High glucose inhibits apoptosis in human coronary artery smooth muscle cells by increasing bcl-xL and bfl-1/A1

HIROYA SAKUMA, MAYUMI YAMAMOTO, MIE OKUMURA, TOSHIHIRO KOJIMA, TAKAKO MARUYAMA, AND KEOGO YASUDA
First Department of Internal Medicine, Gifu University
School of Medicine, Gifu 500-8705, Japan
Received 2 December 2001; accepted in final form 28 March 2002

High glucose inhibits apoptosis in human coronary artery smooth muscle cells by increasing bcl-xL and bfl-1/A1. Am J Physiol Cell Physiol 283: C422–C428, 2002. First published April 18, 2001; 10.1152/ajpcell.00577.2001.—Cardiovascular disease is a serious complication in diabetic patients. To elucidate the precise mechanisms of atherosclerosis in diabetic patients, the effects of high glucose concentration (25 mM) on apoptosis regulation and bcl-2 family protein expression in human coronary artery smooth muscle cells (CASMC) were examined. Treatment with a high level of glucose (25 mM) caused a significant decrease in apoptosis in CASMC compared with the same cells treated with a physiologically normal glucose concentration (5.5 mM) (23.9 ± 2.4% vs. 16.5 ± 1.8%; P < 0.01). With respect to apoptosis regulation, treatment of CASMC with high glucose concentration markedly increased mRNA expressions of bcl-xL and bfl-1/A1 compared with cells treated with normal glucose. High glucose induced phosphorylation of phosphatidylinositol 3-kinase (PI 3-K) and extracellular signal-regulated kinase (ERK)1/2 along with bcl-xL and bfl-1/A1 upregulation. These results suggest that high glucose suppresses apoptosis via upregulation of bcl-xL and bfl-1/A1 levels through PI 3-K and ERK1/2 pathways in CASMC. High glucose-induced increase in the expression of antiapoptotic proteins may be important in the development of atherosclerosis in diabetic patients.

diabetes mellitus; atherosclerosis; apoptosis; vascular smooth muscle cells

VASCULAR DISEASE is one of the most serious complications in diabetic patients. It is widely accepted that atherosclerosis is accelerated by the coexistence of diabetes mellitus (11, 31). Hyperglycemia is an important etiologic factor in the development of vascular complications (47).

Increased proliferation of vascular smooth muscle cells (VSMC) is a key feature in the atherosclerotic lesion (15, 35, 38, 41). It is well established that cell growth is a fundamental feature of intimal hyperplasia (34), and it is becoming clear that perturbations in the regulation of apoptosis are equally important (16, 24). Furthermore, apoptosis of VSMC is critically involved in the formation of the fibrous cap and fatty streak that is the lipid-rich core of the atheroma and may therefore contribute to the instability of advanced atherosclerotic plaques (3, 7, 12, 14, 21, 23). Excessive accumulation of VSMC in atherosclerosis suggests reduced apoptosis and excessive cell proliferation in the lesions, because apoptosis and cell proliferation are intimately coupled (10). Although many studies have focused on the mechanisms of VSMC proliferation (34), the regulatory mechanisms of VSMC apoptosis have not been fully elucidated (30).

The role of sugar in atherosclerosis has been investigated in recent studies. Several findings support the concept that hyperglycemia accelerates the development of atherosclerosis (46, 47). Although glucose concentration enhances growth rate in cultured VSMC (17, 33, 49), little is known about the effect of glucose on the regulation of apoptosis in VSMC. Apoptosis is regulated by a genetic program involving the activation and inactivation of specific genes. In particular, dominant protooncogenes such as bcl-2 and tumor suppressor genes such as p53 are potent regulators of apoptosis. Members of the bcl-2 protein family regulate the response of cells to a wide variety of apoptotic signals. At least 15 bcl-2 family members have been identified, and apoptosis is determined by the relative balance of proapoptotic and antiapoptotic members of the bcl-2 protein family (40). bcl-2 proteins were expressed in VSMC (8, 20, 29, 36, 39), and expression of several family members such as bcl-2, bcl-xL, bcl-xs, and bax were modified by apoptosis induction. For example, platelet-derived growth factor (PDGF) reduces bcl-xL and induces bcl-xs (36) and balloon injury induces bcl-xs (20). Recent reports demonstrated that p53 (3), phosphatidylinositol 3-kinase (PI 3-K; Ref. 1), and mitogen-activated protein kinase (MAPK; Refs. 19, 48) are also involved in apoptosis regulation of VSMC. MAPKs, which constitute an ubiquitous group of serine/threonine kinases, are thought to play a crucial role in transmitting transmembrane signals required for cell growth, differentiation, and apoptosis. However, the effect of glucose on the signal transduction
pathway that regulates the apoptosis regulatory genes, MAPKs, and PI 3-K remains to be further elucidated. We have shown that acute extracellular high glucose concentration conditions stimulate cell proliferation through alteration of cell cycle regulation (49). In the present study, we investigated acute effects of extracellular high glucose concentration on apoptosis, expression of bcl-2 family proteins, and activation of PI 3-K and MAPKs.

METHODS

Chemicals. Smooth muscle basal medium (SMBM) and smooth muscle growth medium-2 (SMGM-2) were purchased from BioWhittaker (Walkersville, MD). Dulbecco's phosphate-buffered saline (DPBS) was purchased from Gibco-BRL (Gaithersburg, MD). Fetal bovine serum (FBS) was purchased from Sigma (St. Louis, MO). The annexin V-FITC kit was purchased from Bender Med Systems (Vienna, Austria) for flow cytometry analysis. All other chemicals used in the present study were of reagent grade.

Cells and cell culture. Primary cultured human coronary artery smooth muscle cells (CASMC) were purchased from BioWhittaker. The cells were grown to confluence in SMBM with SMGM-2 containing 5.5 mM glucose, 5% FBS, 50 μg/ml gentamicin, 50 ng/ml amphotericin-B, 5 μg/ml insulin, 2 ng/ml human recombinant fibroblast growth factor (hFGF), and 0.5 ng/ml human recombinant epidermal growth factor (hEGF) at 37°C in a humidified 5% CO₂-95% air atmosphere according to the company's recommendations. Cells were grown to >80% confluence and used within five passages.

DNA fragmentation assay. Cells in secondary cultures maintained in SMGM-2 containing 5% FBS were trypsinized and seeded on six-well plates. When cells reached 80% confluence, culture medium was replaced with medium containing 3% FBS and 5.5 mM glucose (normal), 25 mM glucose (high glucose), or 5.5 mM glucose plus 19.5 mM mannitol (osmotic control corresponding to high-glucose medium) for 72 h. Glucose concentration of culture medium was monitored and adjusted every day. DNA fragmentation assays were performed by the method of Sellins and Cohen (43) as follows. Cells were washed with DPBS, trypsinized, and centrifuged at 110 g for 5 min to remove trypsin. The cell pellets were lysed with 0.4 ml of hypotonic lysis buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) containing 0.5% Triton X-100, and the lysates were centrifuged at 13,000 g for 10 min to separate intact from fragmented chromatin. The supernatants, containing fragmented DNA, were placed in separate microfuge tubes, and both pellet and supernatant were precipitated overnight at 4°C in 12.5% trichloroacetic acid (TCA). The precipitates were sedimented at 13,000 g for 4 min. The DNA in the precipitates was hydrolyzed by heating to 90°C for 10 min in 50 μl of 5% TCA and was quantified by the modified method of Burton (5) as follows. Briefly, 100 μl of diphenylamine (DPA) reagent (150 mg DPA, 150 μl sulfuric acid, 50 μl acetaldehyde (16 g/l stock)/10 ml glacial acetic acid) was added to each tube. After overnight color development, 200 μl of each sample was transferred to the wells of diphenylamine re-
Effect of high glucose concentration on apoptosis of human CASMC: glucose-induced suppression of CASMC apoptosis. To characterize the effect of glucose on apoptosis regulation in human CASMC, DNA fragmentation measurement and flow cytometric analysis were performed. Although DNA fragmentation was observed after exposure to normal glucose concentration, there was a significant decrease in the DNA fragmentation ratio of CASMC cultured at high glucose concentration (72-h exposure; 23.9 ± 2.4% vs. 16.5 ± 1.8%, P < 0.01). Mannitol, an osmotic control (20.8 ± 1.78%), had no significant effect on DNA fragmentation in CASMC (Fig. 1).

DNA fragmentation, a process that results from the activation of endonucleases, is one of the later steps during the apoptotic program. By contrast, flow cytometric analysis with FITC-conjugated annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation. Because annexin V is a Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for phosphatidylserine (PS), it binds to cells with exposed PS. In apoptotic cells, the membrane PS is translocated from the inner to the outer leaflet of the plasma membrane, which is one of the earliest apoptotic morphological features. Flow cytometric analysis data are shown in Fig. 2. An exposure to high glucose concentration suppressed apoptosis in a time-dependent manner relative to normal glucose treatment (72-h exposure; 55.4 ± 6.0% vs. 39.9 ± 6.7%, P < 0.01).

Effect of high glucose on phosphorylation of ERK1/2 and PI 3-K: activation of ERK1/2 and PI 3-K followed by high-glucose exposure in CASMC. To determine whether upregulation of bcl-2 family protein contributed to inhibition of human CASMC apoptosis, we used Western blot and reverse transcription (RT)-PCR. Western blot demonstrated that high glucose upregulates bcl-xL and bfl-1/A1 but not bax. However, bcl-2 was not detected in CASMC (Fig. 3). Treatment of human CASMC with a high glucose concentration for 24 h markedly increased mRNA expression of bcl-xL and bfl-1/A1 compared with treatment with normal glucose (Fig. 4). However, bfl-1/A1 and bcl-xL mRNA levels were not altered by mannitol treatment.

Effect of high glucose on phosphorylation of ERK1/2 and PI 3-K: activation of ERK1/2 and PI 3-K followed by high-glucose exposure in CASMC. Western blot analysis demonstrated that high glucose (25 mM)
treatment for 24 h increased phosphorylation status of ERK1/2 and PI 3-K (Fig. 5). However, the osmotic control (19.5 mM mannitol + 5.5 mM glucose) did not increase the phosphorylation status of ERK1/2 and PI 3-K.

**DISCUSSION**

The present study provides evidence that expression of apoptosis in human CASMC is significantly suppressed by high glucose exposure. Apoptosis, or prog...
Grammed cell death, consists of a distinct form of cell death that displays characteristic alterations in cell morphology and cell fate. From the standpoint of tissue structural remodeling, apoptosis might be considered to be one of the most important mechanisms that counterbalance the effect of cell proliferation by mitotic division (24). Because normal VSMC possess the machinery to undergo apoptosis, apoptosis may be a means of cell number regulation in the vessel wall. Vascular lesions, such as atherosclerosis, arise in part from excessive accumulation of VSMC, which may suggest reduced apoptosis in the lesions. Although the processes of vessel remodeling and cell death appear to be opposing, apoptosis and cell proliferation are intimately coupled (10). For example, Diéz et al. (8) documented that VSMC of spontaneously hypertensive animals show an increased expression of bcl-2 that acts as antiapoptotic protein. In hypertension, VSMC replication is increased but it is not counterbalanced by increased apoptosis, resulting in thickening of the media of arteries and arterioles. This indicates that the factors responsible for increased DNA synthesis also account for the relative resistance against apoptosis. Yamamoto et al. reported (49) that acute high glucose exposure increases VSMC proliferation through cell cycle progression, which may contribute to abnormal wall thickening in the hyperglycemic condition. However, it is not clear whether the counterbalance of VSMC proliferation and apoptosis is altered by high glucose at the early stage of atherosclerosis, when abnormal VSMC proliferation is accelerated by high glucose.

The significance and roles of apoptosis in atherosclerosis may depend on the stage of the plaque, its localization, and the cell types involved. Apoptosis has been demonstrated to occur with increased frequency in human atherosclerotic plaques and may contribute to plaque rupture and thrombosis (3, 12, 14, 21). However, the frequency of apoptosis in the early stage of atherosclerosis and the thickening of arterial wall with accelerated VSMC proliferation are mostly unknown. We hypothesize that the extent of apoptosis at early-stage (committed phase) atherosclerosis might be low compared with advanced-stage (executive phase) atherosclerosis, because apoptosis expression in human atherosclerotic plaques was much different from apoptosis in adjacent nonatherosclerotic intima (23). In cultured endothelial cells (EC), high glucose concentration was found to trigger apoptosis (2). Although different mechanisms protect EC and VSMC against apoptosis in the normal artery, whether VSMC apoptosis is altered by high glucose is not fully elucidated. Therefore, in this report we investigated the acute effect of high glucose concentration on apoptosis regulation in human CASMC. High-glucose-induced suppression of CASMC apoptosis might inversely link with high-glucose-induced cell proliferation and result in the abnormal balance between cell replication and cell death to accelerate cardiovascular disease in diabetes after hyperglycemia-induced EC apoptosis. Glucose-induced alteration of VSMC apoptosis should be important as an initial event of vascular injury and thickening of the media of arteries and arterioles as demonstrated in the abnormal VSMC growth in hypertension (8).

Another finding of the present study is that high-glucose treatment stimulates expression of bcl-xL and bfl-1/A1, members of the bcl-2 gene family, in human CASMC. It is now well established that members of the bcl-2 family are critical regulators of apoptosis in a variety of cell types (40). Although the expression pattern and role of different members of the family appear to be cell specific, precise regulation of the family members in VSMC has not been fully elucidated. In rat VSMC, alterations in bcl-2 expression are accompanied by apoptosis induction (29). When VSMC apoptosis was induced by PDGF (36), bcl-xL expression was reduced and bcl-x expression was increased, suggesting the antiapoptotic role of bcl-xL in VSMC. Pollman et al. (39) demonstrated that vascular lesion formation is associated with an upregulation of antiapoptotic gene bcl-xL within intimal VSMC in animal models and human specimens and that downregulation of bcl-xL expression with antisense oligonucleotides induces VSMC apoptosis and regression of vascular lesions. Igase et al. (20) demonstrated that bcl-xL was not detected in rat carotid artery and balloon injury-induced bcl-x mRNA expression. Although they concluded that the selective induction of bcl-x expression is a key regulator of rat VSMC apoptosis, the specific function of bcl-2 family members may be different in each cell species. Our data indicated that high glucose concentration led to specific induction of bcl-xL in human CASMC, and glucose-induced alteration of bcl-xL is a key step for glucose-induced apoptosis suppression. On the other hand, the mechanism and the role of bfl-1/A1 expression in VSMC are unknown. bfl-1/A1 is expressed in the bone marrow and at a low level in some other tissues, and the expression level of bfl-1/A1 gene and the development of stomach cancer were correlated (6), suggesting the involvement of bfl-1/A1 in the regulation of cell survival. In epithelial cells, bfl-1/A1 suppresses p53-induced apoptosis (9) and may play an important role in cell survival of lymphocytes (28). In our observations, high glucose also stimulates phosphorylation of PI 3-K and ERK1/2 in CASMC. The signaling pathways involved in the antiapoptotic effect of growth factor with intrinsic tyrosine kinase activities are known, such as activation of PI 3-K and ERK1/2 by insulin-like growth factor-1 in PC12 cells (37). Because the PI 3-K and ERK1/2 pathways converge at some point before activation of MAPK kinase, a MAPK activator (22), the mechanisms activated by exposure to high glucose levels remain unclear.

Our observations indicate that apoptosis in human CASMC may be suppressed by high glucose via alterations of high glucose-induced bcl-xL and bfl-1/A1 expression. Recently, it was reported that nuclear factor (NF)-κB is a downstream target of the MAPK cascade (42) and that high glucose increased NF-κB activity and modified tumor necrosis factor (TNF)-α-induced transcriptional factor NF-κB activation in porcine VSMC (50). Glucose-derived advanced glycation end-
products (AGE) have been shown to accumulate in diabetic tissues and have an effect of acceleration of atherosclerosis induced by glucose toxicity (44). Lander et al. (27) demonstrated that AGE activates NF-κB in rat VSMC. Recently, it was demonstrated that bfl-1/A1 is a direct transcriptional target of NF-κB in B lymphocytes (51) and that NF-κB induces bfl-1/A1 expression and suppress apoptosis (45). It is known that the high glucose effect activates NF-κB, which induces its direct transcriptional target, bfl-1/A1, and results in apoptosis suppression induced by alteration of bfl-1/A1 function in apoptosis regulation in human CASMC. On the other hand, several lines of evidence have implicated increased protein kinase C (PKC) activity initiated by hyperglycemia as a key player in the pathological effects of high glucose in diabetic cardiovascular tissues (25). Recently, Hall et al. (13) demonstrated that hyperglycemia inhibits VSMC apoptosis through a PKC pathway. PKC mediates high-glucose-induced inhibition of synthesis of nitric oxide (NO) (32), which induces apoptosis in VSMC (19), and hence glucose-induced PKC elevation might inhibit apoptosis through suppression of NO production. Because bcl-2 regulates an antioxidant pathway for preventing apoptosis (18), it is acceptable that glucose-modified NO production may alter bcl-2 family functions, including bcl-xL and bfl-1/A1, enough to alter apoptosis expressions in human CASMC. These signal pathways involving PKC, AGE, NF-κB, or NO synthesis may act in both cell proliferation and apoptosis. Further precise mechanisms in regulation of apoptosis and cell proliferation modified by high glucose, however, should be elucidated.

In conclusion, these results showed that high glucose concentration mediates an increase of abnormal cell proliferation, and this may be regulated by suppression of apoptosis related to upregulation of bcl-xL and bfl-1/A1 expression through PI 3-K and MAPK pathways in human CASMC. This suggests that glucose-modified expression of antiapoptotic members of the bcl-2 family proteins may play an important role in the development of atherosclerotic lesion leading to cardiovascular disease in diabetic patients. Identification of mechanisms that regulate high glucose concentration-induced apoptosis inhibition might provide a new target for further therapeutic intervention to prevent vascular complications in diabetic patients.

We thank Masao Takemura for kindly providing flow cytometry analysis. We are also grateful to Dr. Denise R. Cooper for valuable discussion. This work was supported by grants from the Uehara Memorial Foundation (M. Yamamoto), Yamanouchi Diabetes Foundation (M. Yamamoto), and the Ministry of Education, Science, Japan (no. 12671105, M. Yamamoto).

REFERENCES


23. Kock MM, De Meyer GRY, Muhring J, Jacob W, Bult H, and Herman AG. Apoptosis and related proteins in different


