High Glucose Inhibits Apoptosis Induced by Serum Deprivation in Vascular Smooth Muscle Cells via Upregulation of Bcl-2 and Bcl-xl

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Apoptosis plays a critical role in normal vascular development and atherosclerosis. To test the hypothesis that diabetic vasculopathy may be due in part to altered apoptosis pathways, we investigated the effects of high glucose treatment on serum withdrawal-induced apoptosis, expression of Bcl-2 family members, and inhibitor of apoptosis protein (IAP)-1 in vascular smooth muscle cells (VSMCs). Treatment with a high concentration of glucose (22 mmol/l) significantly attenuated apoptosis in response to serum withdrawal in cultured rat VSMCs compared with cells treated with a normal glucose concentration (5.5 mmol/l). This attenuation was accompanied by a significant decrease in the caspase-3 activity in comparison with the normal glucose group. Furthermore, exposure of VSMCs to high glucose markedly increased the abundance of Bcl-2 and Bcl-xl mRNAs compared with treatment with normal glucose, while expression of bax and IAP-1 mRNA remained unchanged. Our results suggest that high glucose suppresses serum withdrawal-induced apoptosis in VSMCs by upregulating expression of Bcl-2 and Bcl-xl, suggesting that enhanced expression of antiapoptotic proteins may play an important role in the development of macrovascular complications in diabetes. Diabetes 54:540–545, 2005

Poor control of diabetes is associated with the development of micro- and macroangiopathy (1,2). Hyperglycemia in diabetic patients is believed to play a major role in the development of these vascular complications (3). In diabetic patients with atherosclerotic coronary artery disease, vascular lesions are particularly marked by exuberant vascular smooth muscle cell (VSMC) proliferation. The proliferative lesions may be due to enhanced cell division or decreased cell loss due to apoptosis.

The pathogenesis of macrovascular disease is characterized by the excessive accumulation of cells within the intima and media (4). Although studies (5) have emphasized that this accumulation attributed to alterations in the regulation of cell proliferation, recent studies (6,7) have clearly demonstrated that dysregulated apoptosis also plays a pivotal role in the pathogenesis and progression of macrovascular disease.

Apoptosis is a naturally occurring process through which multicellular organisms dispose of cells. The process involves the sequential activation of cysteine proteases known as caspases, resulting in protein cleavage and breakdown of DNA molecules (8). In response to a variety of stimuli, cells have an intrinsic capacity to activate gene-directed programs that commit the cell to die (9). Much has been discovered about the molecular control of apoptosis along with the identification of caspases. It is now well established that members of the Bcl-2 family are critical regulators of apoptosis in VSMCs (10). While human and animal VSMCs express Bcl-2 (11,12), expression of several other family members such as Bcl-xl, Bcl-xs, and bax have also been reported (12–14) in models of atherosclerosis and injury. In addition, growth factor, withdrawal-induced apoptosis can be inhibited by overexpression of Bcl-2 (15). Together, these findings indicate the potential role of Bcl-2 in regulating VSMC apoptosis. Recently, Erl et al. (16) have also shown that VSMCs grown at high density are protected by an antiapoptotic mechanism that involves increased expression of inhibitor of apoptosis protein (IAP)-1. Taken together, these observations have important implications for understanding the roles of Bcl-2 and IAP-1 in the pathogenesis of vascular disease.

Considering that apoptotic effectors have been implicated in the development of proliferative vascular diseases, we investigated a mechanistic link between hyperglycemia-induced cellular proliferation and regulation of apoptosis in VSMCs. The present study was designed to determine the effects of extracellular glucose on serum withdrawal-induced apoptosis, caspase-3 activity, and cellular expression of Bcl-2 family proteins in VSMCs following exposure to high concentration of glucose.

RESEARCH DESIGN AND METHODS

Cell culture and treatment. Clonal A7r5 rat aortic VSMCs (American Type Culture Collection) at low passage (3–5) were used in this study. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) in the presence of 10% fetal bovine serum (FBS). Cells were cultured on 100-mm plates to near confluence, washed twice with phosphate-buffered saline (PBS), and serum starved for 18–24 h. Cells were then washed three times with PBS and treated with DMEM, DMEM containing 22 mmol/l glucose, or DMEM containing 2.5 mmol/l (normal) glucose in serum-free DMEM. After 18 h, cells were washed once more with PBS, and apoptosis was determined by annexin V staining, TUNEL labeling, or caspase-3 activity.

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5.5 mmol/l glucose (normal glucose), 22 mmol/l glucose (high glucose), or 5.5 mmol/l glucose plus 16.5 mmol/l mannitol (mannitol) for an initial period of 48 h. These glucose concentrations are in a range that is relevant in diabetic humans. Forty-eight hours after plating, the culture medium was replaced with DMEM deprived of fetal bovine serum and supplemented with glucose or mannitol for an additional 48 h to induce apoptosis. Preliminary data showed that there was a trend toward inhibition of apoptosis after 24 h, but this did not reach statistical significance (data not shown). Therefore, we used all cells after a 48 h period of serum withdrawal.

**Determination of apoptosis.** Terminal deoxyribonucleotid transferase-mediated dUTP nick-end labeling (TUNEL) and caspase-3 activity assays were used as quantitative indexes of apoptosis.

**TUNEL assay.** Apoptosis was detected using fluorescein isothiocyanate-labeled TUNEL assay (Roche, Mannheim, Germany). Briefly, cells were seeded onto chamber slides (Nalge Nunc International) in the presence of normal glucose, high glucose, or mannitol. Cells were then fixed with 4% paraformaldehyde and permeabilized by 0.1% Triton X-100 48 h after serum was removed from the medium. Cells were then incubated with fluorescein isothiocyanate-labeled TUNEL reaction mixture at 37°C for 1 h in the dark. The slides were counterstained with propidium iodide for 15 min on ice. Negative controls were obtained by omitting terminal deoxynucleotidyl enzyme and using the same volume of label solution. Positive controls were obtained by treating cells with DNase I (1,000 units/ml, 37°C) before labeling procedures. Samples were analyzed by Laser Scan Cytometer (CompuCyte, Cambridge, MA), and the data are expressed as the percentage of TUNEL-positive cells relative to total cell population in each group.

**Caspase-3 activity assay.** The activity of caspase-3 was determined using fluorometric assay. In brief, cells were lysed in a buffer containing 50 mmol/l Tris-HCl, 150 mmol/l NaCl, and 0.5 mmol/l EDTA 48 h after serum withdrawal in the presence of normal glucose, high glucose, or mannitol. A total of 160 μg of total protein was added to 100 μl reaction buffer (containing 10 mmol/l dithiothreitol) and 5 μl of substrate of DEVD-AFC (Biosource International, Camarillo, CA). Samples were incubated at 37°C for 3 h, and then the enzyme-catalyzed release of AFC was quantified with a fluorescent microplate reader with a 400-nm excitation filter and 505-nm emission filter. The values of high glucose– and mannitol-treated samples were normalized to corresponding normal glucose control, and the amount of change in caspase activity was determined.

**Determination of Bcl-2 family members and IAP-1 by RT-PCR and real-time quantitative RT-PCR.**

**RT-PCR.** Total RNA was isolated from cells 48 h after being cultured in serum-free DMEM containing normal glucose, high glucose, or mannitol, using an RNA isolation kit (Gibco, Gaithersburg, MD). cDNA was synthesized from 3 μg of total RNA with reverse transcriptase (SuperScript II; Gibco-BRL). Real-time PCR was performed at 42°C for 2 min following incubation at 95°C for 5 min. cDNA amplification was carried out according to the following temperature profile: 95°C, 30 s; 60°C, 1 min; and 72°C, 1 min. At the end of 32 cycles, the reaction was prolonged for 10 min at 72°C, and then 5 μl of the reaction was added on a 1.5% agarose gel. The following were the sequences of primers: for IAP-1: 5'-ACACGTGTCCAG-3' and 5'-ACAGTGTCCAG-3'; for bax: 5'-AGTTTACCCCATCCCGAAAG-3' and 5'-AGTTTACCCCATCCCGAAAG-3'; and for G3PDH: 5'-ACACGAGGATGATTGCTGAC-3' and 5'-GCTCGAATTACCGCGGCT-3'.

**Real-time quantitative RT-PCR.** Real-time quantitative RT-PCR was performed on cDNA generated from 0.3 μg of total RNA using reverse transcriptase (SuperScript II; Gibco-BRL). A total of 30 ng of cDNA, 200 mmol/l of both sense and antisense primers, and SYBR Green PCR Master Mix (PE Applied Biosystems) in a final volume of 25 μl were used for PCR. Amplification and detection of specific products were performed with the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems) with the following profile: 1 cycle at 48°C for 30 min, 1 cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. As an internal control, 18S primers 5'-CCGCTTACACATCCCAAGGAA-3' and 5'-GCTGTAATTTACCGCGGCT-3' were used for RNA template normalization.

Fluorescent signals were normalized to an internal reference (ΔCt), and the threshold cycle (Ct) was set within the exponential phase of the PCR. The relative gene expression is calculated by comparing cycle times for each target PCR. The target PCR Ct values were normalized by subtracting the 18S Ct value, which gives the ΔCt (Ct). From this value, the relative expression level between treatments can be calculated using the following equation: relative gene expression = 2^- [(ΔCt sample−ΔCt control)/2].

**Determination of Bcl-2 family proteins by Western blot analysis.** Cells were harvested 48 h after being cultured in serum-free DMEM containing normal glucose, high glucose, or mannitol and lysed in a lysis buffer (10 mmol/l Tris-HCl, 1 mmol/l EDTA, 150 mmol/l NaCl, 1% Triton X-100, and protease inhibitor cocktails). The suspension was sonicated for 10 s and then centrifuged at 300g for 10 min at 4°C. Supernatants were collected, and the protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL). A total of 30 μg protein of cellular extracts were separated by 4–20% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was then incubated with anti-Bcl-2, anti-Bax, or anti-bax antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight and with a secondary antibody at a dilution of 1:8,000 for 60 min. Immune complexes were detected with the enhanced chemiluminescence SuperSignal West Pico System (Pierce, Rockford, IL). Results were quantified with a scanning densitometer using an image analysis system with National Institutes of Health Image 1.55 Software (Image Research, St. Catharines, Canada).

**Statistical analysis.** Data are presented as means ± SE of three independent experiments. Values represent the percentage of TUNEL-positive cells relative to total cell population in each group. In this and subsequent figures, • represents results for cells grown in normal glucose (low), and □ represents cells exposed to mannitol (mannitol). B: Effect of high glucose concentration on caspase-3 activity in VSMCs. Data are expressed as means ± SE of three independent experiments. Values represent the percentage of caspase-3 activity of high glucose or mannitol treatment versus the normal glucose group. *P < 0.05 vs. low; **P < 0.01 vs. low.

**RESULTS**

High glucose concentration inhibits VSMC apoptosis. TUNEL and caspase-3 activity assays were performed to characterize the effect of high glucose treatment on serum withdrawal–induced apoptosis. Results are presented in Fig. 1. Treatment with a high glucose (22 mmol/l) significantly attenuated apoptosis in response to serum withdrawal in cultured rat VSMCs compared with cells treated with a normal glucose (5.5 mmol/l) (13.1 ± 2.7% vs. 22.6 ± 1.8%, n = 3, P < 0.01). Treatment of the cells with mannitol, used as an osmotic control, had no significant effect on apoptosis in rat VSMCs (23.6 ± 2.2%, n = 3, Fig. 1A).

Caspase-3 activity was assayed in VSMCs after exposure...
to normal glucose concentration and is presented in Fig. 1B. There was a significant decrease in the caspase-3 activity (17%) in VSMCs cultured with high glucose when compared with the normal glucose group \((P < 0.05, n = 3)\). In contrast, caspase-3 activity did not change significantly in cells cultured in the presence of mannitol.

**High glucose concentration upregulates the expression of Bcl-2 and Bcl-xl but not bax and IAP-1.** Bcl-2 belongs to a large family of homologous proteins that can either promote or suppress apoptosis. Figure 2 depicts the effect of high glucose treatment on gene expression of Bcl-2, Bcl-xl, and bax, which have been shown to be expressed in VSMCs.

**mRNA levels of Bcl-2, Bcl-xl, bax, and IAP-1.** PCR analysis using four primers detected a single fragment specific for Bcl-2, Bcl-xl, bax, and IAP-1, respectively (Fig. 2A). Treatment of A7r5 cells with high glucose for 48 h markedly increased mRNA expression of Bcl-2 and Bcl-xl compared with treatment using normal glucose. The Bcl-2 and Bcl-xl mRNA levels were not altered by mannitol treatment. mRNA levels of bax and IAP-1 were significantly increased after exposure to high glucose for 48 h when compared with the normal glucose group. However, the gene expression levels of bax and IAP-1 were also increased by mannitol treatment, suggesting that osmolarity changes in the media may be responsible for those increases.

These PCR findings were confirmed by real-time quantitative RT-PCR analysis and presented in Fig. 2B. The relative gene expression level of Bcl-2 increased by 2.62-fold in cells treated with high glucose when compared with the normal glucose group \((P < 0.05, n = 3, \text{Fig. 2B})\). Similarly, the relative gene expression level of Bcl-xl was significantly increased by 2.64-fold in the presence of high glucose \((P < 0.05, n = 3, \text{Fig. 2B})\). As observed with PCR analysis, the relative gene expression levels of bax and IAP-1 were increased by both high glucose and mannitol treatment (Fig. 2B).

**Expression of Bcl-2 family proteins.** In Fig. 3, immunoblot analysis shows that there was an increase in expression of Bcl-2 and Bcl-xl protein in cells treated with high glucose when compared with the normal glucose group (Fig. 3A). The expression of these two proteins was not altered by mannitol treatment. Expression of bax protein was similar among all groups.

Quantitative densitometry from three independent experiments revealed that the protein level of Bcl-2 increased by 1.99-fold \((P < 0.05)\) and 1.12-fold in A7r5 cells treated with high glucose and mannitol, respectively, when compared with normal glucose group (Fig. 3B, \(n = 3\)). The protein expression of Bcl-xl was also increased by 1.91-fold \((P < 0.05)\) and 1.06-fold in cells treated with high glucose and mannitol, respectively, when compared with normal glucose group \((n = 3)\), while the protein expression level of bax remained unaltered (Fig. 3B).

**DISCUSSION**

There are three important and distinctive findings in the present study regarding specific effects of hyperglycemia on serum withdrawal–induced apoptotic mechanisms in rat aortic A7r5 cells: hyperglycemia (1) attenuates the rate of apoptosis, (2) attenuates caspase-3 activity, and (3) increases abundance of transcript and protein levels of Bcl-2 and Bcl-xl, two antiapoptotic proteins.

Apoptosis is an active process of cell death characterized by membrane blebbing, cytoplasmic and nuclear condensation, and internucleosomal DNA cleavage (19). The apoptotic mode of cell death has long been known to be a feature of animal development and to occur in adult tissue in a wide range of normal physiological settings, such as metamorphosis, tissue renewal, and many other conditions (19). However, it can also be activated or suppressed in pathophysiological conditions and in response to toxins, as we have previously reported (19). Recent studies (20,21) have suggested that vascular re-
The majority of studies focusing on the pathogenic mechanism of glucose in promoting vascular lesion formation have highlighted the proliferative effects of glucose on VSMC growth (5). In the present study, we hypothesized that the excessive accumulation of cells in diabetic macrovascular disease involves an inhibition of VSMC death as well as an increase in cell proliferation. In agreement with our hypothesis, we now demonstrate for the first time that treatment with a high concentration of glucose significantly attenuates apoptosis induced by serum withdrawal in cultured rat VSMCs compared with cells treated with a normal glucose concentration. Notably, the model of serum withdrawal for VSMCs to induce apoptosis is well established and currently used by numerous groups (27–30).

To further define the potential molecular mechanisms by which high glucose treatment modulates serum withdrawal–induced apoptosis, we have investigated expression of pro- and antiapoptotic proteins in VSMCs. The regulation of apoptosis within the cell can be simplified into two major pathways (17). One is initiated from death receptors at the cell surface and is mainly regulated by the tumor necrosis factor superfamily and includes, for example, the fas/CD95 receptor. Upon ligand binding to the death receptor, the receptor activates the apoptosis signaling pathway through activation of caspase-8 (31). The other pathway is triggered by a mitochondrial pathway. Genotoxic agents, ischemia, oxidative stress, growth factor withdrawal, and many other stimuli require mitochondria to activate caspases. Unlike caspase-8, caspase-9 is involved in the mitochondrial pathway response to extracellular cues and internal insults. The proapoptotic signals regulate cytochrome c release from mitochondria and further activate caspase-9 through interaction with Apaf-1 (32,33). Both death receptor and mitochondrial pathways converge at the level of caspase-3 activation. Therefore, our new finding that caspase-3 is suppressed by high glucose treatment is of particular mechanistic importance.

Recently, Bcl-2 family proteins have been proved to be critical regulators of apoptosis. Bcl-2 was originally identified as a translocation breakpoint (t14:18) in human B-cell lymphoma (34). It is now clear that Bcl-2 belongs to a large family of homologous proteins that can either promote or suppress apoptosis. The ratio between Bcl-2 to bax or Bcl-xl to bax or bad determines cell survival or death after apoptotic stimuli. The expression pattern and role of different members of the family appear to be cell specific (10). Pollman et al. (35) have recently demonstrated that the antiapoptotic gene Bcl-xl is upregulated within intimal VSMCs in animal models and human specimens of vascular disease. Moreover, they have also shown that downregulation of Bcl-xl expression within intimal cells using antisense oligonucleotides induces VSMC apoptosis and regression of vascular lesions. A study from Perlman et al. (36) also showed that acutely disrupting Bcl-2 expression in VSMCs altered cell cycle activity through the execution of the apoptosis pathway. These findings indicate that Bcl-xl and Bcl-2 are important determinants of VSMC viability and lesion formation. Our study demonstrates that there is an increased expression of Bcl-xl and Bcl-2 by high glucose treatment in VSMCs, while the expression of bax was affected by both high glucose and mannitol treatments. Therefore, we propose that glucose-induced apoptosis suppression in VSMCs is partly due to upregulation of Bcl-xl and Bcl-2. The mechanisms for Bcl-2 and Bcl-xl antiapoptotic function may include the following: binding to Apaf-1 to prevent the activation of caspase cascade, altering mitochondrial...
membrane potential, and inhibiting cytochrome c release from mitochondria (37–39).

Caspase-3 activity was decreased following exposure to high glucose concentration in our study. To date, over a dozen caspases have been identified, and about two-thirds of these have been suggested to function in apoptosis (40). Caspase-3 is an effector enzyme for induction of DNA damage and for ensuring apoptotic cell death. Caspase function is positively or negatively modified by Bcl-2 family proteins, including Bcl-2, Bcl-xl, and bax (17). Our data suggest that upregulation of Bcl-2 and Bcl-xl may play a key role in the reduction of caspase-3 activity in VSMCs.

Recently, IAPs, a family of endogenous cellular caspase inhibitors, have been identified. They can bind directly to pro-caspase-9 and activated caspase-3, preventing apoptosis (17). Erl et al. (16) have shown that VSMCs at high glucose concentration in our study. To date, over a dozen caspases have been identified, and about two-thirds of these have been suggested to function in apoptosis (40).

Caspase-3 is an effector enzyme for induction of DNA fragmentation and higher prevalence of apoptosis, while we only measured total protein amounts of bax, and our data cannot rule out the possibility that bax is affected by high glucose treatment. Interestingly, a similar protective effect of chronic hyperglycemia on cardiomyocytes was also reported in a model of hypoxia-induced apoptosis and necrosis (47).

It is well known that activation of bax appears to involve subcellular translocation from the cytosol to mitochondria. The signal directing the translocation remains to be elucidated. Regulatory proteins such as Bid and 14-3-3 have been reported to be involved in the modulation of cellular function, and localization of bax (48–50) should be further investigated to clarify the exact role of bax in this model, as it may serve as a point for various apoptotic signals.

Taken together, our findings suggest that VSMC exposure to high glucose leads to increased antiapoptotic events by altering the fine balance between the expression of antiapoptotic and proapoptotic proteins. The relative concentration of these proteins is an important determinant of their final impact on cell fate, and promotion of antiapoptotic events may play an important role in the development of macrovascular complications in diabetes.

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