Hyperglycemia Inhibits Vascular Smooth Muscle Cell Apoptosis Through a Protein Kinase C–Dependent Pathway

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Abstract—We hypothesized that the pathogenesis of diabetic vasculopathy involves the abnormal regulation of vascular smooth muscle cell (VSMC) apoptosis. In nondiabetic mice, a reduction in carotid artery blood flow resulted in a significant loss of medial VSMCs via apoptosis (normal flow 84±1 viable VSMCs, reduced flow 70±5 viable VSMCs; n=12, P<0.01). In contrast, flow-induced VSMC apoptosis was markedly attenuated in streptozotocin-induced diabetic mice (normal flow 85±2 viable VSMC, reduced flow 82±4 viable VSMC; n=13, NS). In accord with our in vivo findings, the exposure of cultured rat and human VSMCs to high glucose (17.5 mmol/L) significantly attenuated the induction of apoptosis in response to serum withdrawal (rat VSMCs in normal [5.5 mmol/L] glucose 28±1%, high D-glucose 19±2%; P<0.0001). High glucose also inhibited apoptosis induced by Fas ligand (100 ng/mL) (normal 23±2%, high D-glucose 13±2%; P<0.0006). Supplementation with the nonmetabolized enantiomer l-glucose had no effect. We confirmed reports that high glucose activates protein kinase C (PKC) and demonstrated that PKC blockade with long-term phorbol ester treatment or calphostin C prevented the antiapoptotic effect (P<0.001). Moreover, the upregulation of either PKCa or PKCBII expression was sufficient to inhibit serum withdrawal–induced apoptosis (control 25±2%, PKCa 11±2%, PKCBII 8±2%; P<0.0001), whereas the upregulation of PKCβ had no significant effect. Taken together, these findings demonstrate that hyperglycemia inhibits VSMC apoptosis via a PKC-dependent pathway. (Circ Res. 2000;87:574-580.)

Key Words: diabetes ■ glucose ■ cell death ■ vasculature ■ remodeling ■ blood vessel

It is well established that diabetic patients are at an increased risk of developing atherosclerosis, restenosis after angioplasty, and other forms of vascular disease. However, the molecular mediators that potentiate the process of macrovascular lesion formation in insulin-dependent and non–insulin-dependent diabetics remain to be further defined.

The pathogenesis of occlusive macrovascular disease involves an abnormal accumulation of cells within the vessel wall. The conventional paradigm has emphasized increased cell proliferation as a key feature of macrovascular lesion formation in diabetes. However, an emerging body of evidence suggests that the pathogenesis of vascular disease involves a perturbation in the balance between cell proliferation and cell death. Indeed, recent studies from our laboratory indicate that the upregulation of antiapoptotic genes and the resultant inhibition of vascular smooth muscle cell (VSMC) death represent a critical pathogenic event in the process of intimal lesion formation.

Based on this postulate that the course of vascular disease is governed by the balance between VSMC proliferation and survival, we hypothesized that hyperglycemia may potentiate the process of macrovascular lesion formation by inhibiting VSMC apoptosis. To test this hypothesis, we compared the in vivo apoptotic response of medial VSMCs in a control group of nondiabetic mice and a streptozotocin-induced diabetic cohort. VSMC apoptosis was induced in the intact animal with a well-characterized model of vascular remodeling activated by blood flow reduction. These studies confirmed our hypothesis that flow-induced VSMC apoptosis is markedly attenuated within the vessels of diabetic animals compared with nondiabetic control animals. To further define the cellular mediators of the antiapoptotic effect of diabetes, we studied the effect of elevations in ambient glucose concentrations on the regulation of VSMC death in vitro. These studies confirmed that high glucose inhibited VSMC apoptosis in association with the activation of protein kinase C (PKC). Moreover, blockade of the PKC pathway abolished the antiapoptotic effect of high glucose. Finally, transient transfection experiments demonstrated that up-regulation of PKCa or PKCBII expression alone is a sufficient stimulus to prevent VSMC apoptosis. Taken together, our in vivo and in vitro data suggest a close interrelationship among altered glucose metabolism, the activation of PKC, and the regulation of apoptosis in VSMCs. These findings may have important implications for the role of VSMC apoptosis in the pathogenesis of macrovascular disease in the diabetic population.

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Materials and Methods

Murine Model of Flow Reduction–Induced Apoptosis
A chronic reduction in blood flow induces a shrinkage in vessel dimensions that is preceded by an early phase of medial VSMC apoptosis.12–15 Adult male FVB mice (6–9 wk; 23–28 g) were anesthetized with ketamine (75–95 mg/kg) and xylazine (4–6 mg/kg IP). Using aseptic technique, the left carotid bifurcation was exposed under a dissecting microscope. Flow reduction was achieved by ligation of the left common carotid artery 1 mm proximal to the bifurcation. A sham operation involved the placement of a loose ligature in the identical location.

Tissue Harvest and Analysis
All vessels were harvested 24 hours after the intervention. Four serial sections were stained with Hoechst 33342 (H33342) as previously described.11,16 Characterization of apoptotic cell death with nuclear chromatin within blood vessels has previously been validated through several techniques.11,16 VSMC loss was confirmed through quantification of the total DNA content (PicoGreen DNA Quantitation Reagent; Molecular Probes). No significant thrombus formation or inflammation was observed, consistent with previous reports in which this model was used.12

Diabetic Model
Mice were injected with streptozotocin (200 mg/kg; Sigma Chemical Co) as described previously.17 Mean blood glucose levels in the diabetic cohort that underwent ligation (399 ± 9 mg/dL, ∼22 mmol/L; n = 13) were not significantly different from those of the diabetic sham-operated animals (351 ± 10 mg/dL; n = 12).

Cell Culture
Clonal A7r5 rat aortic VSMCs (American Type Culture Collection), human umbilical artery smooth muscle cells, and human aortic vascular smooth muscle cells (Clonetics) were used in the study. VSMCs were cultured in the presence of either 5.5 mmol/L (99 mg/dL) (normal) or 17.5 mmol/L (315 mg/dL) (high) t-glucose for 48 hours. Apoptosis was induced with serum withdrawal or Fas ligand (100 ng/mL; UBI) in the presence of 2% FBS and a Fas ligand protein enhancer (1.5 µg/mL, mouse IgG; UBI).

Quantification of Apoptosis
Apoptosis was assessed through staining with H33342 and quantification of the percentage of apoptotic nuclei (400 cells counted per sample). Previous work in our laboratory has validated this assay with other techniques.11,16,18 Caspase 3–like activity was assessed with a caspase 3 cleavage activity assay (Biovision) and expressed as arbitrary units of fluorophore activity inhibitable by the DEVD caspase 3 peptide blocker (DEVD-FMK).

PKC Activity and Blockade
Cells were pretreated with 100 nmol/L phorbol-12-myristate-13-acetate (PMA) (Sigma), vehicle (DMSO), or the inactive phorbol ester, 4-α-PMA (BIOMOL), to downregulate the diacylglycerol-sensitive PKC isoforms as described previously.19 Calphostin C (1 µmol/L; Sigma Chemical Co) was administered at a dosage previously documented to achieve near-maximal inhibition of PKC activation.20 PKC activity was quantified through the determination of picomoles of phosphate incorporated into substrate peptide per minute per milligram of protein according to the manufacturer’s protocol (Upstate Biotechnology). PKCa activity was detected with a phosphospecific antibody (Upstate Biotechnology), whereas PKCb and PKCd were assessed with Western blotting on particulate fractions isolated from A7r5 VSMCs, with antibodies from Santa Cruz Biotechnology.

PKC Expression Vector Transfection
A7r5 VSMCs were transiently cotransfected with either wild-type PKCα (GeneStorm; Invitrogen), PKCβ (GeneStorm), a constitutively active PKCβII (gift from Dr P. Buttrick), or an empty control vector, pCDNA3.1 (Invitrogen), along with green fluorescent protein (GFP) (GIBCO), with the use of LipoFECTAMINE PLUS (GIBCO). Apoptosis was induced through serum withdrawal and quantified in the transfected subset as described previously.21

Statistical Analysis
Comparisons between 2 groups were analyzed with a Student’s t test (P < 0.05), whereas comparisons among 3 groups were analyzed with an ANOVA with a Student-Newman-Keuls post hoc test (P < 0.05). Data are presented as mean ± SEM. An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

Results

Hyperglycemia Inhibits Medial VSMC Death in Response to Flow Reduction In Vivo
In accord with previous reports,12–14 our initial results confirmed that a severe reduction in blood flow in nondiabetic animals induced by ligation of the common carotid artery resulted in significant acute medial cell loss of 17% within 24 hours due to apoptotic cell death (Figure 1). Morphological analyses of ligated vessel specimens confirmed that medial cell loss involved the characteristic features of apoptosis (Figure 1B). Sham-ligated vessels and contralateral control vessels exhibited no reduction in cellularity (Figure 1C).

VSMC loss after a reduction in blood flow was also confirmed through an assessment of total DNA content at 24 hours after ligation and was expressed as a ratio of DNA content in the ligated or sham-operated vessel relative to the contralateral vessel (ligated vessel/contralateral vessel 0.8 ± 0.1; sham-operated vessel/contralateral vessel 1.5 ± 0.3; n = 17, P < 0.044).

To assess the effect of diabetes on the regulation of VSMC apoptosis in vivo, FVB mice were rendered diabetic with streptozotocin and compared with the nondiabetic cohort. In contrast to nondiabetic animals, diabetic animals exhibited a significantly attenuated loss in medial VSMC cellularity after flow reduction (ligated-to-nonligated ratio: 0.83 in nondiabetic animals, 0.96 in diabetic animals; P < 0.02) (Figure 2). There was no significant difference between nondiabetic and diabetic animals in the number of medial VSMCs in contralateral or sham-operated vessels. Diabetic animals had a blood sugar level of 350 to 399 mg/dL, which corresponds to ∼19 to 22 mmol/L. These in vivo findings suggested that the diabetic state induced by insulin deficiency inhibits VSMC apoptosis.

High Glucose Inhibits VSMC Apoptosis
It is recognized that insulin deficiency results in a complex in vivo milieu in which hyperglycemia is part of a spectrum of metabolic abnormalities. To test the hypothesis that an increase in ambient glucose concentrations alone was sufficient to inhibit vascular cell apoptosis, we used an in vitro model system. Cultured rat VSMCs were exposed to 5.5 (normal) or 17.5 (high) mmol/L glucose in the presence of serum for 48 hours, followed by serum withdrawal in normal versus high-glucose medium. In accord with our in vivo
findings, elevations in the concentration of extracellular glucose significantly inhibited VSMC apoptosis in response to serum withdrawal (Figure 3). This antiapoptotic effect of high glucose was not mediated by an increase in osmolarity insofar as the nonmetabolized analog L-glucose had no effect on VSMC survival (Figure 3).

To further clarify the time course of the antiapoptotic effect of high glucose, cells were exposed to high glucose for the 20-hour time period of serum withdrawal. Indeed, this relatively brief exposure to high glucose at the time of serum withdrawal was sufficient to inhibit apoptosis (normal glucose 22±1%, high glucose 16±1%; P<0.05).
As an additional verification of our findings, we extended our observations in rat VSMCs to human umbilical artery VSMCs. Using a caspase 3–like activity assay, we initially confirmed that serum withdrawal in 5.5 mmol/L glucose induces a significant increase in caspase 3–like activity in association with apoptosis compared with the serum-treated baseline control (serum-treated baseline 1.00±0.03 caspase 3–like activity units, serum free 1.20±0.02; n=8, P<0.001). Moreover, we documented that high glucose significantly inhibited caspase 3–like activity in human umbilical artery VSMCs (normal glucose serum free 1.20±0.02, high glucose serum free 1.10±0.02; n=8, P<0.02). We recently observed similar results in human aortic VSMCs (data not shown).

Based on evidence that Fas ligand mediates VSMC apoptosis in vivo,22 we also assessed whether the antiapoptotic effect of high glucose had broad applicability to various stimuli. Treatment with Fas ligand resulted in a significant increase in the percentage of VSMCs undergoing apoptosis when exposed to normal glucose (vehicle, 5±1%; Fas ligand, 23±2%; n=8, P<0.00002). Indeed, we observed that high glucose also attenuated the percentage of A7r5 VSMCs undergoing apoptosis in response to Fas ligand (Figure 4).

**Antia apoptotic Mediator Role of PKC**

Previous reports have demonstrated activation of PKC in response to high glucose in VSMCs in vitro and in vivo.19,23–28 It has been postulated that diabetic vasculopathy may be mediated in part by PKC-induced VSMC growth.5 Thus, we hypothesized that PKC may have a bifunctional effect on VSMC fate by inhibiting VSMC apoptosis as well as by stimulating cell growth. We confirmed previous reports that exposure to high glucose results in elevated activity of PKC (Figure 5). To determine whether the glucose-induced activation of PKC was necessary to mediate the antiapoptotic effects of glucose, diacylglycerol-specific PKC isoforms were downregulated through long-term treatment with the phorbol ester PMA. Indeed, downregulation of PKC activity through long-term PMA treatment blocked the antiapoptotic effect of elevated glucose concentrations on serum withdrawal–induced apoptosis (Figure 6). The inactive phorbol ester 4–α-PMA (a negative control) had no significant effect on the glucose-mediated inhibition of apoptosis (Figure 6).

As another means of confirming that PKC activation is a necessary component in the antiapoptotic signaling cascade, we used the pharmacological PKC inhibitor calphostin C. In agreement with the data obtained through PKC downregulation, we observed that pharmacological blockade of PKC activity, as seen in Figure 5, abolished the antiapoptotic effects of high glucose (normal glucose plus vehicle 31±1%,

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**Figure 3.** High glucose significantly inhibits VSMC apoptosis induced by serum withdrawal. VSMCs were exposed to normal (5.5 mmol/L) or high (17.5 mmol/L) d-glucose or the osmolar control (high L-glucose) (5.5 mmol/L d-glucose plus 12 mmol/L L-glucose) for 48 hours in growth medium before and during serum withdrawal. The percentage of apoptotic nuclei was assessed 20 hours after serum withdrawal by staining with H33342. Data are expressed as mean±SEM (n=12 per condition, *P<0.0001).

**Figure 4.** High d-glucose concentrations significantly inhibit VSMC apoptosis induced by Fas ligand. VSMCs were exposed to normal (5.5 mmol/L) or high (17.5 mmol/L) d-glucose for 48 hours in growth medium before and during treatment with Fas ligand (100 ng/mL) in 2% FBS. The percentage of apoptotic nuclei was assessed 20 hours after Fas ligand treatment by staining with H33342. Data are expressed as mean±SEM (n=12 per condition, *P<0.006).

**Figure 5.** High glucose significantly enhances PKC activity. High glucose–induced PKC activity is blocked by long-term PMA treatment or with calphostin C. VSMCs were exposed to 5.5 or 17.5 mmol/L glucose for 22 hours, and PKC activity was assessed by determining the picomoles of phosphate incorporated into substrate peptide per minute per milligram of protein. A subset of VSMCs were pretreated with 100 nmol/L PMA 24 hours before exposure to high glucose or with calphostin C (1 μmol/L) (30-minute pretreatment to high glucose) to downregulate the diacylglycerol-sensitive PKC isoforms. Data are expressed as mean±SEM (n=4 to 7 per condition, *P<0.001).
normal glucose plus calphostin C 31±1%, high glucose plus vehicle 23±2%, high glucose plus calphostin C 31±1%; n=8, P<0.001).

Upregulation of PKCα and PKCβ Is Sufficient to Inhibit VSMC Apoptosis

To determine whether the activation of PKC was a sufficient stimulus to inhibit VSMC apoptosis, we used a gene transfer strategy to transiently upregulate PKCα, PKCβII, or PKCδ. We chose to upregulate these isoforms on the basis of findings from our laboratory and others that these isoforms are preferentially upregulated in VSMCs either in vivo or in vitro in response to elevated glucose levels (Figure 7).19,23–28 As shown in Figure 8, the upregulation of PKCα or PKCβII significantly inhibited apoptosis induced by serum withdrawal compared with transfection with control vector. In contrast, upregulation of PKCδ had no significant effect (control 18±3%, PKCδ 16±1%; n=9, NS). These findings suggest that activation of the PKC pathway is sufficient to protect VSMCs from apoptosis induced by serum withdrawal.

Discussion

Macrovascular disease is the major cause of mortality in the diabetic patient population.1–3 However, the pathogenic mechanisms by which disturbances in glucose metabolism promote vascular lesion formation have remained poorly defined. The majority of studies focusing on this pathophysiological process have emphasized the proliferative effects of the altered metabolic milieu on VSMC growth.5,6 However, we hypothesized that a significant component of the abnormal accumulation of cells in diabetic macrovascular disease involves an inhibition of VSMC death as well as increased cell proliferation. In accord with our hypothesis, the present study is the first to demonstrate that the well-described induction of VSMC death triggered by a reduction in blood flow is markedly attenuated in insulinopenic diabetic mice compared with nondiabetic control mice. These findings suggest an intriguing link between abnormal glucose metabolism and the regulation of apoptosis within the vessel wall.

To more fully delineate the component of the diabetic state responsible for the antiapoptotic effect, we used an in vitro model system. In parallel with our in vivo findings, exposure to high ambient concentrations of extracellular glucose prevented VSMCs from undergoing apoptosis induced by either serum withdrawal or Fas ligand. Experimental controls with the nonmetabolized enantiomer D-glucose confirmed that this antiapoptotic effect required intracellular metabolism of glucose and did not reflect the potential influence of osmolar stimuli. We cannot rule out the possible influence of other circulating factors in the streptozotocin-diabetic model as...
of these mediators as determinants of VSMC survival in the context of diabetes.

The ability of altered glucose metabolism to influence the regulation of apoptosis was only recently defined. Hyperglycemia has been shown to induce apoptosis in preimplantation embryos as well as cultured endothelial cells and pericytes. To our knowledge, the present study is the first to demonstrate an effect of hyperglycemia on VSMC fate. Intriguingly, the antiapoptotic response we observed is diametric to previous reports in other cell types. It is well established that the regulation of apoptosis by various stimuli is often cell specific and contextual. Moreover, it is conceivable that the intracellular signaling pathways that regulate the ability of glucose to influence cell fate may also be cell type specific. Indeed, recent studies in our laboratory and others have documented that vasoactive substances and cytokines may have opposing effects on cell fate in endothelial cells versus VSMCs.

Previous work from our laboratory is consistent with the postulate that the inhibition of VSMC apoptosis may be a necessary condition for vascular lesion formation. In accord with this postulate, the present study demonstrates for the first time that the vasculopathic effect of diabetes is associated with the inhibition of VSMC apoptosis in vivo. Future investigations beyond the scope of the present study will be needed to dissect the role of hyperglycemia-induced inhibition of VSMC apoptosis in the process of vascular remodeling. We speculate that hyperglycemia induces an expansion of vascular lesions via several cellular mechanisms, including increased extracellular matrix production and VSMC proliferation, in addition to the inhibition of VSMC apoptosis. It is intriguing that glucose-mediated activation of certain signaling pathways, such as PKC, may have bifunctional effects in the promotion of cell growth as well as the inhibition of cell death.

In conclusion, our findings indicate that hyperglycemia inhibits VSMC apoptosis in vitro and in vivo. This antiapoptotic effect of high glucose is mediated in part via the activation of PKC. We have further demonstrated that PKC functions as a necessary and sufficient signaling element that links perturbations in cellular glucose metabolism to the regulation of VSMC fate. These studies may have important implications for understanding diabetic macrovascular biology and could lead to novel therapeutic strategies to reduce the incidence or acceleration of vascular complications in the diabetic population.

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References


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