant PG is PGI_2. PGI_2 relaxes isolated vascular strips and is a potent endogenous inhibitor for platelet and leukocyte activation. PGI_2 not only prevents platelets from sticking together but also disperses existing aggregates in vitro and in the circulation of humans (11,12). The effects of PGI_2 are opposed by TxA_2, a major product of platelets (10,11). Suppressed PGI_2 (12–15) and/or increased TxA_2 or PGH_2 (16–19) have been reported in the early stage of diabetes (1,4). For example, PGI_2 production by blood vessels from patients with diabetes is depressed and urinary and circulating levels of 6-keto-PGF1α are reduced in patients with proliferative retinopathy (13,14). Furthermore, decreased PGI_2 has been linked to platelet hyperaggregability, increased adhesiveness, and increased release of PGH_2/TxA_2 in diabetic patients (13,14). However, how diabetes leads to an imbalance of TxA_2/PGI_2 remains elusive.

Our previous studies (20–24) had demonstrated that $ONOO^-$, when given exogenously or when produced endogenously, is able to induce endothelial dysfunction via a mechanism dependent on the inhibition and nitration of PGIS at tyrosine 430 (25) and consequent stimulation of Tx receptor (TPr) (5,22). In the present study, we hypothesized that high glucose might, via $ONOO^-$, alter the balance of PGI_2/TxA_2 by tyrosine nitration of PGIS. Using

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Received for publication 13 April 2006 and accepted in revised form 15 August 2006.

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BCA, bovine coronary artery; BH_4, tetrahydrobiopterin; COX, cyclooxygenase; eNOS, endothelial nitric oxide synthase; hSOD, human superoxide dismutase; L-NAME, L-nitroarginine methyl ester; $O_2^{\cdot -}$, superoxide anion; $ONOO^-$, peroxynitrite; PEG, polyethylene glycolated; PG, prostaglandin; PGI_2, prostacyclin; PGIS, PGI_2 synthase; SOD, superoxide dismutase; STZ, streptozotocin; TPr, thromboxane receptor; Tx, thromboxane.

DOI: 10.2337/db06-0505

isolated bovine coronary arteries (BCAs) and streptozotocin (STZ)-induced diabetic mice, we have, for the first time, demonstrated an endothelial nitric oxide synthase (eNOS)-mediated PGIS nitration in diabetes in vivo.

RESEARCH DESIGN AND METHODS

Male mice overexpressing human Cu,Zn superoxide dismutase (SOD) (hSOD-TG; TgHS-51), eNOS knockout mice ($eNOS^{-/-}$), and their littermates, C57BL6 mice, 7 weeks of age, were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in temperature-controlled cages with a 12-h light/dark cycle and given free access to water and normal diet. Mice were randomly divided into control or diabetic groups. STZ was prepared in 0.05 mol/l citrate buffer, pH 4.5 (50 $mg \cdot kg^{-1} \cdot day^{-1}$ for both C57BL6 and $eNOS^{-/-}$ but 70 $mg \cdot kg^{-1} \cdot day^{-1}$ for SOD-TG), and was intraperitoneally injected each day for 5 days. Control nondiabetic animals received the citrate buffer (pH 4.5) solvent. The diabetic state was confirmed the following week by glucose determination on tail vein blood. Body weight, water, and food intake were monitored routinely. Two weeks after STZ injection, the mice were killed with inhaled isoflorane. Mice hearts and aortas were removed and immediately frozen in liquid nitrogen. The animal protocol was reviewed and approved by the Institute Animal Care and Use Committee.

L-nitroarginine methyl ester (L-NAME), PGH_2, 1S[1α, 2B(5Z),3b,4α]-7-[3-[2(phenylamino) carbonyl] hydrazino methyl [-7-oxabicyclo(2.2.1)hept-2-yl-5-heptenoic acid] (SQ29548), indomethacin, sepiapterin, and the enzyme-linked immunoassay kits for 6-keto-PGF1α, TxB2, PGF2α, and PGE_2 were obtained from Cayman Chemicals (Ann Arbor, MI). Protein-A sepharose CL-4B was obtained from Pharmacia. A monoclonal antibody against 3-nitrotyrosine was purchased from Upstate Biotechnology Incorporated (Waltham, MA). Antibodies against eNOS and caveolin-1 were obtained from Transduction Laboratory. Rabbit anti-PGIS antisera were kindly provided by Dr. T. Klein (Altana Pharm, Konstanz, Germany). Secondary antibodies were from Pierce. Enhanced chemiluminescence kits and nitrocellulose membranes (Hybond-C) were purchased from Amersham. Other chemicals, if not indicated, were acquired from Sigma (St. Louis, MO) with highest quality.

Isolation and isometric measurement of tension in BCAs. BCAs of the left ventricle were isolated and assayed as described previously (22–24). Briefly, experiments were started by obtaining, from each spiral, a reference response of vasoconstriction relaxation and prostanoid release after addition of angiotensin II (50 nmol/l) over a time period of 30 min. Subsequently, the spiral was rinsed several times with prewarmed (37°C) Krebs-Ringer buffer and supplemented with normal glucose (5.5 mmol/l), high glucose (30 mmol/l), or mannitol (24.5 mmol/l mannitol plus 5.5 mmol/l glucose). At the times indicated, incubation was terminated by removing glucose-containing medium and rinsed several times with Krebs-Ringer buffer. After the tension returned to the baseline, the vessel was stimulated with the same concentration of angiotensin II. Indomethacin (10^{-5} mol/l) or nonselective NOS inhibitor, L-NAME (10^{-4} mol/l), or polyethylene-glycolated (PEG)-SOD (300 units/ml) were added during incubation and supplemented to the organ bath after removing elevated glucose- or mannitol-containing medium, as well as during the second stimulation with angiotensin II. The media from the first and the second stimulation with angiotensin II were collected and stored at −20°C for prostanoid analysis using enzyme-linked immunoassay kits according to the manufacturer's instructions. During the experiments, care had been taken to avoid any injury to the endothelium. In some experiments, the endothelial layer was deliberately removed after dissection by intraluminal perfusion with 0.5% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1 propane sulfonate in Krebs-Ringer buffer for 40 s followed by repeated washings with Krebs-Ringer buffer.

Assay of PGIS activity. PGIS activity was assayed by the stable metabolite of PGI_2, 6-keto-PGF1α, after incubating cells with its substrate, PGH_2 (10^{-5} mol/l for 3 min), as described previously (22–25).

Detection of aortic $O_2^{\cdot -}$. Aortic $O_2^{\cdot -}$ in isolated mouse aorta was measured by lucigenin chemiluminescence (5 μmol/l). Measurements on intact arteries with lucigenin at 5 μmol/l have been corroborated with electron spin resonance and were not complicated by its redox cycling (26).

High-performance liquid chromatography detection of 3-nitrotyrosine. Aortic proteins were isolated and hydrolyzed by pronase digestion (72 h at 56°C). 3-nitrotyrosine was quantified by a high-performance liquid chromatography/ultraviolet detector coupled with electrochemical detections, as previously described (22–24).

Immunoprecipation and Western blots. Immunoprecipitation and Western blots were performed as described previously (21–25).

Quantification of Western blot. The intensity (area × density) of the individual bands on Western blots was measured by densitometry (Model GS-700, Imaging Densitometer; Bio-Rad). The background was subtracted from the calculated area.

Statistical analysis. Results were analyzed by using a two-way ANOVA. Values are expressed as means ± SD for the number of assays. A P value <0.05 was considered statistically significant.

RESULTS

Hyperglycemia blunts PGI_2-dependent relaxation and releases PGH_2-mediated vasospasm in BCAs. We have shown (22–24) that in BCAs, PGI_2 is responsible for ~80% of angiotensin II–induced relaxation, whereas NO accounts for ~20%. Stimulation of BCAs with angiotensin II (50 nmol/l) triggered a rapid rise of tension (constriction) followed by a PGI_2-mediated relaxation (22–24). Thus, BCAs appeared to be an ideal system for studying the effects of high glucose on PGI_2-dependent relaxation.

Using this well-characterized system, we next investigated whether high glucose mimicked the effects of $ONOO^-$ in vivo. Freshly isolated BCAs were exposed to normal glucose (control, 5 mmol/l D-glucose), high glucose (30 mmol/l D-glucose), or osmotic control (5 mmol/l D-glucose plus mannitol) for 0.5–4 h. As shown in Fig. 1*B*, high glucose did not alter angiotensin II–triggered vasoconstriction. Similar to the vessels exposed to submicromolar concentrations of $ONOO^-$ (22), exposure of BCAs to high glucose for 4 h significantly impaired angiotensin II–induced relaxation (Fig. 1*A* and *B*) and subsequently triggered a sustained vasoconstriction after a transient and small relaxation (Fig. 1*B*).

We next investigated if high-glucose–impaired relaxation was due to a reduction of PGI_2, the major vasodilator in BCA. Short exposure of BCAs to high glucose (<30 min) slightly increased the release of 6-keto-PGF1α (data not shown). In parallel with impaired relaxation, prolonged exposure (1–4 h) of BCAs to high glucose significantly inhibited angiotensin II–induced PGI_2 release (Fig. 1*A*). In contrast, exposure of BCAs to mannitol for 4 h did not inhibit angiotensin II–induced PGI_2 release (Fig. 1*A*).

We next determined if the second vasospasm caused by high glucose was due to an overproduction of PGH_2, which acts upon TPr (27,28). As shown in Fig. 1*B*, concurrent administration of SQ29548 (10 μmol/l), a potent TxA_2/PGH_2 receptor antagonist, and high glucose abolished high-glucose–induced vasospasms. Furthermore, both indomethacin, a COX inhibitor, and SQ29548 restored angiotensin II–induced relaxation without altering PGI_2 reactivity. However, CGS13080, a potent TxA_2 synthase inhibitor, had no effect (Fig. 1*C*). Taken together, these results suggest that high glucose increased the release of COX-derived products, likely PGH_2, resulting in vasospasm.

Our previous studies demonstrated that tyrosine nitration and inactivation of PGIS leads to an accumulation of PGH_2, resulting in vasospasm by TPr activation in isolated BCAs exposed to either $ONOO^-$ (22) or hypoxia reoxygenation (23). Although both indomethacin and TPr blockade were effective in preventing vasospasm in vascular rings exposed to high glucose indicating PGH_2 (Fig. 1*B* and *C*), a quantification of this precursor is necessary because other prostanoid and hydroxyeicosatetraenoic acids are capable of stimulating TPr.

PGH_2 is an unstable prostaglandin that is converted into PGF2α by mild reducing agents such as $SnCl_2$ (22,23). Therefore, to estimate the PGH_2 released, we calculated the difference between the PGF2α peak of the samples from cells in the presence of 200 μg/mL $SnCl_2$. As depicted in Fig. 1*D*, the levels of PGF2α were significantly elevated

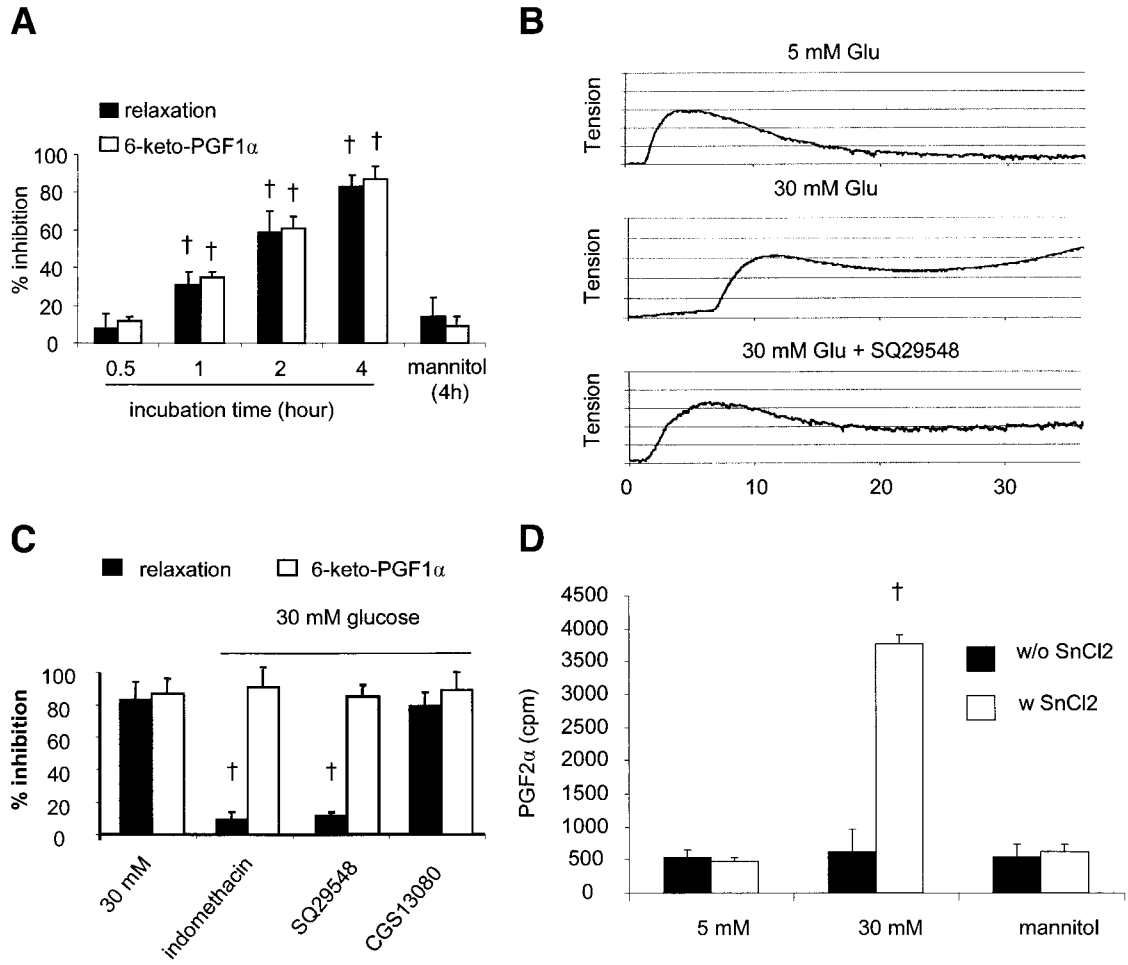

FIG. 1. High glucose impairs PGI2-dependent vasorelaxation and causes PGH2-mediated vasoconstriction in isolated BCAs ex vivo. *A*: Effect of high glucose and mannitol on angiotensin II–induced relaxation and PGI_2 release. After having obtained a reference response to angiotensin II, BCAs were exposed to 30 mmol/l glucose for the time indicated. The incubation was stopped by removing high glucose, and the vessels were stimulated with angiotensin II again. 6-keto-PGF1α, a stable metabolite of PGI_2, was analyzed by enzyme-linked immunoassay. The results are calculated as percentage of inhibition (5 mmol/l D-glucose) and are expressed as means ± SD (n = 12; †P < 0.01, 5 vs. 30 mmol/l D-glucose). *B*: Representative recording for angiotensin II–induced relaxation in isolated BCAs ex vivo. *C*: High glucose triggers PGH2-dependent vasospasm. After having obtained a reference response to angiotensin II, BCAs were exposed to 30 mmol/l glucose in the presence of indomethacin (10 μmol/l), SQ29548 (10 μmol/l), or CGS13080 (10 μmol/l) for 4 h as indicated. The incubation was stopped by removing the glucose-containing media, and the vessels were stimulated again in the presence of the same amount of agent in 30 mmol/l glucose. 6-keto-PGF1α s was analyzed by enzyme-linked immunoassay. The results are expressed as means ± SD (n = 8; †P < 0.01, 30 mmol/l glucose vs. 30 mmol/l glucose plus reagents). *D*: High glucose increases the formation of PGH2 in BCAs ex vivo. Isolated BCAs were exposed to either 5 mmol/l glucose, 30 mmol/l glucose, or mannitol for 4 h at 37°C with or without SnCl2, as described in RESEARCH DESIGN AND METHODS. PGF2α in incubation media was assayed by enzyme-linked immunoassays. The results are expressed as means ± SD (n = 4; †P < 0.05, 30 vs. 5 mmol/l glucose).

in BCAs exposed to high glucose compared with BCAs with either 5 mmol/l glucose or mannitol, which indicated that high glucose increased the release of PGH_2 in isolated BCAs.

Together with other PGs and Tx, both TxA_2 and PGI_2 are produced by the two rate-limiting COXs, COX-1 and -2, which form the PG endoperoxide PGG_2. Therefore, we next determined if the reduction of PGI_2 by high glucose was due to reduction of either COX-1 or -2. As shown in Fig. 2*A*, neither high D-glucose nor mannitol altered the levels of COX-1. Conversely, high glucose but not mannitol increased the detection of COX-2 (Fig. 2*A*). Furthermore, exposure of BCAs to high glucose significantly increased PGE_2, whereas the levels of both PGF2α and TxB_2 remained unaffected (Fig. 2*B*). This suggested that high glucose might increase the levels and activity of COX-2. Thus, high-glucose–induced PGI_2 inhibition was not due to a reduction of either COX-1 or -2.

$ONOO^-$-dependent tyrosine nitration of PGI_2 synthase. We next determined if high glucose increased PGIS nitration in BCAs. To this end, PGIS was first immunoprecipitated with a polyclonal antibody against PGIS and then Western blotted with a monoclonal antibody against 3-nitrotyrosine. Compared with a weak staining in BCAs exposed to 5 mmol/l glucose or mannitol, high glucose significantly increased PGIS nitration (Fig. 3*A* and *B*).

To further identify the source(s) of $O_2^{\cdot -}$ and $ONOO^-$, we determined the levels of PGIS nitration in presence of PEG-SOD; apocynin, a selective inhibitor for NAD(P)H oxidase assembly (29); allupurinol, a potent inhibitor for xanthine oxidase (30); or L-NAME, a nonselective inhibitor for NOS. As shown in Fig. 3*A* and *B*, concurrent administration of both L-NAME and PEG-SOD significantly suppressed high-glucose–enhanced PGIS nitration, suggesting that $ONOO^-$ was involved in high-glucose–enhanced PGIS nitration. In contrast, neither allopurinol nor apocynin

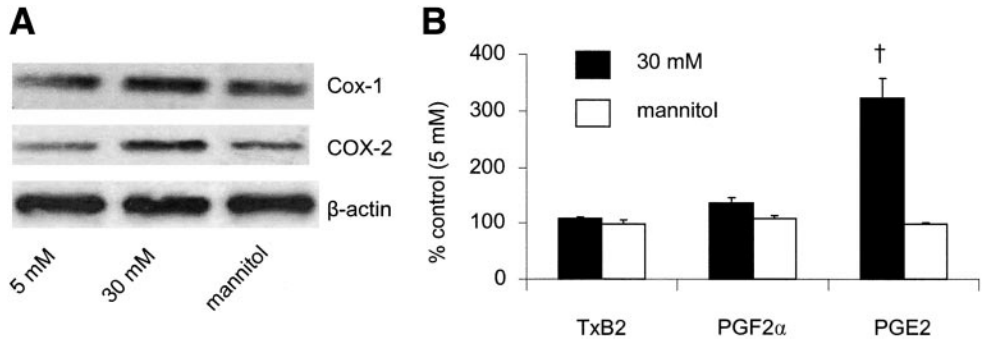

FIG. 2. Effects of high glucose on the expression of COX and prostaglandin production in isolated BCAs. *A*: High glucose increases the detections of COX-2. Isolated BCAs were exposed to normal glucose (5 mmol/l, control), high glucose (30 mmol/l), or mannitol for 4 h as described in RESEARCH DESIGN AND METHODS. The levels of COX-1 and -2 were detected in Western blots using the specific antibodies. The blot is a representative of three blots obtained from three independent experiments. *B*: High glucose increases PGE_2 synthesis. Isolated BCAs were exposed to normal glucose (5 mmol/l, control), high glucose (30 mmol/l), or mannitol for 4 h. At the end of incubation, the media were collected and analyzed for PGs, as indicated in RESEARCH DESIGN AND METHODS. The results are expressed as means ± SD (n = 8; †P < 0.01, 5 vs. 30 mmol/l glucose).

altered high-glucose–enhanced PGIS nitration (Fig. 3*A* and *B*), implying that eNOS might be responsible for high-glucose–enhanced PGIS nitration.

Identification of eNOS as the source of $O_2^{\cdot -}$. We next determined the source of $O_2^{\cdot -}$ and $ONOO^-$ in BCAs exposed to high glucose. As shown in Fig. 3*C*, high glucose but not mannitol significantly increased the release of $O_2^{\cdot -}$. Cocurrent administration of apocynin, allopurinol, or rotenone did not alter high-glucose–enhanced $O_2^{\cdot -}$ production. In contrast, either tiron, a potent $O_2^{\cdot -}$ scavenger, or L-NAME abolished high-glucose–enhanced $O_2^{\cdot -}$ (Fig. 3*C*). Theses results coincided with the selective inhibition of L-NAME on high-glucose–enhanced PGIS nitration (Fig. 3*A* and *B*), confirming that eNOS might be a source of $O_2^{\cdot -}$ and $ONOO^-$.

Effects of elevated glucose on PGIS nitration and $O_2^{\cdot -}$ in endothelium-denuded vessels. Further evidence for eNOS-derived $ONOO^-$ came from endothelium-denuded BCAs. To establish the contributions of eNOS in high-glucose–enhanced oxidant stress and PGIS nitration, endothelium was mechanically removed to delete eNOS located within endothelium. Removal of the endothelium

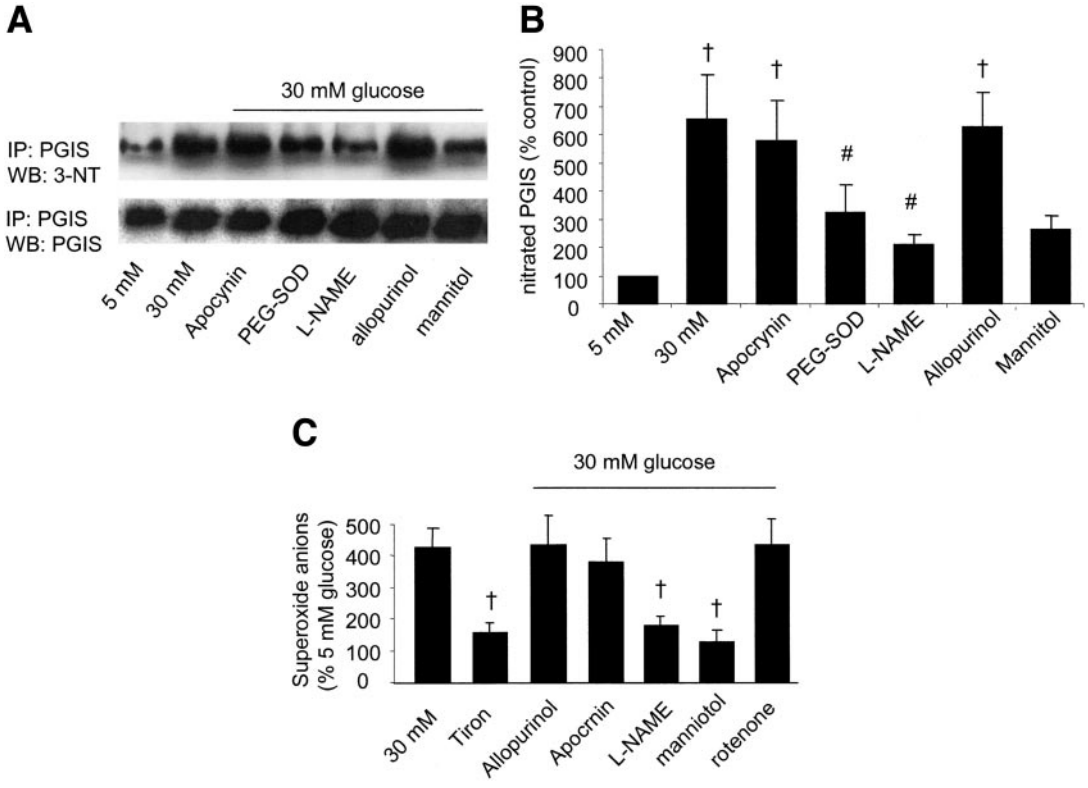

FIG. 3. High glucose increases superoxide release and tyrosine nitration of PGIS in isolated BCAs ex vivo. *A*: High glucose increases tyrosine nitration of PGIS in BCAs ex vivo. PGIS was precipitated by PGIS polyclonal antibodies in the vessel homogenates after the treatments indicated. Proteins were separated by electrophoresis and Western blotted with monoclonal antibody against 3-nitrotyrosine (3-NT). The results represent four blots from four independent assays. *B*: Densitometric analysis (density × areas) of nitrated PGIS. The results are expressed as means ± SD (n = 4; †P < 0.01, 30 vs. 5 mmol/l glucose; #P < 0.05, 30 mmol/l glucose vs. 30 mmol/l glucose plus reagents). *C*: High glucose increases $O_2^{\cdot -}$ in BCAs. After incubation with 5 mmol/l glucose, 30 mmol/l glucose, or mannitol for 4 h with or without the indicated reagents, BCAs were incubated with lucigenin chemiluminescence (5 μmol/l). The results, calculated as percent increase over 5 mmol/l glucose, were expressed as means ± SD (n = 10; †P < 0.05, 30 mmol/l glucose vs. 30 mmol/l glucose plus reagents).

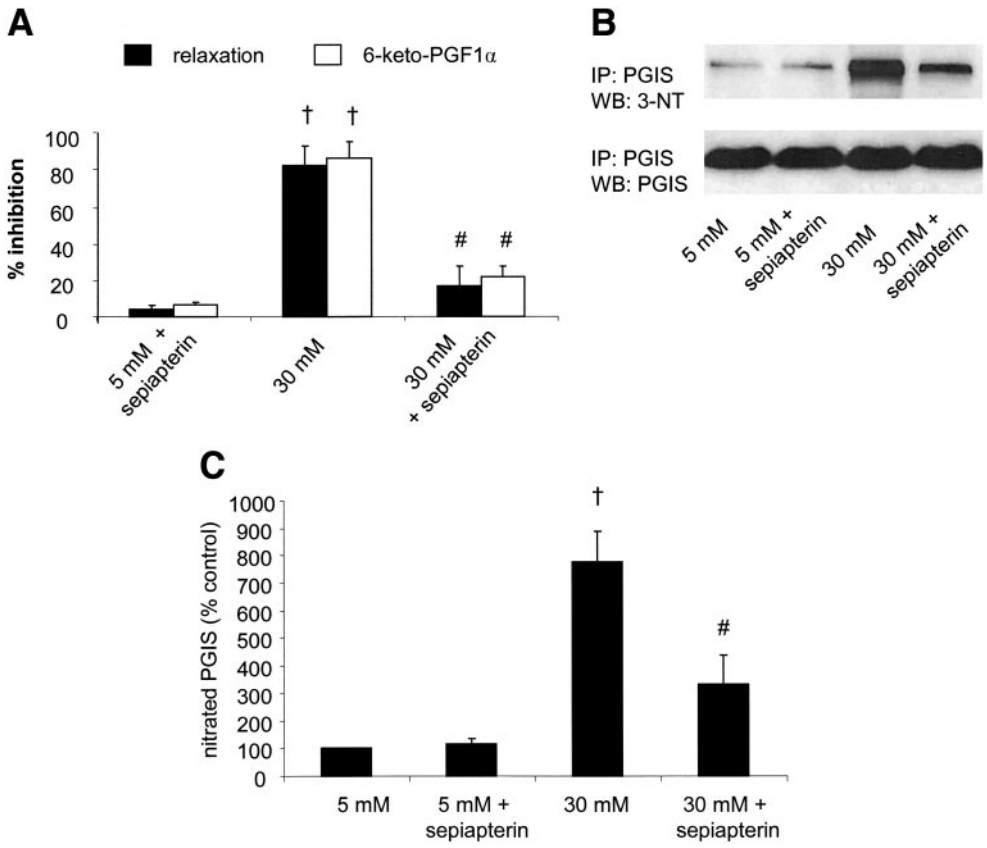

FIG. 4. Sepiapeterin prevents high-glucose–induced PGIS nitration ex vivo. *A*: Sepiapterin prevents high-glucose–induced inhibition on angiotensin II–induced PGI_2 release and relaxation. Angiotensin II–induced PGI_2 and relaxation were measured as described above. The results are expressed as means ± SD (n = 8; †P < 0.01, 5 vs. 30 mmol/l glucose; #P < 0.01, 30 mmol/l glucose vs. 30 mmol/l glucose plus reagents). *B*: Sepiapterin prevents high-glucose–enhanced PGIS nitration. Nitrated PGIS was detected as described in RESEARCH DESIGN AND METHODS. The blot is a representative of four blots obtained from four independent experiments. 3-NT, 3-nitrotyrosine. *C*: Densitometric analysis (density × areas) of nitrated PGIS. The results are expressed as means ± SD (n = 4; †P < 0.05, 5 vs. 30 mmol/l glucose; #P < 0.05, 30 mmol/l glucose vs. 30 mmol/l glucose plus sepiapterin).

blunted angiotensin II–induced PGI_2 release (−91 ± 4%). In addition, exposure of endothelium-denuded vessels to high glucose for up to 4 h altered neither angiotensin II–stimulated PGI_2 nor $O_2^{\cdot -}$ release (data not shown), implying that intact endothelium was required for high-glucose–enhanced $O_2^{\cdot -}$ and PGIS nitration.

Supplementation of sepiapterin attenuates high-glucose–induced PGIS nitration. There is evidence that under conditions such as lack of its essential cofactor, tetrahydrobiopterin (BH_4), eNOS releases $O_2^{\cdot -}$ instead of NO (eNOS uncoupling) (31,32). Sepiapterin is a precursor of BH_4 and can be converted into BH_4 through a savage pathway (33,34). We next determined if supplementation of sepiapterin altered high-glucose–enhanced oxidant stress and PGIS nitration. As expected, concurrent administration of sepiapterin, which had no effect on angiotensin II–triggered vasorelaxation in BCAs exposed to 5 mmol/l glucose, not only abolished high-glucose–impaired vasorelaxation and PGI_2 release (Fig. 4*A*) but also prevented high-glucose–enhanced PGIS nitration (Fig. 4*B* and *C*). Together, these results indicated that high glucose, via eNOS, increased PGIS nitration by reducing the levels of BH_4, the essential cofactor of eNOS.

High glucose increases coimmunoprecipitation of caveolin-1, eNOS, and PGIS. We next investigated why eNOS-derived $ONOO^-$ targeted PGIS. PGIS is mainly localized in endoplasmic reticulum, but there is evidence that PGIS is colocalized with caveolin-1 within caveolae (35). We first examined if high glucose increased the translocation of PGIS from endoplasmic reticulum into caveolae, where eNOS is localized (36,37). Exposure of BCAs to either high glucose or chemically synthetized $ONOO^-$ (50 μmol/l) significantly increased the association of caveolin-1 with PGIS (Fig. 5*A* and *B*). The same blots were stripped and Western blotted against eNOS. As expected, both high glucose and $ONOO^-$ simultaneously increased the association of eNOS with caveolin-1 (Fig. 5*A* and *B*). These results indicated that $ONOO^-$ generated by high glucose increased the colocalization of both PGIS and eNOS within caveolae.

We next studied the effects of sepiapterin on high-glucose–enhanced association of eNOS with PGIS. BCAs were exposed to high glucose with or without sepiapterin. After incubation, eNOS was immunoprecipiated and Western blotted with either PGIS or eNOS. As expected, high glucose drastically increased the association of eNOS with PGIS (Fig. 5*C* and *D*). Interestingly, concurrent administration of sepiapterin attenuated high-glucose–enhanced association of PGIS with eNOS (Fig. 5*C* and *D*).

eNOS-dependent tyrosine nitration of PGIS in STZ-induced diabetes. It was interesting to investigate if diabetes increased PGIS nitration in vivo and, if so, the contributions of eNOS-derived oxidants. To this end, three types of animals, mice overexpressing hSOD ($hSOD^{+/+}$, scavenging $O_2^{\cdot -}$ to prevent $ONOO^-$), eNOS-KO mice (lack of eNOS-derived NO to prevent $ONOO^-$), and their litter-

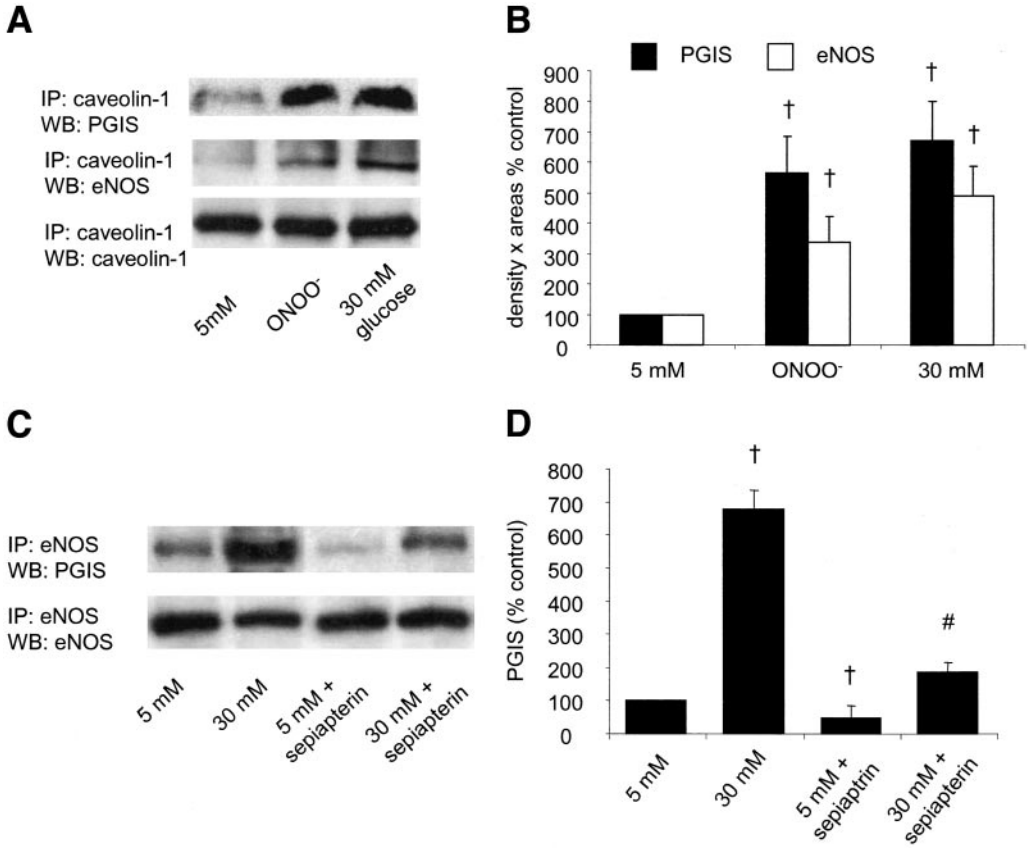

FIG. 5. Sepiapterin attenuates high-glucose–enhanced association of PGIS with eNOS with caveolin-1. *A*: High glucose increases the association of caveolin-1 with both eNOS and PGIS. Isolated BCAs were exposed to normal glucose (5 mmol/l, control), high glucose (30 mmol/l), or mannitol for 4 h. Caveolin-1 was immunoprecipitated (IP) by using the specific antibody and Western blotted (WB) using the specific antibodies. The blot is representative of three or four blots obtained from three or four independent experiments. *B*: Densitometric analysis (density × areas) of both PGIS and eNOS in the immunoprecipitates of caveolin-1. The results are expressed as means ± SD ($n = 3$; †$P < 0.05$, 5 mmol/l glucose vs. $ONOO^-$ or 30 mmol/l glucose). *C*: Supplementation of BH_4 attenuates high-glucose–enhanced association of eNOS and PGIS. After incubation, eNOS was immunoprecipitated and Western blotted using the specific antibodies as indicated. The blot is a representative of three blots obtained from three independent experiments. *D*: Densitometric analysis (density × areas) of eNOS-associated PGIS. The results are expressed as means ± SD ($n = 3$; †$P < 0.05$, 5 vs. 30 mmol/l glucose; #$P < 0.05$, 30 mmol/l glucose vs. 30 mmol/l glucose plus sepiapterin).

mates, C57BL6 mice, were made diabetic by STZ injections. Mice with $hSOD^{+/+}$ appeared resistant to STZ; therefore, a higher dose of STZ was given to $hSOD^{+/+}$ mice to achieve comparable levels of serum glucose. Two weeks after STZ injections, serum glucose levels in STZ-injected mice were significantly elevated compared with mice given vehicle (451 ± 31 vs. 129 ± 11 mg/dl for C57BL6, 437 ± 21 vs. 113 ± 8 mg/dl for $hSOD^{+/+}$, and 461 ± 34 vs. 135 ± 15 mg/dl for $eNOS^{-/-}$; $P < 0.001$, $n = 9$–11). The levels of serum glucose were not significantly different in all STZ-injected mice. In all three groups, the body weights of diabetic animals were significantly lower (11%) than those of nondiabetic controls (17.0 ± 0.6 vs. 19.1 ± 1.1; $P < 0.05$, $n = 10$). The diabetic animals had similar levels in heart weight (72.4 ± 3.1 vs. 71.1 ± 0.9 mg) compared with untreated diabetic mice.

As shown in Fig. 6*A*, diabetes increased $O_2^{\cdot -}$ in isolated arteries from diabetic mice. $O_2^{\cdot -}$ release was significantly elevated in diabetic C57BL6 mice aortas compared with those in the sham-treated mice (Fig. 6*A*). In hSOD mice, the basal aortic $O_2^{\cdot -}$ release was significantly lower than those in C57BL6 mice (Fig. 6*A*). Basal aortic $O_2^{\cdot -}$ was elevated in $eNOS^{-/-}$ mice compared with C57BL6 mice, which might be due to lack of NO. Interestingly, diabetes increased $O_2^{\cdot -}$ levels in $hSOD^{+/+}$ mice to a much less significant extent than in diabetic C57BL6 mice (Fig. 6*A*). Unlike in C57BL6 mice, diabetes did not increase $O_2^{\cdot -}$ in $eNOS^{-/-}$ mice. $O_2^{\cdot -}$ release in diabetic $eNOS^{-/-}$ mice was significantly less than that in diabetic C57BL6 mice (Fig. 6*A*).

Concomitant production of both $O_2^{\cdot -}$ and NO results in the formation of reactive nitrogen such as $ONOO^-$. Since $ONOO^-$ has a half-life of <1 s at physiological conditions, we next assayed the contents of 3-nitrotyrosine in mouse aortic homogenates, a footprint of reactive nitrogen species including $ONOO^-$. As shown in Fig. 6*B*, diabetes significantly increased the levels of 3-nitrotyrosine in C57BL6 but significantly less in both $eNOS^{-/-}$ and $SOD^{+/+}$ mice. Since neither NO nor $O_2^{\cdot -}$ alone causes 3-nitrotyrosine formation in vivo, these results implied that diabetes enhances 3-nitrotyrosine, likely via the formation of $ONOO^-$.

We next determined if $ONOO^-$ formed in diabetes altered PGIS activity by nitrating the enzyme. As shown in Fig. 7*A*, diabetes significantly suppressed PGIS activity, whereas overexpression of either hSOD or $eNOS^{-/-}$ prevented diabetes-induced PGIS inhibition. In parallel, diabetes drastically increased PGIS nitration (Fig. 7*B* and *C*) in STZ-injected C57BL6 mice but to a lesser extent in mice either lacking $eNOS^{-/-}$ or overexpressing hSOD (Fig. 7*B* and *C*). These results further support the notion that

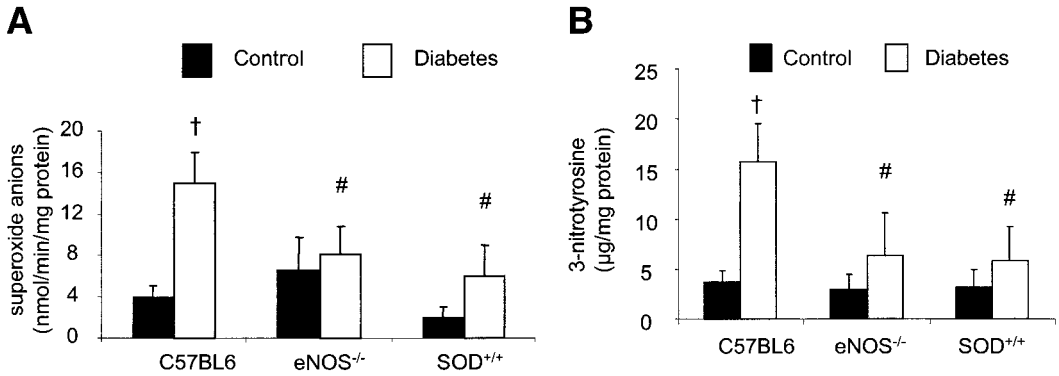

FIG. 6. Increased detection of superoxide and 3-nitrotyrosine in diabetes. *A*: Diabetes increases the detection of $O_2^{\cdot -}$. $O_2^{\cdot -}$ was detected as described in RESEARCH DESIGN AND METHODS. The results are expressed as means ± SD (n = 9; †P < 0.05, control C57BL6 vs. diabetic C57BL6; #P < 0.05, diabetic C57BL6 vs. diabetic hSOD$^{+/+}$ or eNOS$^{-/-}$). *B*: Diabetes increases the formation of 3-nitrotyrosine. 3-nitrotyrosine was detected using high-performance liquid chromatography as described in RESEARCH DESIGN AND METHODS. The results are expressed as means ± SD (n = 9; †P < 0.05, control C57BL6 vs. diabetic C57BL6; #P < 0.05, diabetic C57BL6 vs. diabetic hSOD$^{+/+}$ or diabetic eNOS$^{-/-}$).

diabetes increases PGIS nitration in vivo, likely via $ONOO^-$.

DISCUSSION

In the present study, we have, for the first time, demonstrated eNOS-dependent PGIS nitration in isolated BCAs when stimulated with angiotensin II in diabetes in vivo. The evidence can be summarized as follows. First, high glucose significantly impaired PGI_2-dependent relaxation in isolated BCAs and increased $O_2^{\cdot -}$ release after a 1- to 4-h exposure. In addition, high glucose, but not mannitol, significantly increased PGIS nitration, although the levels of PGIS expression were not changed. Second, removal of endothelium (depletion of eNOS) prevented high-glucose–induced $O_2^{\cdot -}$ release and PGIS nitration, suggesting that the oxidant was mainly from vascular endothelium. Third, our previous studies (22–24) have demonstrated that this enzyme is tyrosine nitrated by low concentrations of exogenous $ONOO^-$. In this study, we found that either inhibition of eNOS with L-NAME or scavenging $O_2^{\cdot -}$ with SOD attenuated high-glucose–enhanced PGIS nitration, which implied that reactive nitrogen species, likely $ONOO^-$, are involved in high-glucose–enhanced PGIS nitration. Fourth, inhibition of eNOS by L-NAME abolished

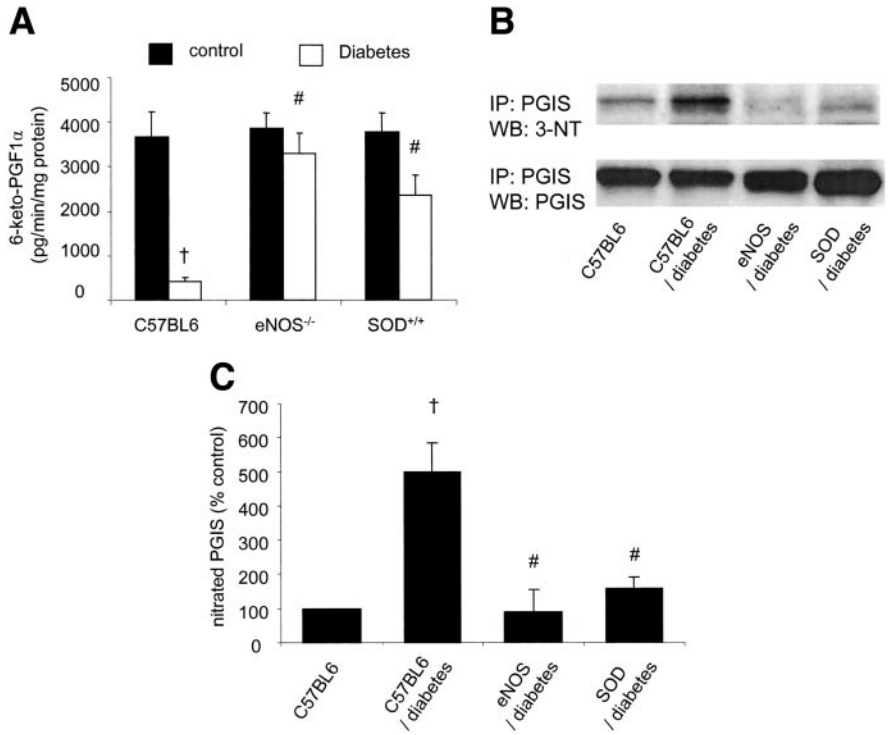

FIG. 7. $ONOO^-$-dependent tyrosine nitrated PGIS in diabetes in vivo. *A*: Either overexpression of hSOD or eNOS deficiency attenuates diabetes-induced PGIS inhibition in diabetes. PGIS activity in the aortic homogenates was assayed as described in RESEARCH DESIGN AND METHODS using the conversion of its substrate PGH_2. The results are expressed as means ± SD (n = 9; †P < 0.05, C57BL6 vs. diabetic C57BL6; #P < 0.05, diabetic C57BL6 vs. diabetic hSOD$^{+/+}$ or diabetic eNOS-KO). *B*: Either overexpression of hSOD or lack of eNOS attenuates diabetes-enhanced PGIS nitration. The results are expressed as means ± SD (n = 9; †P < 0.05, C57BL6 vs. diabetic C57BL6; #P < 0.05, diabetic C57BL6 vs. diabetic hSOD$^{+/+}$ or diabetic eNOS-KO). 3-NT, 3-nitrotyrosine. *C*: Densitometric analysis (density × areas) of nitrated PGIS caused by diabetes. The results are expressed as means ± SD (n = 9; †P < 0.05, C57BL6 vs. C57BL6 diabetic; #P < 0.05, C57BL6 diabetic vs. diabetic hSOD$^{+/+}$ or diabetic eNOS-KO).

both high-glucose–enhanced $O_2^{\cdot -}$ and PGIS nitration, suggesting that eNOS might be a source of $ONOO^-$. Fifth, sepiapterin restored PGI_2-dependent relaxation by preventing high-glucose–enhanced PGIS nitration. This lends support to eNOS becoming the source of $O_2^{\cdot -}$ and $ONOO^-$ in BCAs exposed to high glucose. Finally, using STZ-induced diabetic mice, we found that both diabetic $eNOS^{-/-}$ mice and $hSOD^{+/+}$ mice exhibited less PGIS nitration and released less $O_2^{\cdot -}$ compared with their diabetic littermates. Conversely, PGIS activity was significantly preserved in both $eNOS^{-/-}$ and $hSOD^{+/+}$ mice. Thus, tyrosine nitration of PGIS is most likely mediated by $ONOO^-$ formed endogenously from NO and $O_2^{\cdot -}$ in diabetes. Although peroxidases such as myeloperoxidase have been reported to cause protein nitration in vivo (38,39), the role of peroxidase-catalyzed PGIS nitration is less likely, since the $hSOD^{+/+}$ mice significantly attenuated diabetes-enhanced PGIS nitration and inhibition. Thus, peroxidase-catalyzed PGIS nitration is less likely because overexpression of hSOD, which provides hydrogen peroxide (H_2O_2) to facilitate peroxidase-catalyzed 3-nitrotyrosine formation, should enhance instead of decrease PGIS nitration in STZ-injected mice. Although we cannot totally exclude the possibility of peroxidase-catalyzed PGIS nitration, our data strongly suggest that $ONOO^-$ is likely to be responsible for the increased PGIS nitration caused by diabetes in vivo.

Another important finding we have presented is that inactivation of PGIS in diabetes results in a consequent TPr activation through its cumulative substrate, PGH_2, on isolated vessels ex vivo (Fig. 1*D*). This consequent TPr stimulation triggers vasospasm. In addition, there is evidence that TPr causes platelet aggregation and pathological changes, such as increased apoptosis and abnormal expression of adhesion molecules in endothelial cells, that are opposed by PGI_2. Since TXA_2 promotes and PGI_2 prevents the initiation and progression of atherogenesis, our results unveil a novel mechanism by which diabetes causes atherosclerosis, i.e., diabetes generates uncontrolled $O_2^{\cdot -}$, resulting in an increased destruction of NO and a concomitant formation of a highly cytotoxic oxidant, $ONOO^-$, that triggers nitration and inhibition of PGIS and results in consequent TPr stimulation. Thus, the present study, for the first time, provides evidence that diabetes via $ONOO^-$ may contribute to the functional defects of the endothelium in pathological situations, not only by a lack of the vasorelaxants NO and PGI_2 but even more directly by causing accumulation of the proatherothrombotic PGH_2. Indeed, infusion of oxygen radical scavengers (SOD, vitamin C, deferoxamine, etc.) improves cold pressor–induced vasorelaxation and abolishes acetylcholine-induced paradoxical vasoconstriction, suggesting that inactivation of NO by reactive oxygen species and subsequent formation of $ONOO^-$ contribute to some extent to the abnormal vasomotion observed in diabetic patients (16–20). In addition, our results might also provide a potential explanation for the paradoxic effects of endothelium-dependent vasorelaxants, such as acetylcholine, that trigger vasoconstriction in human atherosclerotic arteries. Even individuals with significant atherosclerotic risk factors but without clinically manifested atherosclerosis have a decreased vasodilator response in parallel with higher production of vasoconstricting PGs. Such abnormal responses are normalized by inhibition of COX. Thus, the nitration of PGIS we have described in the present study may contribute to the initiation and progression of vascular complications in diabetes as a result of the downregulation of protective actions of both PGI_2 and NO and because nonmetabolized PGH_2 tips the balance toward platelet aggregation, atheroma accumulation, and thrombus formation. The mechanism of diabetes via $ONOO^-$ formation for which we have presented evidence may serve as an explanation for the observed endothelial dysfunction, since $ONOO^-$-dependent tyrosine nitration of PGIS helps to unify and explain several previously proposed pathogenic abnormalities in diabetes, including *1*) decreased availability of NO, *2*) decreased PGI_2, *3*) expression of vasoconstrictors PGH_2/TxA_2, and *4*) increased free radicals (Fig. 8).

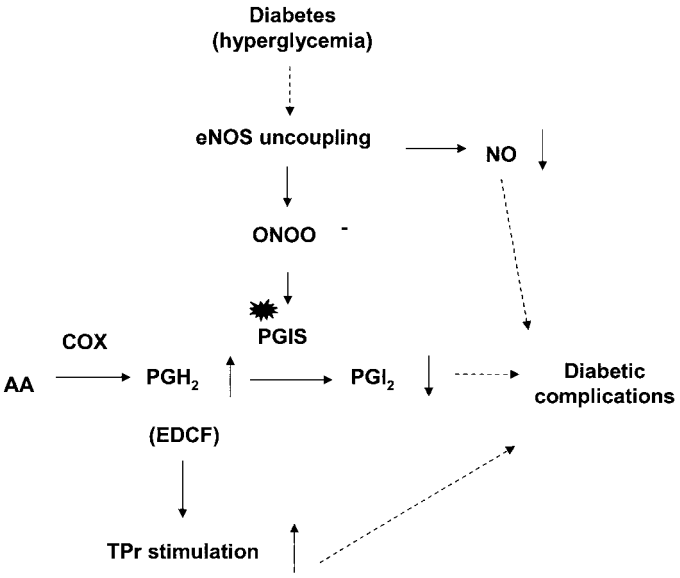

FIG. 8. Proposed mechanism for diabetes-induced PGIS nitration and its implications on the development of vascular complications in diabetes. AA, arachidonic acid.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (HL079584, HL07439, and HL080499) and a Research Award from the American Diabetes Association.

The authors acknowledge Dr. T. Klein for kindly providing an antibody against human PGIS. The also thank Melissa Guzman for editorial assistance.

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