Calcium and Protein Kinase C Mediate High-Glucose-Induced Inhibition of Inducible Nitric Oxide Synthase in Vascular Smooth Muscle Cells

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Abstract—Abnormal vascular smooth muscle cell (VSMC) proliferation is a key feature in diabetes-associated atherosclerotic disease. Since nitric oxide inhibits VSMC tone, migration, adhesion, and proliferation, we examined the effects of high glucose on IL-1β-induced NO release from VSMCs in culture. Confluent smooth muscle cells, pre-incubated with either 5 mmol/L (nM) or 20 mmol/L (mM) glucose for 48 hours, were stimulated with IL-1β. Nitrite was measured in the culture medium after 24 hours. IL-1β-induced a 15-fold increase in NO production in normal glucose medium. Glucose (10 to 30 mmol/L (nM)) significantly reduced the response to IL-1β. High glucose (20 mmol/L (mM)) inhibited IL-1β-evoked NO production by approximately 50%. IL-1β-stimulated [3H]citrulline forming activity of the nitric oxide synthase (NOS) was also significantly lower in high-glucose-exposed cells, and this was reflected in diminished cellular levels of NOS protein. To assess the role of protein kinase C (PKC), membrane PKC activity was measured, and glucose (20 mmol/L (mM)) significantly increased it. Immunoblotting of the membranes revealed a glucose-induced increase in the PKC II isoform. 1,2-Dioctanoyl-glycerol, a PKC activator, mimicked the high-glucose effect on IL-1β-induced NO release, while staurosporine, a PKC inhibitor, reversed it. The role of calcium in the glucose-mediated inhibition of cytokine-induced NO release was determined by treatment with BAPTA, an intracellular chelator of calcium. BAPTA partially reversed the inhibitory effects of glucose. Increasing intracellular calcium by A23187, an ionophore or thapsigargin, an inhibitor of endoplasmic reticulum Ca2+-ATPase, significantly decreased IL-1β-induced NO release and NOS expression. These results indicate that glucose-induced inhibition of IL-1β-stimulated NO release and NOS expression may be mediated by PKC activation and increased intracellular calcium. (Hypertension. 1998;31[part 2]:289-295.)

Key Words: protein kinase C • nitric oxide synthase • muscle • smooth • vascular • calcium • hyperglycemia

NOSs synthesize NO from l-arginine in a tissue-specific and cell-specific manner. In blood vessels, two distinct NOS isoforms have been identified. The eNOS in endothelial cells is constitutively present and regulated by cytoplasmic calcium levels in a transient fashion. In VSMC, induction of a nNOS capable of sustained production of NO occurs in response to a variety of agents and cytokines.

Induction of nNOS in VSMC by cytokines may have an adaptive role in the vascular response to injury. Cytokines, such as interleukin-1β (IL-1β), are released during both atherogenesis and arterial injury. IL-1β induces apoptosis, attenuates platelet aggregation and cell adhesion to vascular walls, and negatively regulates cell adhesion molecules in the vessel wall. Taken together, these observations suggest that induction of nNOS during atherogenesis may reduce pathological VSMC proliferation and plaque formation. Conversely, deterioration of these inductive responses may lead to exaggerated atherogenic changes.

Significantly, accelerated atherosclerosis and hypertension are key abnormalities in diabetes-associated vascular disease. Clinical trials have consistently concluded that hyperglycemia is a primary cause of diabetic vascular complications. The goal of the present study, therefore, was to determine whether hyperglycemia interferes with cytokine induction of VSMC NOS and to study the mechanism of this interference. Some of the toxic effects of hyperglycemia have been attributed to activation of PKC by increased DAG synthesis. Glucose-induced impairment of calcium regulatory processes has also been proposed to play a role. In this study, we examined whether high glucose reduces cytokine-induced NOS activity in VSMC and whether such regulation may be mediated by PKC and calcium.

Materials and Methods

VSMC Preparation and Culture

VSMC were isolated from male Sprague-Dawley rat thoracic aorta by enzymatic dissociation as described previously. Cells were grown in...
Selected Abbreviations and Acronyms

- DAG = 1,2-diacyl-sn-glycerol
- cNOS = calcium/calmodulin-dependent NOS
- DAG = dacylglycerol
- iNOS = 1,2-dioctanoyl-sn-glycerol
- HG = high glucose
- nNOS = calcium-independent NOS
- NG = normal glucose
- NO = nitric oxide
- PKC = protein kinase C
- PMSF = phenylmethylsulfonylfluoride
- VSMC = vascular smooth muscle cells

Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal calf serum, 2 mMol/L (mM) glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, in an incubator at 37°C in 95% humidified air and 5% CO2. Serial passages of VSMC were obtained by treating confluent cultures with 0.2% trypsin, EDTA in Ca2+/Mg2+-free HBSS (Trypsin-HBSS, Sigma Chemical Co.). For experiments, cells in passages 6 to 12 were seeded into 6-well or 24-well culture plates (Falcon), fed every other day, and used at confluence (5 to 7 days). These experiments used VSMC preparations from six different rats, and all six preparations were demonstrated to have the basic phenomenon investigated here (i.e., high-glucose inhibition of IL-1β induction of NO synthesis). The cells were treated with the following media: NG medium serum-free and phenol red-free DMEM with 0.1% BSA (Gibco), HG medium similar to NG medium but supplemented with 5 mM EDTA to raise the glucose concentration to 20 mMol/L (mM), or osmotic control medium, red-free DMEM with 0.1% BSA, and HG medium but supplemented with mannitol (15 mMol/L (mM)), for 48 hours and then were treated with different agents as described.

Assay of NO Synthesis
After treatment with the appropriate media, VSMC were exposed to IL-1β (20 ng/mL, recombinant human IL-1β, Sigma Chemical Co.) for 24 hours. Synthesis of NO was determined by assay of culture supernatant nitrite, a stable reaction product of NO and molecular nitrogen, at 37°C in 95% humidified air and 5% CO2. Serial passages of VSMC were obtained by treating confluent cultures with 0.2% trypsin, EDTA in Ca2+/Mg2+-free HBSS (Trypsin-HBSS, Sigma Chemical Co.). For experiments, cells in passages 6 to 12 were seeded into 6-well or 24-well culture plates (Falcon), fed every other day, and used at confluence (5 to 7 days). These experiments used VSMC preparations from six different rats, and all six preparations were demonstrated to have the basic phenomenon investigated here (i.e., high-glucose inhibition of IL-1β induction of NO synthesis). The cells were treated with the following media: NG medium serum-free and phenol red-free DMEM with 0.1% BSA (Gibco), HG medium similar to NG medium but supplemented with 5 mM EDTA to raise the glucose concentration to 20 mMol/L (mM), or osmotic control medium, identical to NG medium but supplemented with mannitol (15 mMol/L (mM)), for 48 hours and then were treated with different agents as described.

VSMC Lysate Preparation and NO Assay
After appropriate treatments and exposure to IL-1β, VSMC monolayers were washed three times in ice-cold homogenization buffer (25 mMol/L (mM) Tris HCl, 1 mMol/L EDTA/EGTA, pH 7.4). The cells were scraped and lysed in the homogenization buffer containing protease inhibitors (0.1 mMol/L (mM) PMSF, 5 μg/mL aprotinin, 25 μg/mL trypsin inhibitor, and 5 μg/mL leupeptin) by sonication for 10 seconds. The lysates were then spun at 10,000g for 10 minutes, and the resulting supernatant was used for NO assay. NO activity was measured by the conversion of L-[3H]arginine to L-[3H]citrulline as previously described (17). Briefly, 10 μL of the VSMC lysate was incubated with 40 μL of the reaction mixture containing 10 μM L-[3H]arginine (1 μCi), 1 mMol/L NADPH, 3 μM molsidomycin, 1 μM flavin adenine dinucleotide, and 1 μM flavin adenine mononucleotide for 30 minutes at 37°C in a water bath. The reaction was stopped by the addition of 400 μL of ice-cold stop buffer (50 mMol/L (mM) HEPES, 5 mMol/L (mM) EDTA, pH 5.5) and Dowex resin 50W-X8 (Sigma Chemical Co.). The mixture was then spun in a spin filter (Roto-Raf) and radioactivity in the eluate containing L-[3H]citrulline was quantified by liquid scintillation counting. Protein concentrations were measured by using the Bio-Rad protein assay kit. The results are expressed as cpm/μg/min.

Preparation of VSMC Membrane Fractions
Crude membrane fractions from VSMC were prepared as described previously (18) with some modifications. After treatment of VSMCs in NG or HG media for 48 hours, cells were rinsed with ice-cold homogenization buffer (50 mMol/L (mM) Tris HCl, 1 mMol/L (mM) EDTA, 0.2 mMol/L (mM) PMSF, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and 5 mMol/L (mM) β-mercaptoethanol), and scraped into the same solution. The cells were then disrupted by 15 strokes in a tight-fitting glass homogenizer. Nuclei and mitochondria were pelleted at 10,000g for 20 minutes, and the supernatant was then centrifuged at 48,000g for 60 minutes. The membrane pellet was then suspended in a buffer containing 20 mMol/L (mM) Tris-HCl, 0.2 mMol/L (mM) PMSF, 5 μg/mL aprotinin, 5 μg/mL leupeptin and 0.1% Triton X-100. The membrane fractions were used for in vitro PKC kinase assay and immunoblotting.

PKC Assay
Membrane fractions of VSMC were used to measure PKC activity as previously described (19). Aliquots of membrane fractions were incubated in a reaction buffer containing 50 μM Ac-myristoyl basic protein, 20 μM ATP, 1 mMol/L (mM) CaCl2, 20 mMol/L (mM) MgCl2, 4 mMol/L (mM) Tris HCl (pH 7.5), 10 μM phorbol myristate acetate, and 0.28 mg/mL phosphadidylinosine in Triton X-100 micelles and 25 μCi of [γ-32P]ATP (6000 Ci/mmol, DuPont). The reaction was started by the addition of [γ-32P]ATP, and incubation was carried out at 30°C for 10 minutes. Aliquots of the reaction mixture were then spotted on phosphocellulose discs and washed with 1% phosphoric acid four times. The filters were then counted in a scintillation counter, and the results were expressed as pmln/mg of protein.

Western Blotting of iNOS and PKC Isoforms
Equal amounts of membrane or whole cell lysate protein (40 to 50 μg) were separated on a 7.5% SDS-polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell) in Tris-Glycine transfer buffer with 20% methanol in a Bio-Rad Trans-Blot Cell (Bio-Rad). Membranes were blocked overnight at 4°C with 9% instant nonfat dry milk (Carnation) in Tris-buffered saline (TBS in mMol/L (mM) buffer) 20 Tris, 137 NaCl, pH 7.6 containing 0.3% Tween 20, washed in TBS, and incubated with the appropriate primary antibody a monoclonal antibody against iNOS, 1:2500 (Transduction Laboratories) or polyclonal antibodies against PKC α and PKC βII, 1:500 (Santa Cruz Biotech) for 2 hours. The membranes were washed thoroughly (PKC blots received one additional high salt wash (TBS, with 0.5 M NaCl) for 5 minutes) and incubated with horseradish peroxidase–coupled anti-rabbit or anti-mouse IgG antibody (1:8000, for mouse and 1:2500 for rabbit, Amersham) for 1 hour. After thorough washings, the bound antibodies were visualized by enhanced chemiluminescence using the ECL system (Amersham) and exposure to Kodak X-Omat film. Signals on the immunoblot were quantified by using the Ambis densitometry system (version 4.31). Multiple exposures of each blot were performed to ensure that signal was within the linear range of the film.

Statistical Analyses
Results are expressed as mean ±SEM. Statistical analysis used ANOVA or the paired or unpaired Student’s t-test or with Bonferroni correction, as appropriate. Differences were considered significant if P<0.05.

Results
We first examined the effect of high extracellular glucose on IL-1β-stimulated NO production and NO activity in normal glucose conditions (NG medium), IL-1β (20 ng/mL) caused a significant accumulation of nitrite at the end of 24 hours. Pretreatment of VSMC with glucose at a concentration of 20 mMol/L (mM) for 48 hours significantly reduced (n=4)
Figure 1. Effect of high glucose on IL-1β-stimulated (A) nitrite accumulation and (B) NOS activity. Confluent VSMC were exposed to either NG medium (5 mmol/L (mM)) or HG medium (20 mmol/L (mM)) for 48 hours. IL-1β (20 ng/mL) was added, and nitrite accumulation in the culture medium or citrulline-forming activity of the NOS in cell lysates was measured at the end of 24 hours. Bars represent mean ± SEM; n=4 experiments (two each from different subcultures). (A) Bars labeled with different letters are significantly different from each other. ***P<.001, one-way ANOVA. (B) *P<.05, paired Student’s t-test.

the amount of IL-1β-induced NO release when compared to control (NG medium, Fig 1A). In preliminary experiments, the inhibitory effects of glucose was observed to be concentration-dependent, beginning at 10 mmol/L (mM) with maximal inhibition at 30 mmol/L (mM). We therefore chose 20 mmol/L (mM) glucose (HG medium) for all further experiments. Pretreatment of VSMC with equimolar concentration of mannitol (15 mmol/L (mM)), a poorly diffusible hexose, failed to inhibit IL-1β-stimulated NO release, a finding suggesting that increased extracellular osmolality was not responsible for the inhibitory actions of glucose (IL-1β in NG, 17.3±0.7 (nmoles nitrite/well); IL-1β + mannitol; 18.2±0.2 (nmoles nitrite/well)).

HG medium also caused a reduction in NOS enzymatic activity; this reduction was due to a decrease in the amount of iNOS protein in cell lysates. Since iNOS is regulated mainly at the level of expression, we sought to determine whether high glucose inhibits IL-1β-induced iNOS protein expression. As shown in Fig 2, 48 hours of incubation with HG caused a significant reduction (n=4) in iNOS protein levels measured by Western blotting. These data suggest that glucose exerts its inhibitory action at the level of expression of the NOS protein; this is then reflected in decreased NOS activity and NO production.

Elevated extracellular glucose has been reported to provoke increases in DAG and simultaneously to increase the enzyme activity, translocation, and phosphorylation of endogenous substrates of PKC in VSMC. It has been proposed that a reduced cytosolic redox state of NADH/NAD⁺ may be involved in stimulating de novo synthesis of DAG from increased influx of glucose. It is also known that PKC modulates iNOS induction in many different cell types including VSMC. We therefore explored whether activation of PKC mediates some of the effects of glucose. PKC activity was significantly enhanced (n=3) in the membrane fractions of VSMC treated with HG medium for 48 hours (Fig 3A). To determine which PKC isoform(s) might mediate the increase in membrane-bound PKC activity, we measured the amount of two of its isoforms (α and β1) in HG-treated VSMC membranes by Western blotting. PKC α was most abundant but unchanged by glucose. However, HG significantly increased (n=5) levels of the β1 isoform in the membrane fraction (Fig 3B).

We used a membrane-permeable diacylglycerol, DOG (100 μM), as an activator of PKC; as seen in Fig 4, DOG significantly decreased (n=5) the IL-1β-induced NO release. Staurosporine (100 nM), a widely used potent inhibitor of PKC, had no effect on IL-1β-stimulated NO release in cells exposed to NG medium, but it significantly attenuated (n=4) the inhibitory action of HG medium (Fig 4B). Staurosporine per se had no significant effect on nitrite accumulation, a result that is consistent with previous reports.
High Glucose and Nitric Oxide

Glucose-induced increases in DAG levels in vascular tissue may be prevented by concomitant exposure to pyruvate. It is proposed that pyruvate mediates this effect by ameliorating glucose-induced reductions in the cytosolic redox state. We therefore examined whether pyruvate would decrease the inhibitory effects of glucose on iNOS induction. VSMC were treated with or without pyruvate (2.5 mmol/L (mM)) in NG or HG medium for 48 hours, and the cells were treated with IL-1β as before. As is evident in Fig 5, pyruvate partially restored the IL-1β response in HG-treated VSMC while having no significant effect on cytokine-induced NO release from VSMC in NG medium. These results support the notion that iNOS inhibitory effects of glucose are mediated, in part, by activation of PKC.

Many studies have documented that hyperglycemia impairs calcium homeostasis in VSMC, resulting in a calcium overload. In addition to regulating vascular tone, calcium also modulates gene expression: elevated intracellular calcium has been shown to inhibit iNOS induction in human chondrocytes. Therefore, we used BAPTA-AM, a cell-permeable calcium chelator, to investigate the calcium dependency of high-glucose-induced iNOS inhibition. VSMC exposed to NG or HG medium for 48 hours were loaded with BAPTA-AM (50 µM) for 90 minutes at 37°C and then treated with IL-1β as before. When BAPTA-AM enters the cells, the acetoxymethylester groups are cleaved off, trapping BAPTA in the cytosol, where it then significantly reduces and maintains a low cytoplasmic calcium concentration. As seen in Fig 6, intracellular calcium chelation had no effect on IL-1β-stimulated NO release in NG medium and significantly reversed (n=4) the high-glucose effect, although it did not normalize it. These observations were paralleled by changes in the iNOS protein levels (Fig 6B) in these experimental groups. These results suggest that high-glucose-mediated inhibition of cyto-
Figure 6. Effect of BAPTA-AM on glucose-induced downregulation of iNOS. Confluent VSMC were exposed to NG or HG medium for 48 hours and then treated with BAPTA-AM (50 μM) for 90 minutes at 37°C. The cells were then washed three times with serum-free DMEM and treated with IL-1β (20 ng/mL). (A) Nitrite accumulation in the culture medium (B) and iNOS protein in cell lysates were measured after 24 hours. Bars represent mean±SEM; n=4. Bars labeled with different letters are significantly different from each other. ***P<.001, **P<.01, one-way ANOVA. (B) Representative immunoblot of four independent experiments.

Figure 7. Effect of increasing intracellular calcium on (A) IL-1β-stimulated nitrite accumulation and (B) iNOS protein. VSMCs were stimulated with IL-1β (20 ng/mL) in the presence of A23187 (0.5 μM) or Thapsigargin (1 μM). Nitrite accumulation in the culture medium and iNOS protein in cell lysates were measured at the end of 24 hours. (A) bars represent mean±SEM; n=3 performed in quadruplicate. ***P<.001, one-way ANOVA. (B) Representative immunoblot of two independent experiments.

Discussion

In this study, we have demonstrated that high glucose downregulates cytokine-induced NO production by inhibiting the expression of iNOS protein. Similar results have recently been reported, however, the present study examines the activity of NOS and PKC in more detail and investigates the roles of calcium and metabolic alterations in the response. We provide evidence that long-term treatment of VSMC with high glucose increases PKC activity and that pharmacological inhibition of this activity reverses the effect. The high-glucose effects were reproduced by a PKC activator, DOG. The data also indicate that increases in cytosolic calcium may partly mediate the negative effects of glucose. Indeed, raising intracellular calcium by two different mechanisms lowered IL-1β-stimulated NO release and iNOS protein. Therefore, increased glucose inhibits cytokine-stimulated iNOS by two possibly related mechanisms: activation of PKC and enhancement of cytosolic calcium.

Considerable evidence now exists to support the hypothesis that high glucose activates PKC in vascular tissue. Levels of DAG, the endogenous activator of PKC, are increased in vascular tissue of diabetic animal models and in cells exposed to high glucose. Metabolic labeling studies show that DAG concentrations are increased because of de novo synthesis; this involves a progressive acylation of glycolytic triose intermediates, ultimately culminating in the formation of DAG. It has also been proposed that an elevated NADH/NAD ratio favors reduction of dihydroxyacetone phosphate, a key glycolytic intermediate to phosphatidic acid and then to diacylglycerol. This is supported by the finding that combination of pyruvate prevents the glucose-induced increase in DAG levels and PKC activity in granulation tissue, presumably by reversing the NADH/NAD ratio. Pyruvate has also been shown to normalize elevated levels of triose intermediates induced by glucose.

On elevation of DAG, PKC translocates to the plasma membrane and assumes an active conformation. The increased
PKC activity in membranes of VSMC exposed to high glucose in our study reflects such an activation of the kinase. Furthermore, DOG-mediated inhibition of IL-1β-induced NO release does suggest that a diacylglycerol sensitive PKC is involved. The inhibitory effects of DOG were not as robust as those of high glucose, perhaps because of rapid degradation by nonspecific esterases or through operation of additional inhibitory mechanisms in the high-glucose-treated cells (e.g., increased calcium). Pyruvate partially decreased the inhibitory effect of high glucose on IL-1β-stimulated NO release. This finding supports the notion that this may be mediated in part by prevention of glucose-induced accumulation of these intermediates (precursors of DAG synthesis) and subsequent PKC activation.

The PKC family consists of at least 11 isoforms and has been divided into three subfamilies: conventional PKCs (cPKC \( \alpha, \beta, \) and \( \gamma \)), which are dependent on calcium, DAG, and phospholipid for activity, the novel PKCs (nPKC \( \delta, \varepsilon, \eta, \theta, \) and \( \mu \)), which are calcium insensitive but DAG and phospholipid sensitive, and the atypical PKCs (aPKC \( \lambda, \tau, \) and \( \xi \)) which are both calcium and DAG insensitive. PKC isoforms \( \alpha, \beta, \varepsilon, \) and \( \xi \) have been demonstrated in VSMC, and calcium has been shown to translocate all of these isozymes except \( \xi \). However, this effect was sustained more than 24 hours for only the \( \beta \) isoform. These findings, coupled with observations in diabetic rat vascular tissue and cells exposed to glucose for prolonged periods of time, clearly suggest a preferential activation of the PKC \( \beta II \) isoform by high glucose. Our results showing elevated PKC \( \beta II \) isoform in glucosed-treated VSMC concur with previous observations. PKC is known to modulate iNOS expression in different tissues. It positively regulates iNOS expression in hepatocytes, macrophages, and avian osteoblasts but inhibits iNOS induction in mesangial cells. In VSMC, the role of PKC in regulating iNOS is unclear, some reports demonstrating inhibition while others show both.

This ambiguity may be explained by the diversity of PKC isoforms, different endogenous substrates, and subcellular localization. In contrast to our study, high glucose increases NO production in macrophages, mesangial cells, and sert endothelial cells, in a PKC-dependent manner, findings suggesting that cell-specific factors may influence the nature of the response to glucose. The exact mechanism of PKC-mediated inhibition of iNOS still remains to be investigated. PKC might affect the receptor and various signaling cascades initiated by IL-1β. Evidence in the literature indicates that PKC activates the IL-1 receptor in a human transformed B-cell line. PKC might also affect downstream elements of the transduction pathway, such as NFκB, a key transcription factor mediating iNOS induction. Thus, high-glucose-mediated activation of PKC might modulate a number of key processes involved in iNOS induction.

Calcium levels are increased in VSMC treated with high glucose. While calcium predominately upregulates certain genes, it is known to inhibit others. Increased calcium influences iNOS gene expression in certain cell types, inhibiting induction in human chondrocytes but upregulating it in avian osteoblasts, findings suggesting a cell-specific effect. In our experiments, intracellular calcium chelation with BAPTA-AM partially decreased the inhibitory effects of glucose but increasing intracellular calcium by A23187 and thapsigargin inhibited cytokine-induced iNOS expression. These results suggest that an increase in cytosolic calcium and not secretory depletion mediates the inhibitory process. Increased calcium has been reported to decrease iNOS mRNA stability. The increased cytosolic calcium also aids PKC activation by decreasing its affinity for phospholipids. This might also explain the sustained activation of PKC \( \beta II \), a member of cPKC, which requires both calcium and DAG for activation.

To summarize, high glucose inhibits induction of iNOS in VSMC by activating PKC and increasing cytosolic calcium. Loss of the inhibitory actions of NO, an anti-atherogenic molecule, in the diabetic state would favor the development of myointimal proliferation and accelerate the pathogenesis of atherogenesis. This might therefore partly explain the higher rates of restenosis and increased incidence of atherosclerosis in diabetic patients.

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