High Glucose Causes Upregulation of Cyclooxygenase-2 and Alters Prostanoid Profile in Human Endothelial Cells

Role of Protein Kinase C and Reactive Oxygen Species

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**Background**—Prostaglandins generated by cyclooxygenase (COX) have been implicated in hyperglycemia-induced endothelial dysfunction. However, the role of individual COX isoenzymes as well as the molecular mechanisms linking oxidative stress and endothelial dysfunction in diabetes remains to be clarified.

**Methods and Results**—Human aortic endothelial cells were exposed to normal (5.5 mmol/L) and high (22.2 mmol/L) glucose. Glucose selectively increased mRNA and protein expression of COX-2. Its upregulation was associated with an increase of thromboxane A2 and a reduction of prostacyclin (PGI2) release. Glucose-induced activation of PKC resulted in the formation of peroxynitrite and tyrosine nitration of PGI2 synthase. NO release was reduced despite 2-fold increase of endothelial NO synthase expression. Phorbol ester caused an increase of COX-2 and endothelial NO synthase expression similar to that elicited by glucose. These effects were prevented by the PKC inhibitor calphostin C. N-acetylcysteine, vitamin C, and calphostin C prevented ROS formation, restored NO release, and reduced colocalization of nitrotyrosine and PGI2 synthase.

**Conclusions**—Thus, high glucose, via PKC signaling, induces oxidative stress and upregulation of COX-2, resulting in reduced NO availability and altered prostanoid profile. (*Circulation*. 2003;107:1017-1023.)

Key Words: diabetes mellitus ■ nitric oxide ■ stress

Loss of the modulatory role of the endothelium may be a critical and initiating factor in the development of diabetic vascular disease.1 The endothelial activity extends far beyond the control of vascular tone. Indeed, it interferes, mainly via NO release, with key events in the development of atherosclerosis, such as monocyte and leukocyte adhesion, platelet-vessel wall interaction, and smooth muscle proliferation. Hyperglycemia, which is recognized as the culprit in the pathogenesis of diabetic complications, may cause a reduced bioavailability of NO.

Paradoxically, we demonstrated that in human aortic endothelial cells, high glucose increases endothelial NO synthase (eNOS) gene and protein expression.2 However, upregulation of eNOS is associated with increased superoxide anion (O2•−) production,2 suggesting that NO is inactivated by O2•−. The reaction of NO with O2•− produces a potent oxidant peroxynitrite (ONOO−), which has an array of biological actions contributing to impair endothelial function. In diabetic arteries, O2•− may also favor contractions via formation of hydrogen peroxide and hydroxyl radical, which stimulate the production of contractile prostanoids.3

However, the precise molecular mechanisms by which high glucose increases eNOS expression, O2•− production, and vasoconstrictor prostanoids are not known. One possibility may involve activation of protein kinase C (PKC), because hyperglycemia increases diacylglycerol, a strong activator of PKC.4,5 Interestingly, the promoter region of the human eNOS gene contains a phorbol ester responsive element.6 Furthermore, phorbol-12-myristate-13-acetate (PMA) increases expression of the inducible isoform of cyclooxygenase (COX) in many cell types.7-9 In normal blood vessels, activation of PKC by phorbol ester impairs endothelium-dependent relaxation via release of O2•− and vasoconstrictor prostanoids.10

Prostanoids are generated by COX from arachidonic acid. Two isoforms of the enzyme, encoded by distinct genes, have
been isolated in mammalian cells. COX-1 is constitutively expressed in most tissues and is involved in maintenance of cellular homeostasis, including regulation of vascular tone. In contrast, under normal conditions, COX-2 is expressed at low or undetectable levels but is readily upregulated by inflammatory, mitogenic, and physical stimuli. However, the mechanisms regulating COX expression in endothelial cells exposed to high glucose are unknown. It remains unclear which of the 2 COX isoforms plays a prominent role in the stimulated synthesis of vasoconstrictor prostanooids under this condition. This prompted us to investigate whether cumulative effects of oxidative stress along with hyperglycemia may interact with COX expression or activity and, hence, alter endothelial function.

**Methods**

**Cell Culture**

Human aortic endothelial cells (HAECs) were obtained from Clonetics (San Diego, Calif) and grown in gelatin-coated flasks in optimized endothelial growth medium (Clonetics) supplemented with 10% FCS. The cells were detached by exposure to trypsin/EDTA for ~120 seconds in HEPES buffered saline and reseeded in collagen-coated 6-cm cell-culture dishes or 24-well multiwells. Cells were first grown to confluency in humidified air, 5% CO₂ at 37°C. Confluent cells were maintained in endothelial growth medium containing 2% FCS. They were incubated with control (5.5 mmol/L) and elevated glucose concentration (22.2 mmol/L). Cells between the third and sixth subpassages were used.

**Amplification of COX-1 and COX-2 mRNA**

**Semiquantitative RT-PCR**

Total RNA was isolated by the acid guanidinium thiocyanate procedure and reverse-transcribed with the use of the SuperScript preamplification system (GIBCO BRL). cDNA was used as a template in PCR. cDNA aliquots were amplified with primers specific for human COX-1 and COX-2 (5'-TGC CCA GCT CCT GCC CCG CCT CTT-3' and 5'-GTC CAT CAA CAC AGG CGC CTC TTC-3'; 5'-TTC AAT GGA GAG GTG TAG GTA AGG AAA ATT GCT-3' and 5'-GTC CAT CAA CAC AGG CGC CTC TTC-3', respectively). PCR products (303, 305, and 593 bp for COX-1, COX-2, and housekeeping gene GAPDH, respectively) were separated by electrophoresis on 1.5% agarose gels and transferred to Hybond-N filter (Amersham).

**Southern Blotting**

COX-1, COX-2, and GAPDH probes were obtained by using genomic DNA from blood cells as a template. Complementary DNA probes were [a-32P]-dCTP labeled with a random primer DNA 45-mer served as the hybridization probe. The membranes were visualized by the ECL kit (Amersham Life Sciences). Densitometric measurements were performed by Fotodyne visionary documentation system (Fotodyne, Bio Cell Consulting Research).

**Western Blot**

COX-1, COX-2, prostacyclin (PGI₂) synthase (PGIS), eNOS, and p22(phox) proteins were analyzed by Western blot with anti-human COX-1 and COX-2 (Santa Cruz Biotechnology Inc), PGIS (Cayman Chemicals), eNOS (Transduction Laboratories), and p22(phox) (kindly provided by Dr Imajoh-Ohmi, Department of Bacterial Infection, University of Tokyo, Japan) antibodies as described. The membranes were visualized by the ECL kit (Amersham Life Sciences). Densitometric measurements were performed by Fotodyne visionary documentation system (Fotodyne, Bio Cell Consulting Research).

**NO Measurements**

Direct in situ measurements of NO were carried out as described. Immediately before NO measurements, the active tip of the L-shaped porphyrinic NO microsensor was placed directly on the surface of the endothelial cell monolayer. For maximal stimulation of eNOS, calcium ionophore A23187 was injected into the cell culture dish to yield a final concentration of 1 μmol/L.

**Prostanoid Determination**

Thromboxane B₂ and 6-Keto prostaglandin F₁α were measured in the culture medium after stimulation with calcium ionophore A23187 (300 nmol/L) by radioimmunoassay as described (Amerham Life Science).

**Intracellular Reactive Oxygen Species**

The intracellular formation of reactive oxygen species (ROS) was detected with the fluorescent probe CM-H₂DCFDA (Molecular Probes). Cells were loaded with 10 mmol CM-H₂DCFDA, incubated for 45 minutes at 37°C, and analyzed in an HTS 7000 Bio Assay Fluorescent Plate Reader (Perkin Elmer) using the HTSoft program. ROS production was determined from an H₂O₂ standard curve (10 to 200 nmol/mL⁻¹).

**Immunocytochemistry of 3-Nitrotyrosine and PGIS**

For examination of 3-nitrotyrosine formation and PGIS, endothelial cells were grown on culture slides (Becton & Dickinson). After incubation, cells were fixed. After blocking, cells were incubated with a 1:30 dilution of monoclonal antibody against nitrotyrosine (Upstate Biotechnology) and with a 1:300 dilution of polyclonal antibody against PGIS (kindly provided by Dr Tanabe, University Medical School, Osaka, Japan). Thereafter, fluorescence-labeled secondary antibodies (Molecular Probes) with the AlexaTM dye 568 and 488 for PGIS and 3-nitrotyrosine, respectively, were applied for 1 hour (1:200). Then the slides were rinsed with PBS and embedded in Immu-Mount for fluorescence microscopy with a Leica DMIRB equipped with a digital spot camera (Visitron System).

**Nitration of PGIS**

Nitration of PGIS in cell extracts was assessed by immunoprecipitation and Western blotting, as described.

**Cell Fractionation and PKC Activity**

PKC activity was measured by using the PKC enzyme assay system (Amersham) after cell fractionation into a cytosolic and a membrane fraction. Cells were harvested and sonicated, and samples were centrifuged at 100 000g for 1 hour at 4°C. The supernatant was used as the cytosolic fraction. The pellet was resuspended in 40 mL of buffer containing 1% Triton X-100 and 0.1% SDS. Cell debris were separated by centrifugation, and the supernatant was used as the membrane fraction.

**Statistical Analysis**

Results are expressed as mean±SEM, and n indicates number of experiments. Statistical evaluation of the data was performed with Student’s t test for simple comparison between 2 values when appropriate. For multiple comparisons, results were analyzed by ANOVA followed by Fisher’s test. P<0.05 was considered statistically significant.
selective upregulation of COX-2 expression was also observed by Western blotting (data not shown).

Treatment with PMA (1 μmol/L) caused a selective increase of COX-2 expression (Figure 1B). Interestingly, coinubication of endothelial cells with the PKC inhibitor calphostin C (300 nmol/L) reduced glucose and PMA-induced upregulation of COX-2 mRNA (Figure 1B). Glucose-induced increases of COX-2 mRNA and protein expression were associated with a higher release of thromboxane B2 after stimulation of endothelial cells with calcium ionophore A23187 (300 nmol/L; Figure 2A). Although high glucose increased PGIS expression (Figure 2B), the levels of 6-keto-prostaglandin F1α, a stable metabolite of vasodilator PGI2, were reduced (Figure 2A).

**Effect of Glucose on PKC Activity**
Glucose increased membrane-bound PKC activity at 1 hour (191% versus control; n=4; P<0.05; data not shown).

Glucose-induced activation of PKC was not affected by N-acetylcysteine (NAC; 50 μmol/L) or vitamin C (100 μmol/L). Mannitol had no significant effect on PKC distribution.

**Effect of Glucose on NO Pathway**
We also determined the effects of glucose on eNOS protein expression and compared the results with the effect of PMA (Figure 3A). Densitometric analysis showed an almost 2-fold upregulation of eNOS protein in cells exposed to high glucose, whereas mannitol did not affect the expression of the enzyme (Figure 3A). PMA induced a similar upregulation of eNOS (Figure 3A). Incubation of endothelial cells with calphostin C reduced eNOS protein expression almost to control values. The inhibitor alone had no significant effect (data not shown).

Despite the glucose-induced 2-fold increase of eNOS expression, NO release after stimulation with calcium iono-
phore A23187 (10 μmol/L) was reduced in endothelial cells exposed to high glucose (n=6; P<0.05; Figure 3B). However, in the presence of vitamin C or NAC, peak NO concentrations could be restored to control values (Figure 3B). Calphostin C blunted the inhibitory effect of glucose on NO concentrations (Figure 3B). Furthermore, in the presence of direct O$_2^-$ scavengers polyethylene glycol-superoxide dismutase (500 u/mL) or SOD mimetic, 4-hydroxy-tempo (10 μmol/L) NO production was greater than control values (n=4; P<0.05; data not shown).

Glucose-Induced ROS Generation and NAD(P)H Oxidase Expression
High glucose increased ROS intracellular formation, as assessed by the fluorescent probe CM-H$_2$DCFDA (Figure 4A).

Incubation of the cells with NAC or vitamin C prevented this increase in fluorescence (Figure 4A). When we compared the effects of high glucose with those of PMA, we observed a similar increase of ROS generation (24893±3996 and 27450±4327 relative intensity for glucose and PMA, respectively). Thus, inhibition of PKC abolished glucose-induced generation of ROS (Figure 4A). Furthermore, diphenyleneiodonium (1 μmol/L) blunted the stimulatory effect of glucose on ROS generation (Figure 4A). By contrast, indomethacin (10 μmol/L) did not affect glucose-induced ROS generation (Figure 4A). High glucose significantly increased the expression of the NAD(P)H oxidase subunit p22phox (Figure 4B).

Glucose and Nitration of PGIS
Endothelial cells exposed to high glucose exhibited a markedly enhanced specific immunostaining with a monoclonal antibody to 3-nitrotyrosine compared with control cells (Figures 5A and 5B), which was reduced by coincubation with NAC, vitamin C, or calphostin C (data not shown). By contrast, immunostaining with polyclonal anti-PGIS antibody remained unchanged in control and glucose-treated cells (Figures 5C and 5D). However, to determine whether 3-nitrotyrosine and PGIS colocalize in endothelial cells exposed to high glucose, a double-staining approach was adopted. The same cells show a good colocalization of green
and red spots that is represented in yellow in the merged image (Figure 5F). Nitration of PGIS was also identified by immunoprecipitation and Western blotting (Figure 5G).

Discussion
In the present study, we demonstrate for the first time that high glucose causes PKC-dependent upregulation of inducible COX-2 and eNOS expression as well as selective increase of thromboxane production and reduced NO release. Hence, activation of the PKC pathway represents a proximal node in the intracellular signaling leading to hyperglycemia-induced oxidative stress and endothelial dysfunction. Several lines of evidence support this conclusion. Glucose augmented membrane-bound PKC activity. Furthermore, PMA increased COX-2 and eNOS expression to a similar extent as high glucose. The effects of glucose and PMA were totally reversed by the PKC inhibitor calphostin C. Despite glucose-induced PGIS upregulation, prostacyclin release was decreased. By contrast, thromboxane levels were enhanced. Indeed, glucose-induced increase of PKC activity resulted in the formation of the NO/O₂⁻ reaction product ONOO⁻ and tyrosine nitration of PGIS. NAC, vitamin C, and calphostin C restoring the balance between NO release and ROS formation reduced colocalization of 3-nitrotyrosine and PGIS.

The effects of high glucose on PKC activation are mediated via an intracellular increase of diacylglycerol levels. Activation of PKC by phorbol esters leads to an impairment of endothelium-dependent relaxations in intact arteries similar to that observed in diabetes. In agreement with our results, the adverse effects of elevated glucose levels on acetylcholine-induced relaxations of rabbit aorta and rat pial arterioles can be restored by PKC inhibitors. Moreover, in vivo treatment with PKC inhibitors ameliorates vascular complications in diabetic rats. PKC also plays a role in mediating increased O₂⁻ production in streptozotocin-induced diabetes. However, the precise molecular mechanisms underlying PKC-mediated endothelial dysfunction have been thoroughly addressed in this study.

We demonstrate that high glucose, via PKC, induces oxidative stress and upregulation of COX-2, resulting in reduced NO availability and altered prostanoid profile (Figure 6). Indeed, the present study provides first direct evidence for an involvement of PKC in the activation of cyclooxygenase (COX) pathway by glucose. The elevation of glucose significantly increased the expression of COX-2 mRNA and protein levels but did not affect COX-1. This effect was also elicited by PMA as well as abolished by calphostin C. Most interestingly, glucose-induced COX-2 upregulation was associated with a shift in the balance of vasodilatory (6-keto-PGF₁α) and vasoconstricting (TXB₂) eicosanoids produced by the endothelial cells in favor of the latter. Prostaglandin H₂ is the common precursor for prostaglandins, thromboxane, and PGI₂. PGI₂ is synthesized from prostaglandin H₂ by a specific enzyme, PGIS. Exposure to high glucose increased PGIS protein expression. However, increased formation of NO/O₂⁻ reaction product was associated with tyrosine nitration of PGIS. Tyrosine nitration is a mechanism of selective inactivation of PGIS by peroxynitrite. Thus, the same molecular mechanism leading to inactivation of NO also caused inactivation of PGIS (Figure 6). Our data provide the molecular background to several observations that have implicated an overproduction of vasoconstrictor prostanoids in diabetes-induced endothelial dysfunction. In the rabbit aorta, the impaired relaxations are restored by nonselective COX blocker, prostanoid receptor antagonists, as well as SOD, suggesting that vasoconstritor prostanoids and ROS are the underlying cause of endothelial dysfunction. The glucose-induced release of vasoconstritor prostanoids is also prevented by PKC inhibitors. A recent experimental study has shown an important role of COX-2-derived prostanoids in the pathogenesis of diabetic renal hemodynamic changes.
In patients with NIDDM and IDDM, vitamin C increases endothelium-dependent vasodilation in the forearm circulation. In this study, we demonstrate that despite a paradoxical upregulation of eNOS, NO release was reduced, and coinubcation with antioxidants restored the balance between NO and ROS formation. Accordingly, NO release was greater than control in the presence of polyethylene glycol-superoxide dismutase and 4-hydroxy-tempo, suggesting that the reduced availability is mainly attributable to scavenging of NO. However, other recent studies have also shown a reduction of eNOS activity under hyperglycemic conditions. Diphenyleneiodonium, an inhibitor of flavin-containing enzymes including NAD(P)H oxidase, blunted the stimulatory effect of glucose on ROS generation, indicating that this enzyme may contribute to glucose-induced ROS generation. To additionally support this hypothesis, we found a marked increase in the NAD(P)H oxidase subunit p22phox expression. Because calphostin C prevents glucose-induced ROS formation and PKC activates NAD(P)H oxidase in vascular tissue, it is likely that the increased NAD(P)H oxidase–derived \( \text{O}_2^- \) production is at least in part mediated by PKC. However, the possibility that eNOS is also a source of \( \text{O}_2^- \) production cannot be excluded based on our present data. By contrast, we ruled out that COX-2 is a source of ROS. Indeed, targeting COX-2 by indomethacin, a nonselective inhibitor, did not affect glucose-induced ROS generation.

In conclusion, our results show that a single unifying PKC-dependent mechanism is the triggering step by which hyperglycemia induces oxidative stress and in turn endothelial dysfunction. Indeed, high glucose via PKC signaling promotes oxidative stress as well as increased expression of COX-2, resulting in reduced NO availability and altered

Figure 5. Colocalization of 3-nitrotyrosine and PGIS. Green signal (3-nitrotyrosine) and red signal (PGIS) localization in HAECs exposed to control (A and C) and high glucose (B and D). The merged image (yellow) of the 2 signals is shown in panel F for glucose-treated cells and in panel E for control cells. Nitration of PGIS was also identified by immunoprecipitation and Western blotting (G), representative blot, and densitometric quantification in protein homogenates. Data are mean±SEM (n=3). *P<0.05 vs control.
Figure 6. Schematic representation of intracellular signaling leading to hyperglycemia-induced oxidative stress, reduced NO availability, and altered prostanoid profile.

prostanoid profile. These findings may be relevant in understanding the intracellular signaling associated with vascular disease in diabetes mellitus and provide new pharmacological targets to prevent the development and progression of diabetic complications.

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References
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