

High Glucose via Peroxynitrite Causes Tyrosine Nitration and Inactivation of Prostacyclin Synthase That Is Associated With Thromboxane/Prostaglandin H₂ Receptor–Mediated Apoptosis and Adhesion Molecule Expression in Cultured Human Aortic Endothelial Cells

Ming-Hui Zou, Chaomei Shi, and Richard A. Cohen

Loss of the modulatory role of the endothelium may be a critical initial factor in the development of diabetic vascular diseases. Exposure of human aortic endothelial cells (HAECs) to high glucose (30 or 44 mmol/l) for 7–10 days significantly increased the release of superoxide anion in response to the calcium ionophore A23187. Nitrate, a breakdown product of peroxynitrite (ONOO⁻), was substantially increased in parallel with a decline in cyclic guanosine monophosphate (GMP). Using immunochemical techniques and high-performance liquid chromatography, an increase in tyrosine nitration of prostacyclin (PGI₂) synthase (PGIS) associated with a decrease in its activity was found in cells exposed to high glucose. Both the increase in tyrosine nitration and the decrease in PGIS activity were lessened by decreasing either nitric oxide or superoxide anion, suggesting that ONOO⁻ was responsible. Furthermore, SQ29548, a thromboxane/prostaglandin (PG) H₂ (TP) receptor antagonist, significantly reduced the increased endothelial cell apoptosis and the expression of soluble intercellular adhesion molecule-1 that occurred in cells exposed to high glucose, without affecting the decrease in PGIS activity. Thus, exposure of HAECs to high glucose increases formation of ONOO⁻, which causes tyrosine nitration and inhibition of PGIS. The shunting of arachidonic acid to the PGI₂ precursor PGH₂ or other eicosanoids likely results in TP receptor stimulation. These observations can explain several abnormalities in diabetes, including 1) increased free radicals, 2) decreased bioactivity of NO, 3) PGI₂ deficiency, and 4) increased vasoconstriction, endothelial apoptosis, and inflammation via TP receptor stimulation. *Diabetes* 51: 198–203, 2002

From the Vascular Biology Unit, Whitaker Cardiovascular Institute, Department of Medicine, Boston University School of Medicine, Boston, Massachusetts.

Address correspondence and reprint requests to Ming-Hui Zou, MD, PhD, Vascular Biology Unit X708, Boston University School of Medicine, 650 Albany St., Boston, MA 02118. E-mail: mhzou@medicine.bu.edu.

Received for publication 10 May 2001 and accepted in revised form 12 October 2001.

ELISA, enzyme-linked immunosorbent assay; GMP, guanosine monophosphate; HAEC, human aortic endothelial cell; L-NAME, N^G-monomethyl-L-arginine methyl ester; NO, nitric oxide; ONOO⁻, peroxynitrite; PBS, phosphate-buffered saline; PG, prostaglandin; PGI₂, prostacyclin; PGIS, PGI₂ synthase; sICAM, soluble intercellular adhesion molecule; SOD, superoxide dismutase; TP, thromboxane/PGH₂; TxA₂, thromboxane.

Macro- and microvascular diseases are the principal causes of morbidity and mortality in patients with type I and II diabetes. Diabetic vascular dysfunction is a major clinical problem that predisposes patients to a variety of cardiovascular diseases (1–3). Hyperglycemic episodes, which complicate even well-controlled cases of diabetes, are closely associated with the development of vascular disease (1–3). Endothelial dysfunction, as evidenced by the increased release of free radicals, diminished activity of nitric oxide (NO), decreased release of prostacyclin (PGI₂), and enhanced endothelial production of thromboxane/prostaglandin H₂ (TxA₂/PGH₂, or TP) precedes the structural vascular alterations (1–3). These pathogenic events affect micro- and macrovasculature and are observed in animal models of diabetes and in the coronary and peripheral circulation in patients with type 1 and type 2 diabetes (1–3). However, the underlying mechanisms remain poorly understood.

Recent evidence indicates that the endothelial dysfunction associated with diabetes (or, in in vitro studies, in response to high glucose) is the local formation of free radicals and oxidants (2–4). This hypothesis is supported by evidence that many biochemical pathways associated with hyperglycemia (e.g., glucose auto-oxidation, polyol pathway, eicosanoid synthesis, and protein glycation) can increase the production of superoxide anion (O₂⁻) (2–5). However, O₂⁻ itself is chemically inert, but when it combines with NO at a diffusion-limited rate, it becomes a highly reactive species, peroxynitrite (ONOO⁻) (6–8). ONOO⁻ can initiate both nitrosative and oxidative reactions in vitro and in vivo with proteins, lipids, and DNA. A characteristic reaction of ONOO⁻ is the nitration of protein-bound tyrosine residues (6–8).

The capacity of blood vessels to generate PGI₂ is essential to the integrity of the endothelium (9). PGI₂ is generated by PGIS from the PG endoperoxide PGH₂, which is generated by cyclooxygenase, the rate-limiting step for PGI₂ release (9). The inactivation of PGIS causes an accumulation of its precursor, PGH₂, which activates TP receptors (10). The activation of TP receptors triggers vasoconstriction (10,11), platelet aggregation (11), in-

creased expression of leukocyte adhesion molecules (12,13), and apoptosis (14).

We have previously reported that ONOO^- , when given exogenously or when produced endogenously, is able to induce endothelial dysfunction via a mechanism that depends on the inhibition and nitration of PGIS (15,16). PGIS nitration has been observed in cytokine-treated cultured cells (17), in atherosclerotic lesions (18), and after ischemia-reperfusion (19). ONOO^- inactivates PGIS by heme-thiolate-catalyzed tyrosine nitration (16,20). As a result of the inactivation of the enzyme by exogenous ONOO^- and reduced production of PGL_2 , there is increased formation of vasoconstrictor prostanoids (15), similar to that observed in vasculature exposed in vitro to high glucose (2,3) or obtained from diabetic animals (21–23).

The present study shows that exposure of cultured human aortic endothelial cells (HAECs) to high glucose increases the release of $\text{O}_2^{\cdot-}$ and the production of ONOO^- , which results in tyrosine nitration of PGIS. This is associated with increased endothelial cell apoptosis and adhesion molecule expression, which is mediated via TP receptors. These results provide the first evidence that PGIS nitration is of pathophysiological importance in the development of diabetic endothelial dysfunction, leading to increased TP receptor activation, endothelial cell apoptosis, and adhesion molecule expression.

RESEARCH DESIGN AND METHODS

Both poly- and monoclonal antibodies against 3-nitrotyrosine were bought from Upstate Biotechnologies (Lake Placid, NY). Dialyzed pronase E was from Boehringer Mannheim (Indianapolis, IN). L-Glucose, mannitol, calcium ionophore A23187, cytochrome c, superoxide dismutase (SOD), catalase, tyrosine, 3-nitrotyrosine, nitrate reductase, and pig gelatin were obtained from Sigma (St. Louis, MO). Monoclonal antibodies against PGIS and PGH_2 as well as enzyme-linked immunosorbent assay (ELISA) kits to measure 6-keto-PGF 1α , and cyclic guanosine monophosphate (GMP) were obtained from Cayman Chemicals (Ann Arbor, MI). Human soluble intercellular adhesion molecules (sICAMs) ELISA kits were bought from R&D systems (Minneapolis, MN). Cell culture media were obtained from Gibco BRL (Grand Island, NY). Other chemicals and organic solvents of highest grade were obtained from local suppliers.

Cell culture. HAECs (American Type Culture Collection, Rockville, MD) were cultured in Ham's F12 medium, 10% fetal bovine serum, 100 $\mu\text{g}/\text{ml}$ heparin, and 30 $\mu\text{g}/\text{ml}$ endothelial cell growth supplement. Cells were seeded in 60 \times 15-mm culture dishes precoated with 0.1% pig gelatin. When they reached confluence, the cells were maintained in 1% fetal calf serum and exposed to normal glucose (5.5 mmol/l) or high glucose (30 or 44 mmol/l) for 7 days, during which the medium was changed every 2 days. Control groups (to account for media hyperosmolarity) were exposed to mannitol (24.5 or 39.5 mmol/l) in normal medium containing glucose (5.5 mmol/l). After incubation, the media were collected and assays were conducted as described below. For measurements of $\text{O}_2^{\cdot-}$ and cyclic GMP, the growth media was replaced by phosphate-buffered saline (PBS) containing no glucose. Unless otherwise noted, these measurements were performed on cells or the PBS bathing cells, which were stimulated by the calcium ionophore A23187 (10^{-5} mol/l, 2 h).

Detection of $\text{O}_2^{\cdot-}$ by SOD-inhibitable cytochrome c reduction. The release of $\text{O}_2^{\cdot-}$ was assayed by the SOD-inhibitable cytochrome c reduction assay, as described previously (24,25). Cytochrome c was present throughout the 2-h incubation of cells stimulated with A23187.

Determination of NO_x . The level of nitrite (NO_2^-) and nitrate (NO_3^-) in the culture medium exposed to cells during the last 2 days of the exposure period were measured by using the Griess reaction. For total NO_x , NO_3^- was first converted to NO_2^- by adding nitrate reductase.

Determination of cyclic GMP. After incubation in the presence of A23187 for 2 h in PBS, cells were quickly scraped into PBS and sonicated twice on ice. The supernatants were immediately separated by centrifugation at 10,000g for 5 min at 4°C and assayed for cyclic GMP by using ELISA kits according to the instructions provided by the supplier.

Assay of PGIS activity and endogenous 6-keto-PGF 1α release. PGIS activity was assayed by incubating cells with PGH_2 (10^{-5} mol/l for 3 min), and PGIS activity was assessed by levels of 6-keto-PGF 1α in the cell supernatant. In addition, for assessment of endogenous PGL_2 formation, levels of 6-keto-PGF 1α were measured in cells stimulated with the calcium ionophore 23187 (10^{-5} mol/l for 2 h) as described previously (10,15–19).

Immunoprecipitation and Western blot analysis. Nitration of PGIS in cell extracts (0.5 mg protein) was identified by immunoprecipitation and Western blotting, as previously described (16–19). The corresponding bands in Western blots were calculated by using a phospho-imaging system (Bio-Rad) as the integrated values from the area \times density. The value obtained for protein from cells exposed to 5 mmol/l glucose were taken as 100%.

Protein hydrolysis. After incubation, the cells were scraped into ice-cold 0.1 mol/l sodium acetate (pH 7.2) and sonicated twice on ice after being washed twice with ice-cold PBS (pH 7.4). The samples were hydrolyzed with dialyzed pronase E in the presence of 10 mmol/l CaCl_2 for 60–72 h at 55°C, as described previously (26).

HPLC-electrochemical/UV detection of 3-nitrotyrosine. A Varian Prostar pump (Walnut Creek, CA) equipped with a reverse-phase column (25 \times 0.46 cm i.d., Ultrasphere 5- μm octyldecylsulfate column) was used under isocratic conditions with 50 mmol/l aqueous sodium acetate (pH 3.1) containing 10% methanol at a flow rate of 1 ml/min, which was continually sparged with helium. After optimization of the electrochemical detector (Varian Star 9080) conditions by hydrodynamic voltammetry, the oxidation potential for 3-nitrotyrosine was set at 0.85 mV, and the absorption was measured at 365 nm with a photodiode array detector (Varian). 3-Nitrotyrosine was quantified by its absorption at 365 nm, by its electrochemical detector response, and by co-elution with 3-nitrotyrosine standard. 3-Nitrotyrosine in samples was completely eliminated by reduction with sodium dithionite, as expected.

Measurement of sICAMs. After incubation, the culture media from the last 2 days of the exposure period were collected. The levels of sICAMs in culture media were measured by using ELISA kits, as instructed by the provider.

Quantitative DNA fragmentation analysis. Endothelial cell DNA fragmentation was assayed with the diphenylamine assay, as reported elsewhere (27). The percentage of DNA fragmentation was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet.

Statistics. Results are expressed per milligram protein, as measured by the Bio-Rad protein assay kits in cells scraped from the culture dishes after each experiment. One-way analysis of variance and Student's *t* tests were used where appropriate, and *P* < 0.05 was regarded as significant.

RESULTS

High glucose increases the endothelial cell release of $\text{O}_2^{\cdot-}$. As shown in Fig. 1A, exposure of HAECs to high glucose (30 mmol/l) significantly increased the release of $\text{O}_2^{\cdot-}$ in the cells stimulated with the calcium ionophore A23187. Mannitol (30 mmol/l) did not mimic the effects of high glucose.

High glucose increases NO_3^- , a breakdown product of ONOO^- . As shown in Fig. 1B, the release of total NO_x (NO_2^- and NO_3^-) increased modestly in the culture media collected from the last 2 days from cells exposed to high glucose. However, the increased release of NO_x was largely caused by an increased formation of NO_3^- , a breakdown product of ONOO^- (7,8). As a result, the $\text{NO}_3^-/\text{NO}_2^-$ ratios were significantly increased in high glucose-exposed cells, suggesting an increased formation of ONOO^- in cells exposed to high glucose.

High glucose decreases cyclic GMP. Compared with NO, ONOO^- is a much less potent activator of guanylyl cyclase (28), thus the formation of ONOO^- decreases cyclic GMP. As expected, cells cultured in high glucose and then exposed to PBS containing A23187 showed a significant decrease in cyclic GMP formation compared with cells exposed to control glucose or to high mannitol, consistent with reduced NO bioactivity in cells exposed to high glucose (Fig. 1C).

High glucose inhibits PGIS activity. Further studies were conducted to determine whether the increased production of ONOO^- in HAECs exposed to high glucose

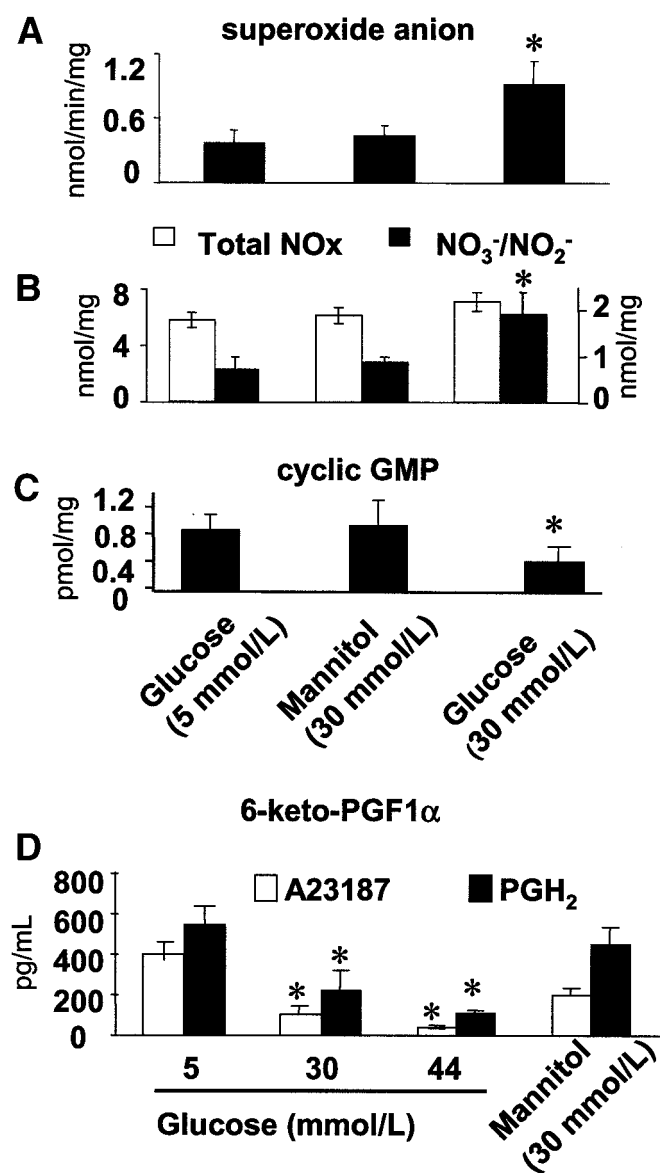


FIG. 1. High glucose increases the release of $O_2^{\cdot-}$ and NO_3^- but decreases cyclic GMP and PGI_2 . HAECs were exposed to high glucose (30 and 44 mmol/l), normal glucose (5 mmol/l), or mannitol (25 mmol/l plus 5 mmol/l glucose) for 7 days. **A:** Increased $O_2^{\cdot-}$ release in cells exposed to high glucose ($n = 12$). $O_2^{\cdot-}$ was assayed by SOD-inhibitable cytochrome c reduction in cells stimulated with A23187 for 2 h following culture in various glucose concentrations. **B:** High glucose increases total NO_x ($n = 18$) and NO_3^- ($n = 14$) measured in the media collected from the last 2 days in culture. NO_3^- was first converted into NO_2^- by nitrate reductase, and then NO_2^- was assayed as described in RESEARCH DESIGN AND METHODS. **C:** Decreased cyclic GMP levels in HAECs cultured in high glucose ($n = 12$). Cyclic GMP is expressed as 0.1 pmol/mg protein. **D:** High glucose inhibits PGI_2 release and PGIS activity. High glucose significantly decreased levels of 6-keto- $PGF_{1\alpha}$ released into the supernatant during 2-h stimulation with A23187 and PGIS activity, as determined by levels of 6-keto- $PGF_{1\alpha}$ measured after exposing cells to PGH_2 for 3 min ($n = 12$). 6-Keto- $PGF_{1\alpha}$ is expressed as picograms per milligram protein. Asterisks indicate a significant difference from control with 30 or 44 mmol/l glucose. There were no significant effects of mannitol (25 mmol/l) plus glucose (5 mmol/l).

inhibited PGIS activity. As shown in Fig. 1D, high glucose significantly inhibited calcium ionophore-stimulated endogenous PGI_2 release (as measured by levels of its degradation product 6-keto- $PGF_{1\alpha}$) as well as PGIS activity (as measured by the conversion of exogenous PGH_2

into 6-keto- $PGF_{1\alpha}$). In contrast, mannitol did not affect A23187-stimulated PGI_2 release or cellular PGIS activity. **High-glucose tyrosine nitrates PGIS.** As shown in Fig. 2A, growth in high glucose increased PGIS immunoprecipitates obtained with antibodies against 3-nitrotyrosine (Fig. 2A). In addition, increased 3-nitrotyrosine was also observed in Western blots of immunoprecipitates obtained with PGIS antibodies (Fig. 2B). Because high glucose did not affect the expression of PGIS (data not shown), these results can only be explained by increased tyrosine nitration of PGIS in cells exposed to high glucose. Exposure of HAECs to mannitol was not associated with nitration of PGIS (Fig. 2A and B).

We further used HPLC-electrochemical/UV detection to confirm the identity of and quantify 3-nitrotyrosine. Homogenates of HAECs or immunoprecipitates obtained with anti-PGIS antibody were digested with pronase E,

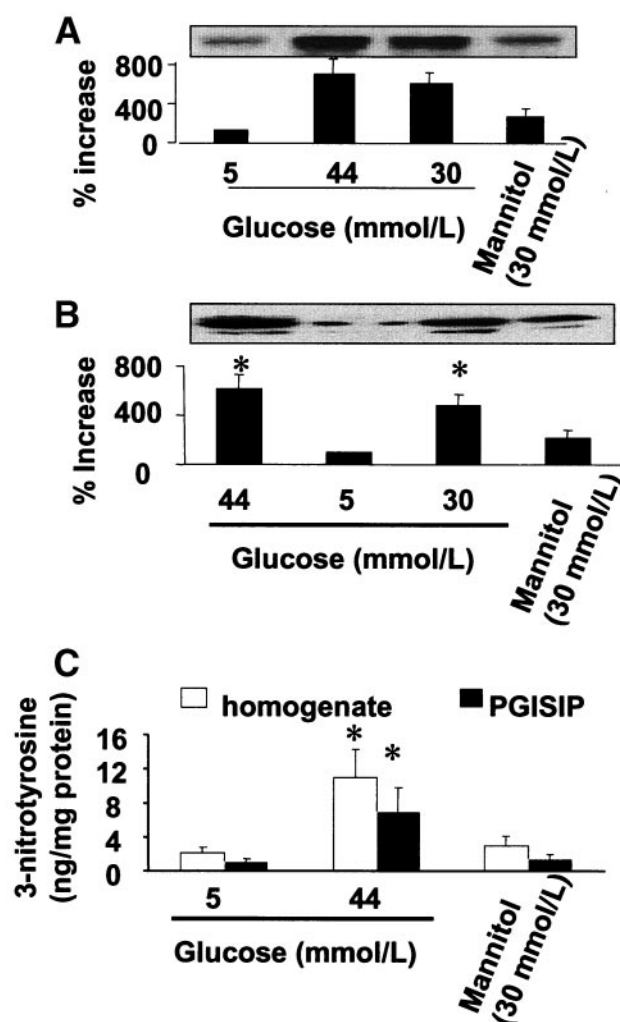


FIG. 2. Tyrosine nitration of PGIS in cells exposed to high glucose. **A:** Immunoprecipitation with 3-nitrotyrosine (3-NT) antibody, Western-blotted with PGIS antibody ($n = 4$). **B:** Immunoprecipitation with PGIS antibody, Western-blotted with 3-NT antibody ($n = 5$). Immunoprecipitation, Western blots, and density analysis were performed as described in RESEARCH DESIGN AND METHODS. **C:** Identification of PGIS as a main 3-nitrotyrosine-positive protein in cells exposed to high glucose. Cells exposed to high glucose had increased 3-nitrotyrosine content detected both in homogenates ($n = 8$) and in PGIS immunoprecipitates ($n = 6$). *Significant difference from control with 30 or 44 mmol/l glucose. There were no significant effects of mannitol (25 mmol/l) plus glucose (5 mmol/l).

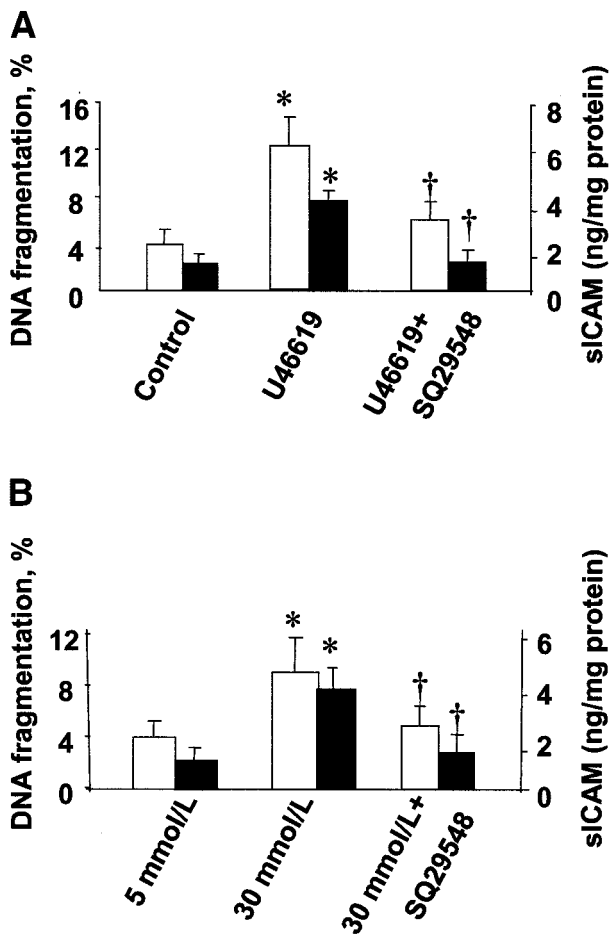


FIG. 3. High glucose increases endothelial cell apoptosis and sICAM expression via TP receptors. **A:** Activation of TP receptors increases endothelial cell DNA fragmentation and sICAM shedding. HAECs were exposed to U46619 (10 μ mol/l) for 18 h in the absence or presence of SQ29548 (10 μ mol/l), a selective TP receptor blocker. *Significant difference between control and U46619. †Significant difference between U46619 and U46619 plus SQ29548 ($n = 12$). **B:** SQ29548 reduces increased apoptosis and sICAM shedding in HAECs exposed to high glucose. HAECs were exposed to high glucose (30 mmol/l) in the absence or presence of SQ29548 (10 μ mol/l) ($n = 12$). Asterisks indicate a significant difference between 5 and 30 mmol/l glucose ($n = 15$). No significant effects of mannitol (25 mmol/l) plus glucose (5 mmol/l). †Significant difference between 30 mmol/l and 30 mmol/l plus SQ29548 ($n = 12$). □, Apoptosis; ■, sICAMs.

and 3-nitrotyrosine was identified by co-elution with standard 3-nitrotyrosine and its sensitivity to reduction by sodium dithionite. As shown in Fig. 2C, exposure to high glucose (44 mmol/l) significantly increased 3-nitrotyrosine content in both HAEC homogenates and immunoprecipitates obtained with PGIS antibodies. The data in Fig. 2C also indicate that 3-nitrotyrosine detected in cell homogenates was largely derived from nitrated PGIS, indicating that PGIS is the main target of ONOO⁻ in cells exposed to high glucose.

Inhibition of PGIS activity is associated with increased apoptosis and sICAM shedding mediated by TP receptors. As shown in Fig. 3A, U46619, a TP-receptor agonist, significantly increased endothelial cell DNA fragmentation and sICAM shedding. The effect of U46619 was antagonized by SQ29548, a selective TP receptor antagonist. Figure 3B shows that increased endothelial cell apoptosis and sICAM shedding occurred in cells exposed

to high glucose. SQ29548 partially blocked these effects of high glucose. These results indicate that growth in high glucose, likely via inhibition of PGIS and consequent shunting to its substrate, PGH₂, or other eicosanoids activates TP receptors and causes endothelial cell apoptosis and sICAM expression.

If tyrosine nitration is responsible for inactivation of PGIS, and this in turn is responsible for TP receptor stimulation, then preventing PGIS nitration should also prevent TP receptor stimulation. Accordingly, studies were done to determine the effects of scavenging O₂^{•-} or inhibiting the synthesis of NO, either of which should decrease ONOO⁻ formation. Figure 4A shows that treatment of HAECs with polyethylene glycol-conjugated SOD (300 units/ml) throughout the exposure to high glucose decreased the nitrotyrosine immunoreactive protein in immunoprecipitates obtained with anti-PGIS antibodies. N^G-monomethyl-L-arginine methyl ester (L-NAME; 0.5 mmol/l) also decreased tyrosine nitration of PGIS, but there was no apparent effect of the xanthine oxidase inhibitor, oxypurinol (0.01 mmol/l).

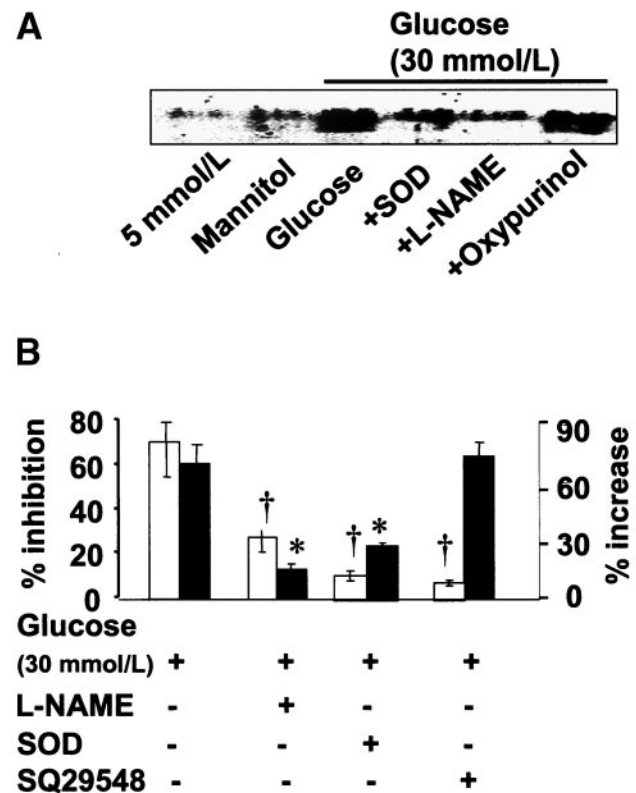


FIG. 4. SOD or L-NAME decreased tyrosine nitration and inactivation of PGIS as well as increased sICAM expression in HAECs exposed to high glucose. **A:** PGIS immunoprecipitates were stained with anti-3-nitrotyrosine antibodies. HAECs exposed to high glucose and treated with polyethylene glycol-SOD (300 units/ml) or L-NAME (0.5 mmol/l) had decreased amounts of 3-nitrotyrosine-positive protein detected. Similar blots were obtained in four separate experiments. **B:** PGIS activity, as determined by the concentration of 6-keto-PGF_{1 α} present in the media obtained during the last 2 days of culture of HAECs, was significantly decreased by ~60% in HAECs exposed to high glucose. *The inhibition was significantly lower in HAECs exposed to high glucose and treated with either SOD or L-NAME, but inhibition was not significantly lower with SQ29548 (10⁻⁵ mol/l). †The levels of sICAMs were significantly increased by ~80% in HAECs exposed to high glucose compared with cells exposed to 5 mmol/l glucose, but this increase was significantly lower in cells exposed to high glucose and treated with SOD, L-NAME, or SQ29548. ■, 6-Keto-PGF_{1 α} ; □, sICAMs.

Figure 4B shows that either SOD or L-NAME treatment also largely prevented the inhibition of PGIS activity in high glucose media, as reflected by 6-keto-PGF_{1α} levels measured in the media incubated with the cells during the last 2 days of culture. In addition, sICAM levels obtained in the same media from cells incubated in high glucose and treated with SOD or L-NAME increased significantly less than those incubated with high glucose alone. Note that treatment of cells with the TP antagonist SQ29548 (10⁻⁵ mol/l) had no significant effect on the inhibition of PGIS caused by high glucose, but it prevented the increase in sICAMs in HAECs exposed to high glucose.

DISCUSSION

Vascular cells are capable of generating ONOO⁻ because of their capacity to simultaneously release O₂^{•-} and NO. Previous studies have demonstrated that high glucose triggers O₂^{•-} formation in vascular cells (4,24,25,29). Potential sources of this O₂^{•-} could be NAD(P)H oxidases (29), xanthine oxidase (2), mitochondria (4), or NO synthase (30). In the present study, exposure of HAECs to high glucose significantly increased the release of O₂^{•-}, as previously reported (24,25). The reaction of NO and O₂^{•-} results in ONOO⁻, which is much less potent than NO in stimulating guanylyl cyclase (28). Indeed, cyclic GMP content was significantly decreased in HAECs exposed to high glucose, suggesting increased inactivation of NO by O₂^{•-}. Levels of NO₃⁻, a stable breakdown product of ONOO⁻ (7–9), were significantly increased, whereas those of NO₂⁻, a product of the reaction of NO with molecular oxygen, declined. This indicated that a major change in the fate of NO occurred in HAECs exposed to high glucose, characterized by its rapid reaction with O₂^{•-} to produce ONOO⁻. These results suggest that an increase of O₂^{•-} and ONOO⁻ explains the decrease in NO bioactivity in cells exposed to high glucose.

Convincing evidence for ONOO⁻ formation in cells exposed to high glucose comes from the identification of tyrosine-nitrated PGIS. Although other pathways of tyrosine nitration have been also suggested (31), 3-nitrotyrosine is regarded as the “footprint” of ONOO⁻ formation *in vivo*. Here, we examined whether tyrosine nitration of PGIS occurred because this enzyme is tyrosine nitrated by low concentrations of ONOO⁻ (15–17). Increased staining with an antibody against 3-nitrotyrosine was found in the immunoprecipitates obtained with antibodies against PGIS in cells exposed to high glucose, although the levels of PGIS expression were not changed. Tyrosine nitration of PGIS was further established by finding that PGIS-positive staining was concomitantly present in the immunoprecipitates obtained with antibody against 3-nitrotyrosine. Moreover, a substantially increased 3-nitrotyrosine content measured by HPLC was found both in cell homogenates and in immunoprecipitates obtained with PGIS antibodies from cells grown in high-glucose media. Remarkably, 3-nitrotyrosine in PGIS was the main component of 3-nitrotyrosine detected in cells exposed to high glucose. Indeed, Western blots with a polyclonal antibody against 3-nitrotyrosine of the immunoprecipitates obtained with a monoclonal antibody against 3-nitrotyrosine yielded a single major band with the molecular weight of

PGIS (data not shown), further indicating that 3-nitrotyrosine mainly originated from PGIS.

In previous studies, we demonstrated that nitration of PGIS with exogenous ONOO⁻ inactivates the enzyme. In the present study, PGIS nitration in HAECs exposed to high glucose was accompanied by inactivation of the enzyme. The tyrosine nitration of PGIS and the decrease in its activity was in large part prevented by treating the cells with either SOD or L-NAME, a finding consistent with the proposal that ONOO⁻, produced in HAECs exposed to high glucose from O₂^{•-} and NO, tyrosine-nitrates and inactivates PGIS.

Inactivation of PGIS results in the accumulation of PGH₂ within vascular tissues and vasoconstriction via the TP receptor, a finding common to blood vessels in diabetes (2,10). Activation of TP receptors can trigger platelet aggregation, thrombus, vasospasm, cell apoptosis, and expression of adhesion molecules. We further addressed whether activation of TP receptors occurred as a consequence of PGIS inactivation and contributed to endothelial cell apoptosis and adhesion molecule expression in HAECs exposed to high glucose. In line with the results obtained with TP receptor agonists (12,14), activation of TP receptors by U46619 significantly increased endothelial apoptosis and shedding of sICAMs from cells into the media. The effect of U46619 on apoptosis and sICAMs was antagonized by SQ29548, indicating that the TP receptor mediated these effects. Furthermore, SQ29548 significantly inhibited the increased sICAM and endothelial apoptosis found to occur in HAECs exposed to high glucose, indicating that activation of TP receptors was at least partially responsible. Whereas the role of TP receptors can be adequately explained by increased levels of the PGI₂ precursor PGH₂ as a result of PGIS inactivation, further studies will be necessary to determine whether increased formation of other eicosanoids that are reported to act upon TP receptors, such as 8-isoprostanes or 15-hydroxyeicosatetraenoic acid, might contribute to high glucose-induced apoptosis and expression of adhesion molecules (32).

Nitration of PGIS by ONOO⁻ likely represents an important mechanism for diabetic vascular complications. Recent evidence demonstrates intense 3-nitrotyrosine staining in vascular endothelium and villous stroma of placentas from diabetic patients when compared with normal control subjects (33). The intimal localization of 3-nitrotyrosine staining indicates that endothelium-derived NO is inactivated by O₂^{•-} to produce the cytotoxic oxidant ONOO⁻. Although the identity of nitrated proteins in these tissues remains to be investigated, our preliminary results in diabetic apolipoprotein E-deficient mice suggest increased tyrosine nitration of PGIS *in vivo* (M.-H.Z., unpublished data).

In summary, exposure of HAECs to high glucose results in increased O₂^{•-}, ONOO⁻, and tyrosine nitration of PGI₂ synthase. Associated activation of TP receptors increases endothelial cell apoptosis and adhesion molecule expression. Oxidant stress and nitration of PGIS could contribute to the initiation and progression of vascular complications of diabetes by several mechanisms, including decreased protective actions of both NO and PGI₂, and because the increased TP receptor stimulation favors endothelial apo-

ptosis and expression of adhesion molecules. The evidence presented here that nitration of PGIS via ONOO⁻ occurs in endothelial cells exposed to high glucose helps to unify several previously proposed pathogenic vascular abnormalities in diabetes, including 1) decreased activity of NO, 2) decreased PGI₂, 3) vasoconstriction mediated by TP receptors, and 4) increased oxygen-derived free radicals.

ACKNOWLEDGMENTS

This work was supported by a grant from the American Heart Association and the Juvenile Diabetes Foundation International (JDF) and by a National Institutes of Health-JDF sponsored grant (PO1-HL96005-05). We are grateful to Dr. T. Klein for kindly providing an antibody against human PGIS.

REFERENCES

- Ruderman NB, Williamson JR, Brownlee M: Glucose and diabetic vascular disease. *FASEB J* 6:2905–2914, 1992
- Cohen RA: Dysfunction of the vascular endothelium in diabetes mellitus. *Circulation* 87:67–76, 1993
- Cosentino F, Lüscher TF: Endothelial dysfunction in diabetes mellitus. *J Cardiovasc Pharmacol* 32:S54–S61, 1998
- Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M: Normalizing mitochondrial superoxide production blocks three pathways of hyperglycemic damage. *Nature* 404:787–790, 2000
- Bucala R, Tracey KJ, Cerami A: Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. *J Clin Invest* 87:432–438, 1991
- Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA: Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A* 87:1620–1624, 1990
- Beckman JS, Chen J, Ischiropoulos H, Crow JP: Oxidative chemistry of peroxynitrite. *Methods Enzymol* 233:229–240, 1994
- Beckman JS, Koppenol WH: Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. *Am J Physiol* 271:C1424–C1437, 1996
- Moncada S, Vane JR: Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A₂ and prostacyclin. *Pharmacol Rev* 30:292–331, 1979
- Zou MH, Jendral M, Ullrich V: Peroxynitrite induces prostaglandin endoperoxide dependent-vasospasm in bovine coronary arteries via nitration of prostacyclin synthase. *Br J Pharmacol* 126:1283–1292, 1999
- Ogletree M, Harris DN, Greenberg R, Haslanger MF, Nakane M: Pharmacological actions of SQ29548, a novel selective thromboxane antagonist. *J Pharmacol Exp Ther* 234:435–441, 1985
- Ishizuka T, Kawakami M, Hidaka T, Matsuki Y, Takamizawa M, Suzuki K, Kurita A, Nakamura H: Stimulation with thromboxane A₂ (TxA₂) receptor agonist enhances ICAM-1, VCAM-1 or ELAM-1 expression by human vascular endothelial cells. *Clin Exp Immunol* 112:464–470, 1998
- Cayatte AJ, Du Y, Oliver-Krasinski J, Lavielle G, Verbeuren TJ, Cohen RA: The thromboxane receptor antagonist S18886 but not aspirin inhibits atherogenesis in apo E-deficient mice: evidence that eicosanoids other than thromboxane contribute to atherosclerosis. *Arterioscler Thromb Vasc Biol* 20:1724–1728, 2000
- Gao Y, Yokota R, Tang S, Ashton AW, Ware JA: Reversal of angiogenesis in vitro, induction of apoptosis, and inhibition of Akt phosphorylation in endothelial cells by thromboxane A₂. *Circ Res* 87:739–745, 2000
- Zou MH, Ullrich V: Peroxynitrite formed by simultaneous generation of nitric oxide and superoxide selectively inhibits bovine aortic prostacyclin synthase. *FEBS Lett* 382:101–104, 1996
- Zou MH, Martin C, Ullrich V: Tyrosine nitration as a mechanism of selective inactivation of prostacyclin synthase by peroxynitrite. *Biol Chem* 378:707–713, 1997
- Zou MH, Klein T, Pasquet JP, Ullrich V: Interleukin-1 β decreases prostacyclin synthase activity in rat mesangial cells via endogenous peroxynitrite. *Biochem J* 336:507–512, 1998
- Zou MH, Leist M, Ullrich V: Selective nitration of prostacyclin synthase and defective vasorelaxation in atherosclerotic bovine coronary arteries. *Am J Pathol* 154:1559–1565, 1999
- Zou MH, Bachschmid M: Hypoxia-reoxygenation causes coronary vasospasm via tyrosine nitration of prostacyclin synthase. *J Exp Med* 190:135–139, 1999
- Zou MH, Daiber A, Peterson JA, Shoun H, Ullrich V: Rapid reactions of heme-thiolate proteins with peroxynitrite as a basis for the inhibition of prostacyclin synthase nitration. *Arch Biochem Biophys* 376:149–155, 2000
- Shimizu K, Muramatsu M, Kakegawa Y, Asano H, Toki Y, Miyazaki Y, Okumura K, Hashimoto H, Ito T: Role of prostaglandin H₂ as an endothelium-derived contracting factor in diabetic state. *Diabetes* 42:1246–1252, 1993
- Dai FX, Diederich A, Skopec J, Diederich D: Diabetes-induced endothelial dysfunction in streptozotocin-treated rats: role of prostaglandin endoperoxides and free radicals. *J Am Soc Nephrol* 4:1327–1336, 1993
- Tesfamariam B, Jakubowski JA, Cohen RA: Contraction of diabetic rabbit aorta caused by endothelium-derived PGH₂-TxA₂. *Am J Physiol* 257:H1327–H1333, 1992
- Cosentino F, Hishikawa K, Katusic ZS, Lüscher TF: High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. *Circulation* 96:25–28, 1997
- Graier WF, Simecek S, Kukovetz WR, Kostner GM: High D-glucose-induced changes in endothelial Ca²⁺/EDRF signaling are due to generation of superoxide anions. *Diabetes* 45:1386–1395, 1996
- Sodum RS, Akerkar SA, Fiala ES: Determination of 3-nitrotyrosine by high-pressure liquid chromatography with a dual-mode electrochemical detector. *Anal Biochem* 280:278–285, 2000
- von Knethen A, Callsen D, Brune B: Superoxide attenuates macrophage apoptosis by NF- κ B and AP-1 activation that promotes cyclooxygenase-2 expression. *J Immunol* 163:2858–2866, 1999
- Wolin MS, Davidson CA, Kaminski PM, Fayngersh RP, Mohazzab-H KM: Oxidant-nitric oxide signaling mechanisms in vascular tissue (Review). *Biochemistry (Mosc)* 63:810–816, 1998
- Griendling KK, Sorescu D, Ushio-Fukai M: NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 86:494–501, 2000
- Xia Y, Dawson VL, Dawson TM, Snyder SH, Zweier JL: Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. *Proc Natl Acad Sci U S A* 93:6770–6774, 1996
- Eiserich JP, Hristova M, Cross CE, Jones AD, Freeman BA, Halliwell B, van der Vliet A: Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 391:391–397, 1998
- Yura T, Fukunaga M, Khan R, Nassar GN, Badr KF, Montero A: Free-radical-generated F₂-isoprostane: stimulates cell proliferation and endothelin-1 expression on endothelial cells. *Kidney Int* 56:471–478, 1999
- Lyall F, Gibson JL, Greer IA, Brockman DE, Eis ALW, Myatt L: Increased 3-nitrotyrosine: in the diabetic placenta: evidence for oxidative stress. *Diabetes Care* 21:1753–1758, 1998