Phosphorylation of p38 Mitogen-Activated Protein Kinase Downstream of Bax-Caspase-3 Pathway Leads to Cell Death Induced by High D-Glucose in Human Endothelial Cells

Hironori Nakagami,[1] Ryuichi Morishita,[1,2] Kei Yamamoto,[1] Shin-ichi Yoshimura,[3] Yoshiaki Taniyama,[1] Motokuni Aoki,[1] Hiroaki Matsubara,[4] Shokei Kim,[5] Yasufumi Kaneda,[2] and Toshio Ogihara[1]

Because high D-glucose significantly stimulates endothelial cell death, we examined the molecular mechanisms of high D-glucose–induced endothelial apoptosis. Treatment of human aortic endothelial cells with high D-glucose (25 mmol/l), but not mannitol and L-glucose, resulted in a significant decrease in cell number and a significant increase in apoptotic cells as compared with a physiological concentration (5 mmol/l). Interestingly, high D-glucose treatment significantly increased bax protein, accompanied by translocation of bax protein from cytosol to mitochondria-enriched heavy membrane fraction. In contrast, the expression and distribution of bcl-2 protein were not altered by high D-glucose. In addition, the activity of caspase-3 proteases was increased after exposure to high glucose, whereas caspase inhibitors prevented endothelial cell death induced by high D-glucose. On the other hand, p38 mitogen-activated protein kinase (MAPK) was markedly phosphorylated and showed sustained phosphorylation after stimulation. A specific inhibitor of p38 MAPK, SB 203580, and the overexpression of kinase-inactive p38 MAPK significantly attenuated cell death induced by high D-glucose in human aortic endothelial cells, whereas at 6 h after high D-glucose treatment, SB 203580 and overexpression of kinase-inactive p38 MAPK did not attenuate caspase-3 activation induced by high D-glucose. Importantly, caspase inhibitors significantly attenuated the sustained phosphorylation of p38 MAPK induced by high D-glucose. Thus, we finally focused the MAPK kinase (MEK) kinase 1 (MEKK1) to further examine the crosstalk between p38 MAPK and the bax-caspase proteases pathway. High D-glucose treatment induced MEKK1 cleavage, whereas caspase inhibitors significantly attenuated the cleavage. Importantly, kinase-inactive MEKK1 also blocked the phosphorylation of p38 MAPK induced by high D-glucose. Here, we demonstrated that high D-glucose induced apoptosis in human endothelial cells through activation of the bax-caspase proteases pathway and through phosphorylation of p38 MAPK mediated by MEKK1. Phosphorylation of p38 MAPK downstream of the bax-caspase pathway may play a pivotal role in endothelial apoptosis mediated by high D-glucose. *Diabetes* 50:1472–1481, 2001

Diabetes is characterized by the premature development of microvascular and macrovascular disease (1–4). In addition, hyperglycemia is an independent risk factor for the development of cardiovascular disease. Currently, it is apparent that dysfunction of endothelial cells may promote abnormal vascular growth, such as that seen in atherosclerosis and arteriosclerosis. However, little is known about how endothelial dysfunction occurs in diabetes. Although D-glucose or insulin affects cell growth through the expression of numerous growth factors, such as vascular endothelial and hepatocyte growth factors in some organs (5–7), we especially focused on the effects of high D-glucose on endothelial cell death using an in vitro culture system. The fact that glucose uptake by vascular cells is largely insulin-independent renders vascular cells vulnerable to glucose-induced injury when the extracellular glucose concentration is elevated (1–4). Previously, we and others (7–9) have demonstrated that high D-glucose treatment stimulated endothelial cell death in a culture model. Proliferation and cell death are considered two mechanically related phenomena. An emerging body of evidence has revealed that cells are programmed to commit suicide by default and require specific extracellular factors to survive (10,11). In particular, a recent report has documented the presence of apoptosis in endothelial cells treated with high D-glucose (12). Nevertheless, the molecular events linking high D-glucose with the apoptotic machinery of the cell have not been elucidated in endothelial cells.

On the other hand, a variety of key events in apoptosis focus on mitochondrial function, including the release of caspase activators (such as cytochrome c), changes in electron transport, loss of mitochondrial transmembrane

From the Departments of [1]Geriatric Medicine and [2]Gene Therapy Science, Osaka University Medical School, Suita; the [3]Department of Neurosurgery, Gifu University School of Medicine, Gifu; the [4]Department of Second Internal Medicine, Kansai Medical College, Moriguchi; the [5]Department of Pharmacology, Osaka City Medical College, Osaka, Japan.

Address correspondence to Ryuichi Morishita, M.D., Ph.D. Associate Professor, Department of Geriatric Medicine, Osaka University Medical School, Yamada-oka, Suite 565, Japan. E-mail: morishit@gts.med.osaka-u.ac.jp.

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Ac-DEVD-MCA, acetyl-L-aspartic-L-glutamic-L-valyl-L-asparatic acid-4-methyl-coumary-7-amide; CBB, Coomassie brilliant blue R; CNP, C-type natriuretic peptide; DSF, defined serum-free; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal–related kinase; FMK, fluoromethyl ketone; HM, heavy membrane; JNK, c-Jun kinase; LDH, lactate dehydrogenase; LM, light membrane; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; MEKK1, MEK kinase 1; PBS, phosphate-buffered saline; PG12, prostaglandin 12; PI, propidium iodide; PMSF, phenylmethylsulfonyl fluoride; RIPA, radioimmunoprecipitation assay; SAPK, stress-activated protein kinase; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate.

potential, altered cellular oxidation-reduction, and participation of pro- and antiapoptotic bcl-2 family proteins. The different signals that converge on mitochondria to trigger or inhibit these events and their downstream effects delineate several major pathways in physiological cell death. It is now well known that the effectors of apoptosis are represented by a family of intracellular cysteine proteases known as caspases (13). Caspase activation by certain types of stimulation depends on the presence of cytochrome c released from mitochondria during apoptosis (14), and it is inhibited by the presence of bcl-2 on these organelles (15,16). In addition to caspases, it is becoming increasingly clear that signal transduction pathways involving specific protein kinases are involved in mediating apoptosis. Specifically, the c-Jun kinases (JNKs) and p38 kinases have been proposed to mediate apoptosis (17–19). Indeed, a number of reports have challenged the notion that the activation of JNKs and/or p38 kinases is sufficient to induce apoptosis (20–25). The integration and balance of the JNK and p38 pathways probably contribute to commitment to apoptosis (18,26). In this study, we focused on the molecular mechanisms of endothelial cell apoptosis induced by high D-glucose, particularly *1*) the modulation of antiapoptotic and proapoptotic molecules, *2*) mitochondrial function including apoptosis-related genes and caspases, and *3*) the signal transduction system including phosphorylation of mitogen-activated protein kinase (MAPK).

RESEARCH DESIGN AND METHODS

Cell culture. Human aortic endothelial cells (passage 3) and bovine aortic endothelial cells (passage 1) were obtained from Clonetics (San Diego, CA) and cultured in modified MCDB131 medium supplemented with 5% fetal calf serum, 50 μg/ml gentamicin sulfate, 50 ng/ml amphotericin-B, 10 ng/ml epidermal growth factor, and 1 mmol/l hydrocortisone in the standard fashion (27). Cells were incubated at 37°C in a humidified atmosphere of 95% O_2:5% CO_2 with medium changes every 2 days. These cells showed the specific characteristics of endothelial cells on the basis of immunohistochemical examination and morphologic observation. Briefly, human aortic endothelial cells tested positive for von Willebrand factor and for uptake of diacetylated LDL. All cells were used within passages 5–8.

Counting of cell number. An index of cell proliferation was determined using a WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) cell-counting kit, which is similar to the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Wako, Osaka, Japan) (5,6,28). Endothelial cells were seeded onto uncoated 96-well tissue culture plates (Corning, NY). In the preparation of experiments for determination of cell count, the cells were grown to subconfluence. After subconfluence, the medium was changed to defined serum-free (DSF) medium. DSF medium was supplemented with insulin (5×10^{-7} mol/l), transferrin (5 mg/ml), and ascorbate (0.2 mmol/l), as previously described (29). The cells were then incubated overnight. On day 1, the medium was changed to fresh DSF medium with D-glucose (5–25 mmol/l). On day 3, an index of cell proliferation was determined using sulfonated tetrazolium salt, WST-1, because this compound produces a highly water-soluble formazan dye that makes the assay procedure easier to perform (28).

Fluorescence microscopy. Cells were stained with Hoechst 33258 (10 μmol/l) and propidium iodide (PI) (10 μmol/l) for 10 min and analyzed under a nonconfocal fluorescence microscope (Olympus BX60) with excitation at 360 nmol/l, as previously reported (30). As Hoechst 33258 stains all nuclei and PI stains nuclei of cells with a disrupted plasma membrane, nuclei of viable, necrotic, and apoptotic cells were observed as blue intact nuclei, red round nuclei, and fragmented (or condensed) nuclei, respectively, under fluorescence microscopy. Individual nuclei were visualized at ×400 to distinguish the normal uniform nuclear pattern from the characteristic condensed coalesced chromatin pattern of apoptotic cells (30). Although chromatin undergoes condensation during mitosis, these cells can be readily distinguished from apoptotic cells by their uniform and equatorial pattern of chromatin condensation compared with the randomly coalesced pattern typical of apoptotic cells.

DNA fragmentation assessed by enzyme-linked immunosorbent assay. As an alternative assay of apoptosis, we used the measurement of cellular DNA fragmentation using a cellular DNA fragmentation enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim, Mannheim, Germany) (31,32). We confirmed that an increase in apoptotic cells is associated by increased absorbance. Briefly, 10,000 apoptotic cells/well are reported to reflect an absorbance of 1.5 under the manufacturer's recommended conditions. Regarding the sensitivity of DNA fragmentation ELISA, its results correlated well with the results of the conventional ^{3}H-thymidine–based DNA fragmentation assay. In our experimental conditions, an increase in absorbance of 0.2 reflected an increase in cell number to 2,000 apoptotic cells/well.

Lactate dehydrogenase assay. Extent of cell death was assessed using a kit to measure released lactate dehydrogenase (LDH) activity from dead cells, because loss of cell membrane integrity was observed in both necrotic and apoptotic cells (33). LDH activity in the culture medium was determined using a commercially available kit (Wako) exactly as described by the manufacturer. In the preparation of experiments for the LDH assay, the cells were grown to subconfluence. After subconfluence, the medium was changed to DSF medium. The cells were then incubated overnight. On day 1, the medium was changed to fresh DSF medium containing D-glucose (5–25 mmol/l) or mannitol (25 mmol/l).

Analysis of protein levels by Western blotting. Western blotting was performed for analysis of bax, bcl-2, and MAPK kinase (MEK) kinase 1 (MEKK1) proteins. Endothelial cells were seeded onto 15-cm dishes (Corning). Endothelial cells were grown to confluence and made quiescent by incubation in DSF medium 24 h before treatment. After treatment, the cells were extracted with radioimmunoprecipitation assay (RIPA) buffer (50 mmol/l Tris-Cl, 0.15 mol/l NaCl, 0.1% SDS, 1% deoxycholate, 1% Triton-X, 2 mmol/l Na_3VO_4, and 10 mmol/l NaF). Samples containing 20 or 100 μg protein were run on 10 or 12.5% SDS polyacrylamide gels. Proteins were separated by SDS/PAGE, transferred to nitrocellulose membrane (Hybond ECL; Amersham), and incubated with an antibody to bax (anti-human rabbit IgG, 1:100; Santa Cruz), bcl-2 (anti-human mouse IgG, 1:25; Santa Cruz), and MEKK1 (anti-human rabbit IgG, 1:100; Santa Cruz) at 4°C overnight. Antibodies were diluted in 4% skimmed milk and 0.1% Tween 20 in phosphate-buffered saline (PBS). The membranes were then washed and incubated with a 1:2,000 dilution of mouse or rabbit Ig horseradish peroxidase–conjugated antibody (Amersham). Bound antibodies were detected by enhanced chemiluminescence (Amersham) and Hyperfilm-MP, Amersham). To quantify and compare levels of proteins, the density of each band was measured by densitometry (Shimazu, Kyoto, Japan). It was confirmed by staining with Coomassie brilliant blue R (CBB; Sigma) that amounts of loaded proteins were equal. Staining with CBB revealed identical amounts of protein in all samples for Western blotting (data not shown). Western blotting of α-tubulin using anti–α-tubulin antibody (anti-human mouse IgG, 1:100; Oncogene) was also performed to confirm that amounts of loaded proteins were equal. Western blotting was also performed for analysis of the phosphorylation of p38 MAPK. After treatment, the cells were extracted with lysis buffer (50 mmol/l Tris-Cl, 10 mmol/l EGTA, 10 mmol/l NaF, 150 nmol/l NaCl, 0.1% SDS, 1% deoxycorticosterone, and 1% Triton X-100). Proteins were separated by SDS/PAGE, transferred to nitrocellulose membranes (Hybond ECL, Amersham), and incubated with a polyclonal antibody to phosphospecific p38 MAPK (anti-human rabbit IgG, 1:1,000; New England BioLabs) or p38 MAPK (anti-human rabbit IgG, 1:1,000; New England BioLabs) at 4°C overnight.

Analysis of subcellular fractionation by Western blotting. Endothelial cells were lysed in PBS, resuspended in isotonic buffer A (200 mmol/l mannitol, 70 mmol/l sucrose, 1 mmol/l EGTA, and 1 mmol/l HEPES, pH 7.5) supplemented with protease inhibitors (1 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml soybean trypsin inhibitor, and 10 μg/ml aprotonin), and homogenized using a polytron homogenizer (Brinkmann Instruments) at setting 6.5 for 10 min. Nuclei and unbroken cells were separated at 120*g* for 5 min as the low-speed pellet (P1). This supernatant was centrifuged at 7,500*g* for 10 min to collect the heavy membrane (HM) pellet. This supernatant was centrifuged at 100,000*g* for 30 min to yield the light membrane (LM) pellet and final soluble fraction. Hypotonic lysis was performed by resuspending the cells in hypotonic buffer (10 mmol/l Tris, pH 7.5, 5 mmol/l $MgCl_2$, 1 mmol/l EGTA, 1 mmol/l dithiothreitol) and homogenized with a Dounce homogenizer. Samples containing 20 or 100 μg protein were run on 12.5% SDS polyacrylamide gels and analyzed as described above.

Activity of caspase-3 proteases. Cells were harvested after exposure to high D-glucose for the indicated periods of time and washed three times with PBS and then suspended in buffer containing 50 nmol/l Tris/HCl (pH 7.4), 1 mmol/l EDTA, and 10 mmol/l EGTA. After addition of 10 μmol/l digitonin, cells were incubated at 37°C for 10 min. They were centrifuged at 900*g* for 3 min, and the resulting supernatant (40 μg protein) was incubated with 50 μmol/l enzyme substrate acetyl-L-aspartic-L-glutamic-L-valyl-L-asparatic acid-4-methyl-coumary-7-amide (Ac-DEVD-MCA) at 37°C for 1 h. Levels of released 7-amino-4-methylcoumarin were measured using spectrofluorometers (Hitachi F-3000

and F-2000) with excitation at 380 nm and emission at 460 nm. Excitation and emission slit width were adjusted to 10 and 20 mm, respectively. One unit was defined as the amount of enzyme required to release 0.22 nmol 7-amino-4-methylcoumarin per minute at 37°C.

Transfection. Human aortic endothelial cells were transiently transfected with the plasmid pcDNA1 or a vector encoding a catalytically inactive mutant in which Lys432 was changed to Ala [MEKK1 (K432A)] (18). In addition, kinase-inactive p38 MAPK using the pcDNA3 vector, as described previously (34), was also transiently transfected. Transfection was performed using LipofectAMINE2000 and PLUS regent (Life Technologies).

Materials. SB 203580, a specific inhibitor of p38 MAPK, was obtained from Calbiochem. The tetrapeptide substrate for caspase-3, Ac-DEVD-MCA, was purchased from Peptide Institute (Osaka, Japan). The caspase inhibitors, benzyloxy-carbonyl-Val-Ala-Asp-fluoromethyl ketone z-VAD.FMK and z-DEVD. FMK, were obtained from Enzyme Systems Products (Dubin, CA). Hoechst 33258 (bisbenzimide) staining dye was obtained from Wako. PI was obtained from Molecular Probes (Eugene, OR).

Statistical analysis. All values are expressed as means ± SE. Analysis of variance with subsequent Bonferroni's/Dunnet test was used to determine the significance of differences in multiple comparisons. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Effects of high D-glucose on cell death and apoptosis in human endothelial cells. First, we examined the effect of high D-glucose treatment on apoptosis in human endothelial cells. Consistent with previous reports (7–9), treatment of endothelial cells with a high concentration of D-glucose resulted in a significant decrease in cell number in a dose-dependent manner (D-glucose 5 mmol/l 0.483 ± 0.022 vs. D-glucose 25 mmol/l 0.416 ± 0.020, $P < 0.01$; mannitol 25 mmol/l 0.485 ± 0.024, L-glucose 25 mmol/l 0.480 ± 0.016 absorbance at optical density 450 nm). No significant changes were observed in cell number of endothelial cells treated with a high concentration of mannitol or L-glucose. Therefore, endothelial cell death was attributable to the cytotoxicity of high D-glucose concentration, but not to high osmolarity. After high D-glucose treatment, some cells started to become round and eventually detached from the plate and floated in the medium, leaving many holes in the sheet of confluent cells (data not shown). The floating cells could be recovered with the medium and neither attached to a new plate nor proliferated. In addition, cells treated with high D-glucose exhibited the characteristic features of cell shrinkage, membrane blebbing, and rounding, typical of apoptotic death, under phase-contrast microscopy. Thus, apoptosis was assessed by the morphologic features using double staining with Hoechst 33258 and PI under fluorescence microscopy (30). Simultaneous assessment of nuclear chromatic morphology by Hoechst 33258 staining (blue) and PI staining (red) verified that these cells eventually manifested typical apoptotic condensed and coalesced nuclei (Fig. 1*A*). Apoptotic cells were clearly observed in cells treated with high D-glucose, as shown in Fig. 1*A*. Cells with fragmented or condensed nuclei were classified further based on PI staining as "early" apoptotic cells with membrane integrity and "terminal" apoptotic cells without membrane integrity. These nuclear morphologic changes under fluorescence microscopy correspond well to necrosis and apoptosis defined by electron microscopy in endothelial cells induced by high glucose. As shown in Fig. 1*B*, treatment with high D-glucose significantly increased the number of apoptotic and necrotic cells ($P < 0.05$), while a high concentration of mannitol did not. A significant increase in apoptotic cells was also confirmed by DNA fragmentation ELISA (Fig. 1*C*) ($P < 0.01$), whereas mannitol as an osmotic negative control did not induce apoptosis. In LDH release, treatment of endothelial cells with high concentration of D-glucose also resulted in a significant increase in a time-dependent manner, as compared with a high concentration of mannitol (Fig. 1*D*) ($P < 0.01$).

Analysis of bax and bcl-2 under high D-glucose treatment. Therefore, we further examined the molecular mechanisms of apoptosis induced by high D-glucose treatment. In particular, we focused on the expression of bcl-2 and bax proteins. Bcl-2 and bax are homologous proteins that have opposing effects on cell life and death, with bcl-2 serving to prolong cell survival and bax acting as an accelerator of apoptosis (35). The bcl-2 and bax proteins can form heterodimers in cells (36). Interestingly, D-glucose treatment significantly increased bax protein as assessed by Western blotting, whereas no significant change in bax protein was observed with mannitol treatment (Fig. 2*A* and *B*) ($P < 0.05$). In contrast, bcl-2 protein was not affected by high D-glucose treatment at 25 mmol/l (Fig. 2*C* and *D*). Thus, the ratio of bcl-2 to bax was significantly decreased in cells treated with high D-glucose (5 mmol/l D-glucose 100% vs. 25 mmol/l D-glucose 67.9 ± 8.6%, $*P < 0.01$; mannitol at 25 mmol/l 97.8 ± 10.2%).

Recent studies demonstrated that translocation of bax protein from the cytoplasm to the mitochondorial membrane is critical in apoptosis (37,38). Therefore, we also investigated the effect of high D-glucose treatment on the translocation of these apoptosis-related proteins. A substantial portion of bax protein was found consistently in the soluble S100 fraction representing the cytosol as well as the mitochondria-enriched HM fraction, as documented by mitochondrial markers (porin/VDAC, a marker of outer mitochondorial membrane) (Fig. 3*A*). Of particular importance, after high D-glucose treatment, most of bax protein moved from the cytosol to the HM fraction (Fig. 3*A*). In contrast, bcl-2 protein resided in the mitochondria-rich HM and LM fraction as well as the soluble fraction under normal glucose condition (Fig. 3*B*). Moreover, the distribution of bcl-2 protein was not altered by high D-glucose treatment (Fig. 3*B*).

Activation of caspase-3 proteases induced by high D-glucose treatment. It was recently reported that caspases are important regulators of apoptosis, and the release of cytochrome c from mitochondria activated caspase, especially caspase-3 (13). Interestingly, recent reports revealed that bax induces the release of cytochrome c and that bcl-2 prevents it (15,16). To further analyze the contribution of activation of caspase proteases in the death signal triggered by high D-glucose treatment, the proteolytic activity of caspases was measured using fluorogenic tetrapeptide substrates. Activity of caspase-3 proteases was significantly increased after high D-glucose treatment, and reached a peak at 24 h after exposure to high D-glucose in endothelial cells, as compared with mannitol (Fig. 4*A*). Thus, we next examined the effects of a specific caspase-3 inhibitor, z-DEVD, and a wide-spectrum caspase inhibitor, z-VAD, on the cell death induced by high D-glucose. Importantly, the addition of z-VAD as well as z-DEVD significantly attenuated the cell death induced by high D-glucose ($P < 0.01$) (Fig. 4*B*).

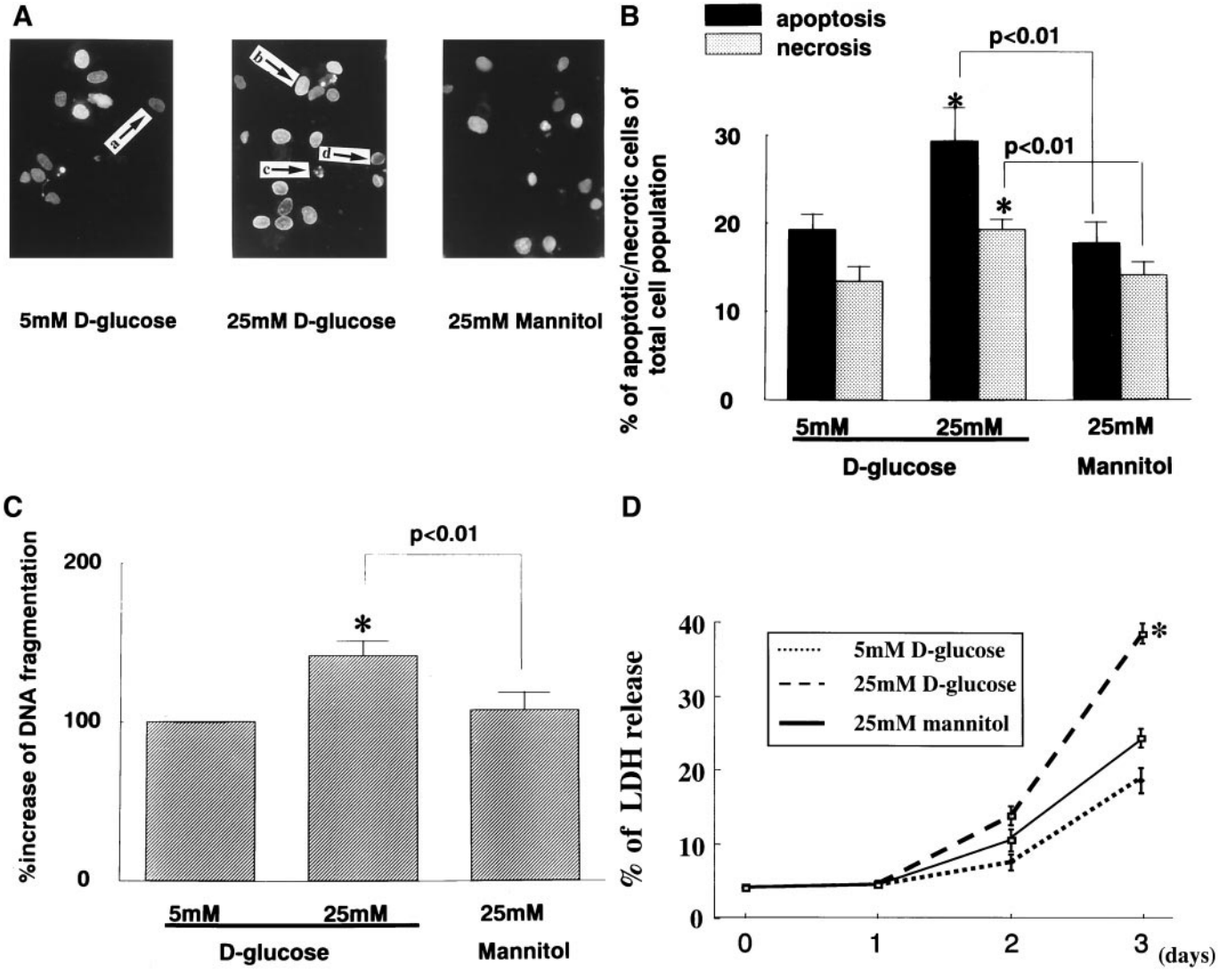

FIG. 1. ***A*****: Morphologic changes in nuclei of human aortic endothelial cells maintained under high glucose conditions at 48 h assessed by Hoechst and PI staining. Endothelial cells maintained under high D-glucose conditions exhibited the characteristic features of cell shrinkage, membrane blebbing, and rounding, typical of apoptotic death.** ***B*****: Increase in apoptotic and necrotic endothelial cells under high D-glucose conditions at 48 h assessed by Hoechst and PI staining. Values are expressed as percentage of apoptotic cells in total cells;** $n = 8$ **per group calculated from eight independent experiments. *$P < 0.01$ vs. 5 mmol/l D-glucose.** ***C*****: Percent increase in DNA fragmentation in endothelial cells treated with D-glucose or mannitol at 48 h assessed by DNA fragmentation assay. Values are expressed as percentage of DNA fragmentation as compared with that under D-glucose (5 mmol/l);** $n = 6$ **per group calculated from six independent experiments. *$P < 0.01$ vs. 5 mmol/l D-glucose.** ***D*****: LDH release in endothelial cells treated with D-glucose or mannitol. Total LDH was measured as peak LDH per well induced by RIPA buffer, and percent of LDH release was measured as a comparison with total LDH. Values are expressed as a percentage of LDH release as compared with that under mannitol (25 mmol/l). *$P < 0.01$ vs. 25 mmol/l mannitol. 5 mM D-glucose = endothelial cells maintained under normal glucose conditions (5 mmol/l); 25 mM D-glucose = endothelial cells maintained under high D-glucose conditions (25 mmol/l); 25 mM mannitol = endothelial cells maintained under high mannitol conditions (25 mmol/l).**

Cross-talk between p38 MAPK and activation of caspase proteases induced by high D-glucose. We also studied how signal transduction pathways, such as those controlled by kinases, modulate critical cellular functions such as cell growth, differentiation, and apoptosis. Three major kinase cascades that culminate in the activation of three different sets of MAPKs, extracellular signal–related kinase (ERK), JNK/SAPK (stress-activated protein kinase), and p38 MAPK, have recently been identified (39,40). Treatment with high D-glucose, as compared with normal glucose, significantly increased phosphorylated p38 MAPK assessed by Western blotting using phosphorylated specific antibody (Fig. 5*A*). p38 MAPK was markedly phosphorylated, with sustained stimulation (Fig. 5*A*), whereas mannitol did not change the level of phosphorylated p38 MAPK (data not shown). In addition, the specificity of phosphorylation of p38 MAPK was confirmed by the observation that no apparent change in total p38 MAPK by high D-glucose was observed (Fig. 5*A*). An osmotic control, mannitol, also did not affect total p38 MAPK in endothelial cells at normal and high concentrations. Importantly, administration of a specific inhibitor of p38 MAPK, SB 203580, completely attenuated endothelial cell death induced by high D-glucose (Fig. 5*B*) ($P < 0.01$). More importantly, administration of a specific inhibitor of p38 MAPK, SB 203580, completely attenuated endothelial cell death induced by high D-glucose in a dose-dependent manner, as assessed by DNA fragmentation and LDH release (Fig. 5*C* and *D*) ($P < 0.01$). In addition, transient overexpression of kinase-inactive p38 MAPK into endothe-

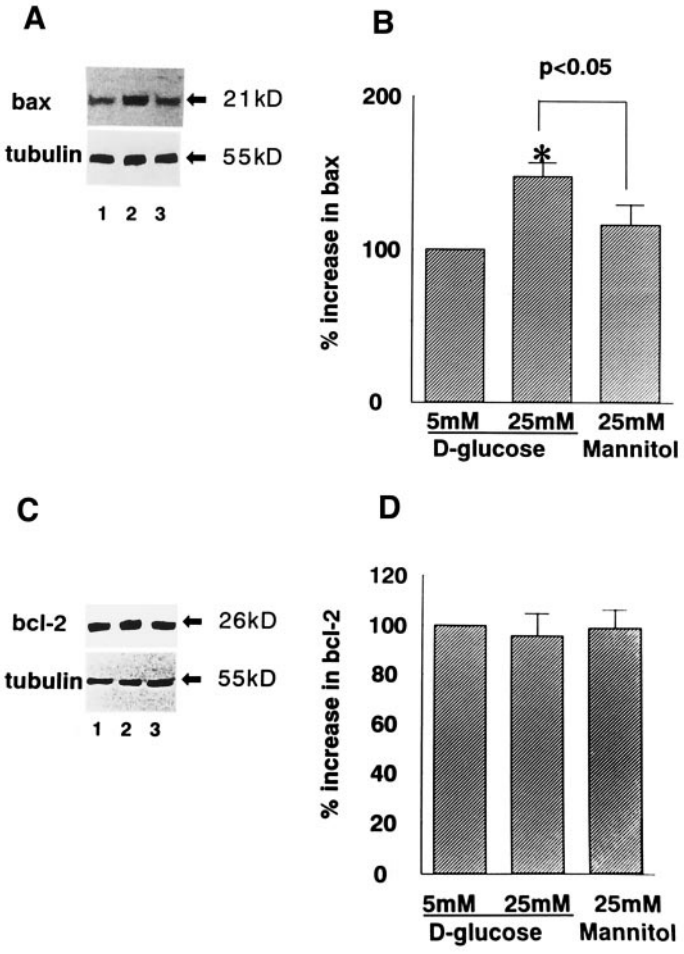

FIG. 2. ***A*****: Typical Western blot of bax and tubulin proteins in endothelial cells treated with D-glucose at 48 h.** ***B*****: Percent change in protein level of bax calibrated from tubulin in endothelial cells treated with D-glucose at 48 h. Values are expressed as percentage of bax/tubulin protein as compared with that under D-glucose (5 mmol/l);** ***n*** **= 6 per group calculated from six independent experiments. *$P < 0.05$ vs. 5 mmol/l D-glucose.** ***C*****: Typical Western blot of bcl-2 and tubulin proteins in endothelial cells treated with D-glucose at 48 h.** ***D*****: Percent change in protein level of bcl-2 calibrated from tubulin in endothelial cells treated with D-glucose at 48 h. Values are expressed as percentage of bcl-2/tubulin protein as compared with that under D-glucose (5 mmol/l);** ***n*** **= 6 per group calculated from six independent experiments.** ***A*** **and** ***C*****: lane 1, 5 mM D-glucose = endothelial cells maintained under normal glucose conditions (5 mmol/l); lane 2, 25 mM D-glucose = endothelial cells maintained under high D-glucose conditions (25 mmol/l); lane 3, 25 mM Mannitol = endothelial cells maintained under high mannitol conditions (25 mmol/l).**

lial cells also attenuated endothelial cell death in LDH releases (Fig. 5*E*) ($P < 0.01$). To confirm this result in another endothelial cell, we also checked the same experiment in bovine aortic endothelial cells. A specific inhibitor of p38 MAPK, SB 203580, and the overexpression of kinase-inactive p38 MAPK also attenuated cell death induced by high D-glucose in bovine aortic endothelial cells (control 1.00 ± 0.23, 25 mmol/l D-glucose 1.855 ± 0.163, 25 mmol/l D-glucose + SB203580 0.994 ± 0.034, and 25 mmol/l D-glucose + dominant-negative p38 MAPK 1.109 ± 0.022; $P < 0.01$) .

To further analyze the cross-talk between p38 MAPK and caspase proteases, we examined the effect of a specific caspase-3 inhibitor, z-DEVD, and a wide-spectrum caspase inhibitor, z-VAD, on the phosphorylation of p38 MAPK induced by high D-glucose treatment. p38 MAPK was phosphorylated after high D-glucose stimulation, whereas addition of z-VAD as well as z-DEVD significantly inhibited the phosphorylation of p38 MAPK at 12 h after high D-glucose treatment (Fig. 6*A*). However, at 30 min after high D-glucose treatment, these caspase inhibitors did not attenuate the phosphorylation of p38 MAPK (Fig. 6*B*). These results demonstrated that phosphorylation of p38 MAPK at late phase (6 h), but not acute phase (30 min), is due to caspase activation. More than likely, phosphorylation of p38 MAPK at acute phase may be due to other stimuli such as stress. For further analysis, we examined the effect of p38 MAPK phosphorylation on caspase-3 activation at 6 h after high D-glucose treatment. SB 203580

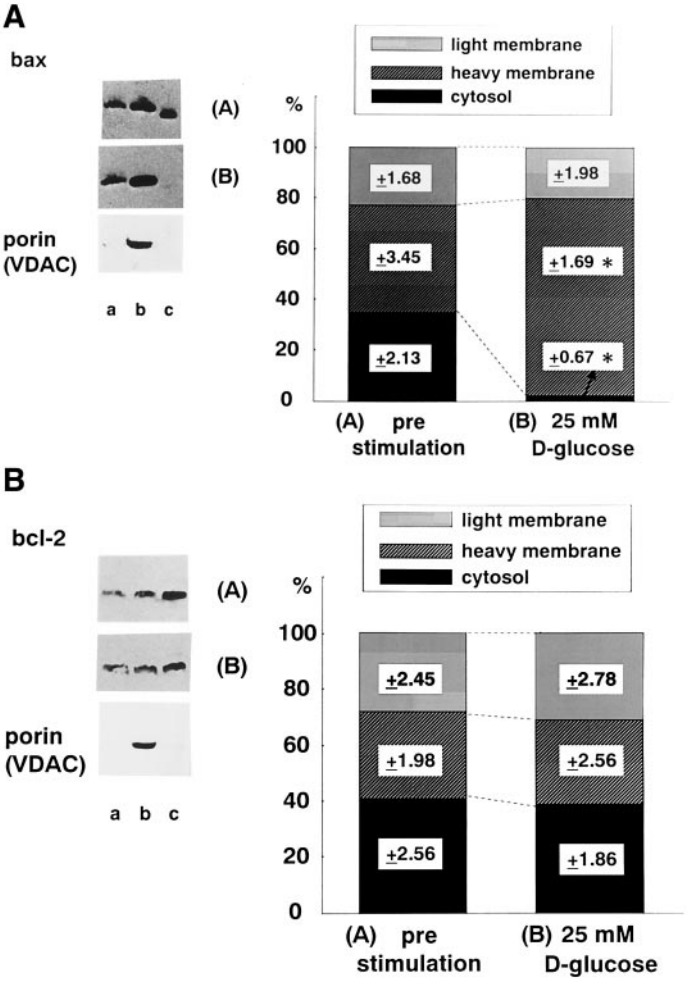

FIG. 3. ***A*****: The left panel shows a typical Western blot of bax protein in the subcellular fraction of endothelial cells treated with D-glucose at 12 h; (*** ***A*** **) = prestimulation; (*** ***B*** **) = 12 h after stimulation with 25 mmol/l D-glucose; a = light membrane fraction; b = heavy membrane fraction; c = cytosolic fraction. The right panel shows the percent change in protein level of bax in the subcellular fraction of endothelial cells treated with D-glucose at 12 h. Values are expressed as percentage of bax protein versus total bax protein;** ***n*** **= 4 per group calculated from four independent experiments. *$P < 0.01$ vs. prestimulation.** ***B*****: The left panel shows a typical Western blot of bcl-2 protein in the subcellular fraction of endothelial cells treated with D-glucose at 12 h. (*** ***A*** **) = prestimulation; (*** ***B*** **) = 12 h after stimulation with 25 mmol/l D-glucose; a = light membrane fraction; b = heavy membrane fraction, c = cytosolic fraction. The right panel shows the percent change in protein level of bcl-2 in the subcellular fraction of endothelial cells treated with D-glucose at 12 h. Values are expressed as percentage of bcl-2 protein versus total bcl-2 protein;** ***n*** **= 4 per group. Values were calculated from four independent experiments. (*** ***A*** **) = prestimulation; (*** ***B*** **) = 12 h after stimulation with 25 mmol/l D-glucose.**

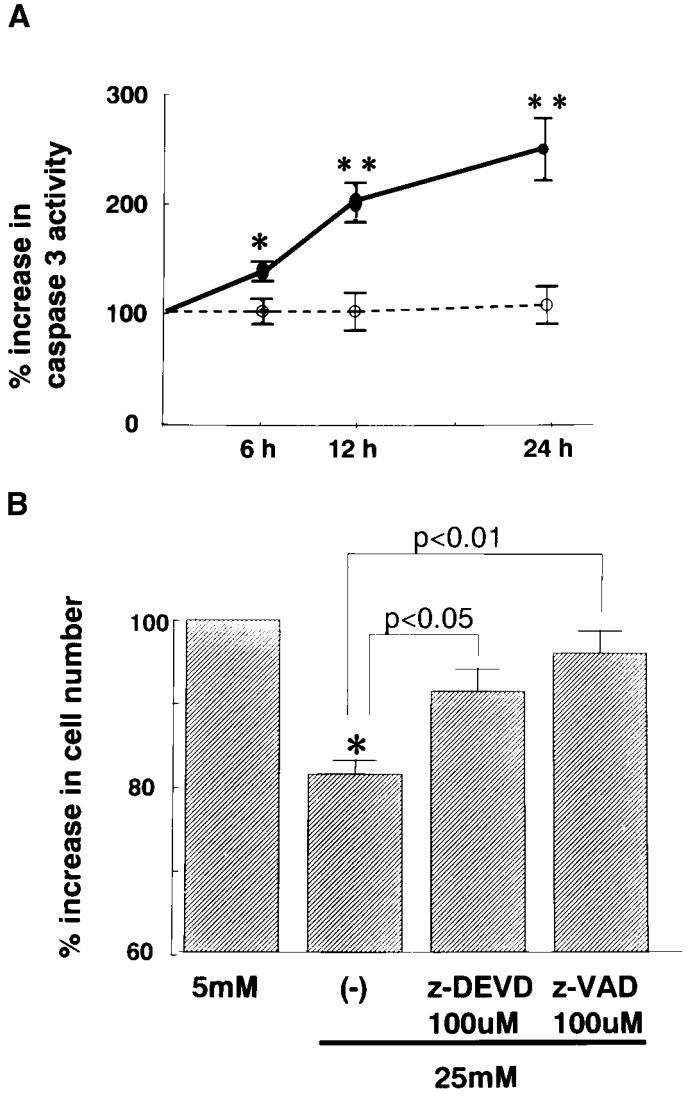

FIG. 4. ***A*****: Percent increase in caspase-3 activity after the stimulation of high D-glucose treatment. Values are expressed as percentage of caspase-3–like activity, as compared with 5 mmol/l D-glucose treatment; $n = 6$ per group. Values were calculated from six independent experiments. 6, 12, 24 h = 6, 12, or 24 h after stimulation with 25 mmol/l D-glucose or mannitol. ●, 25 mmol/l D-glucose; ○, 25 mmol/l mannitol. *$P < 0.05$ and **$P < 0.01$ vs. 25 mmol/l mannitol.** ***B*****: Effect of caspase inhibitors on the number of endothelial cells treated with high D-glucose treatment; $n = 8$ per group. Values were calculated from eight independent experiments. D-glucose 5 mM = endothelial cells maintained under normal glucose conditions (5 mmol/l); D-glucose 25 mM = endothelial cells maintained under high D-glucose conditions (25 mmol/l); D-glucose 25 mM + z-DEVD = endothelial cells maintained under high D-glucose conditions (25 mmol/l) with 100 μmol/l z-DEVD; D-glucose 25 mM + z-VAD = endothelial cells maintained under high D-glucose conditions (25 mmol/l) with 100 μmol/l z-VAD. *$P < 0.05$ and **$P < 0.01$ vs. D-glucose 5 mmol/l.**

(30 μmol/l) and the overexpression of kinase-inactive p38 MAPK did not attenuate caspase-3 activation at 6 h after high D-glucose treatment (Fig. 6*C*). Together, SB 203580 and the overexpression of kinase-inactive p38 MAPK attenuated cell death induced by high D-glucose (Fig. 5*C–E*); p38 MAPK phosphorylation is located downstream of caspase.

Thus, we finally focused on MEKK1 to further examine the cross-talk between p38 MAPK and the bax-caspase proteases pathway, as MEKK1 is a 196-kDa enzyme that is involved in the regulation of apoptosis (41,42). MEKK1 is cleaved at Asp^{874} by caspases, and the cleaved kinase domain of MEKK1 itself stimulates caspase activity leading to apoptosis. Treatment of human aortic endothelial cells with high D-glucose induced cleavage of the 196-kDa MEKK1 protein at concentrations that maximize the apoptotic response indicated by the loss of full-length MEKK1 (Fig. 7*A*). Recent studies demonstrated that in HEK293 cells, the cleavage fragments of endogenous MEKK1 were not consistently detected with other cleaved proteins (42,43). Therefore, the total 196-kDa MEKK1 protein level is considered as the response to apoptotic stimuli. Interestingly, addition of z-VAD as well as z-DEVD significantly attenuated the cleavage of MEKK1 induced by high D-glucose. Thus, to test the hypothesis that the cleavage of MEKK1 contributes to the activation of p38 MAPK, we transfected a catalytically inactive mutant in which Lys^{432} changed to Ala [MEKK1 (K432A)] into endothelial cells before high D-glucose treatment. Importantly, kinase-inactive MEKK1 also blocked the phosphorylation of p38 MAPK induced by high D-glucose (Fig. 7*B*). These data suggest that phosphorylation of p38 MAPK was located downstream of the caspase pathway mediated by MEKK1.

DISCUSSION

Although classical paradigms defining the pathophysiology of vascular disease have focused on the abnormal regulation of cell growth in response to growth factors, it has been recently postulated that the regulation of cell death by apoptosis may be another determinant of vascular structure and lesion formation (44). Therefore, vascular structure has been postulated to be determined in large part by a balance between cell growth and cell death by apoptosis. This process of vascular remodeling plays an important role in determining the natural history of vascular diseases (45). In response to a variety of stimuli and circumstances, cells have an intrinsic capacity to activate a gene-directed program that commits the cell to a suicidal death, described as apoptosis. It has become increasingly clear that the process of cell death by apoptosis is a relatively ubiquitous phenomenon observed in a variety of cell types, including endothelial cells (46). Disruption or dysfunction of endothelial cells, causing loss of multiple endothelium-derived substances (PGI_2, NO, CNP), has been hypothesized to play a pivotal role in the progression and/or development of vascular disease in diabetes. In this study, we demonstrated that high D-glucose treatment induced endothelial cell death through the induction of apoptosis, whereas mannitol and L-glucose, as controls for osmolarity, did not, which is consistent with previous findings (7–9). However, little is known about the molecular mechanisms on D-glucose–induced endothelial cell death. Apoptosis has been suggested to be largely controlled by *1*) modulation of the activity of antiapoptotic and proapoptotic molecules (e.g., bcl-2 and bax) and *2*) triggering of specific signaling pathways (e.g., MAPK cascade). Of importance, the present studies revealed a significant increase in bax, a proapoptotic factor, by high D-glucose treatment. Here, we demonstrated that apoptosis induced by high D-glucose may be attributable to an inappropriate increase in the ratio of bax to bcl-2 induced by high D-glucose. There were two supportive reports that

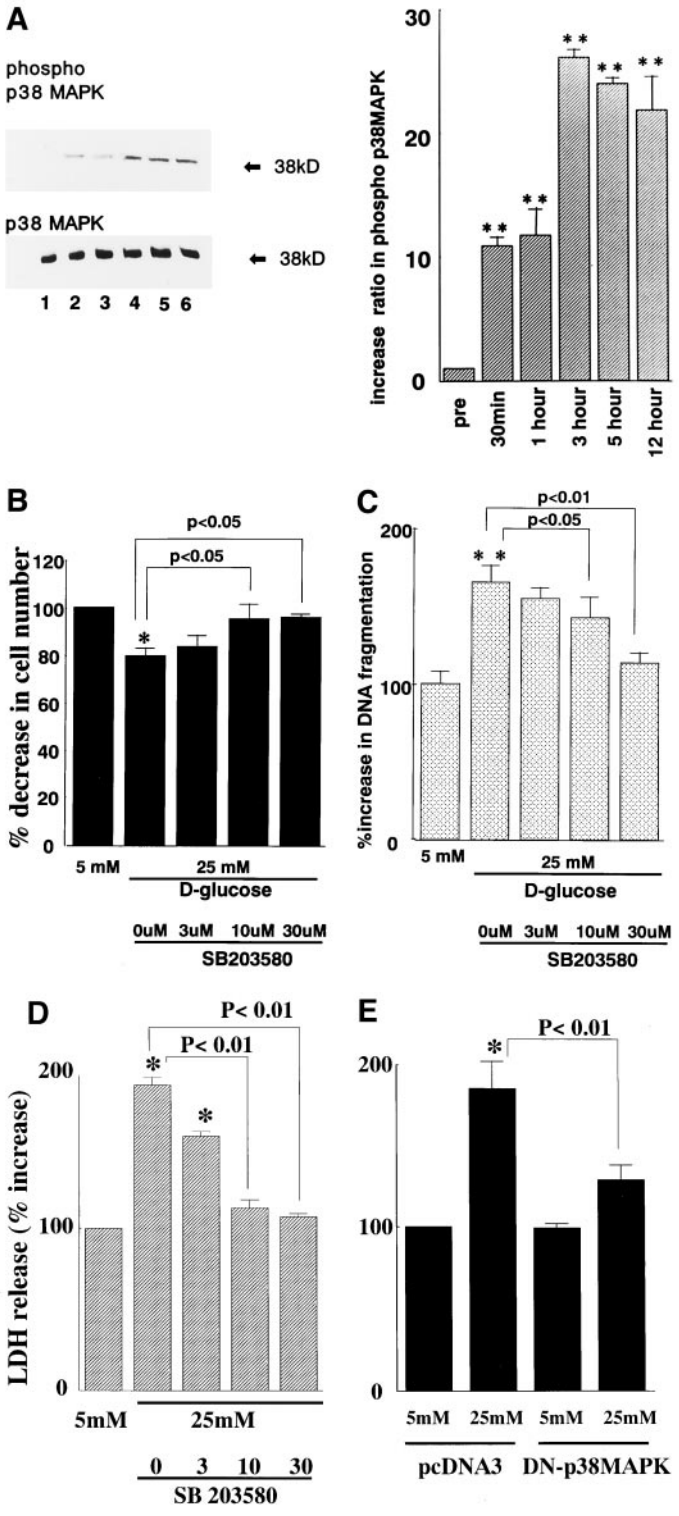

FIG. 5. ***A*****: The left panel shows a typical Western blot of p38 MAPK (lower) and phosphorylated p38 MAPK (upper) in endothelial cells treated with high D-glucose (25 mmol/l).The right panel shows the percent change in phosphorylated p38 MAPK in endothelial cells treated with high D-glucose (25 mmol/l). Values are expressed as a percentage of phosphorylated p38 MAPK compared with that prestimulation; $n = 6$ per group. Values were calculated from six independent experiments: prestimulation (pre) and 30 min, 1 h, 3 h, 5 h, and 12 h after stimulation with high D-glucose (25 mmol/l). **$P < 0.01$ vs. prestimulation. *B*–*D*: Effect of SB 203580 on endothelial cell death (–5 days) (*B*), DNA fragmentation rate (*C*), and LDH release (*D*) induced by high D-glucose (25 mmol/l); $n = 8$ per group. Values were calculated from eight independent experiments. *$P < 0.05$ and **$P < 0.01$ vs. 5 mmol/l D-glucose. *E*: Effect of transfection of kinase-inactive p38 MAPK cDNA on LDH release induced by high D-glucose (25 mmol/l); $n = 8$ per group. Values were calculated from eight independent experiments. *$P < 0.01$ vs. 5 mmol/l D-glucose. *D* and *E*: 5 mM = endothelial cells maintained under normal glucose conditions (5 mmol/l); 25 mM = endothelial cells maintained under high D-glucose conditions (25 mmol/l); SB 203580 = endothelial cells maintained under high D-glucose conditions (25 mmol/l) treated with SB 203580 (0, 3, 10 or 30 μmol/l); pcDNA3 = endothelial cells transfected with pcDNA3 (control plasmid); dn-P38 MAPK = endothelial cells transfected with kinase-inactive p38 MAPK cDNA.**

in vivo bax accelerated death of retinal cells in hyperglycemia (47) and that hyperglycemic conditions increased the expression of bax as early as the preimplantation blastocyte stage in the mouse (48). In the latter report, the blastocysts from bax-deficient mice were protected from glucose-induced apoptosis. This result suggests that bax may be a key modulator of hyperglycemia-induced apoptosis and high-rate congenital malformations and spontaneous miscarriages induced by hyperglycemia at early stages after conception.

Recent studies have also documented that the subcellular localization of these molecules determines the fate of cells, in addition to classical diagrams defining the quantity. Bcl-2 and bax contain a hydrophobic segment at their COOH-terminal end (37,49) that is believed to serve as a membrane anchor. Subcellularly, bcl-2 protein is localized to the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membrane (50). Recently, it was demonstrated that after delivery of death signals to cells in culture, bax moves to the mitochondria and other membrane sites and triggers a catastrophic transformation of mitochondrial function. However, no reports have revealed whether the translocation of bax protein could occur under physiological conditions such as high D-glucose. We demonstrated that after high D-glucose treatment, bax protein inserts into the mitochondrial membranes, while it is located in the cytosol and in association with intracellular membranes including mitochondria under normal glucose condition. Translocation of bax protein into the mitochondrial membrane was accompanied by a significant increase in caspase-3 activity. These results demonstrate that bax translocation from the cytosol to the mitochondrial membrane occurred under physiological conditions, thereby leading to pathological tissue destruction. As it is believed that cytosolic bax fails to homodimerize in the presence of protective levels of bcl-2, exploration of various stimuli of bax translocation might be important to understand the cell death system. Interestingly, we demonstrated that cell death mediated by bax targeting mitochondria could be inhibited by zVAD as well as Z-DEVD. Thus, caspase-3 may play a pivotal role in the process of endothelial cell death induced by high D-glucose. In contrast, the present study failed to demonstrate a change in the distribution of bcl-2 after high D-glucose treatment, whereas bcl-2 has been reported to prevent mitochondrial dysfunction (i.e., cytochrome c release and caspase protease activation).

Finally, we focused on signal transduction pathways, because these pathways regulate cell growth and death. The three MAPK (ERK, JNK/SAPK, and p38 MAPK) pathways have been implicated in the control of apoptosis. ERK is activated by mitogens and survival factors, whereas JNK/SAPK and p38 are stimulated by stress signals (39,40). Although previous findings indicated that the

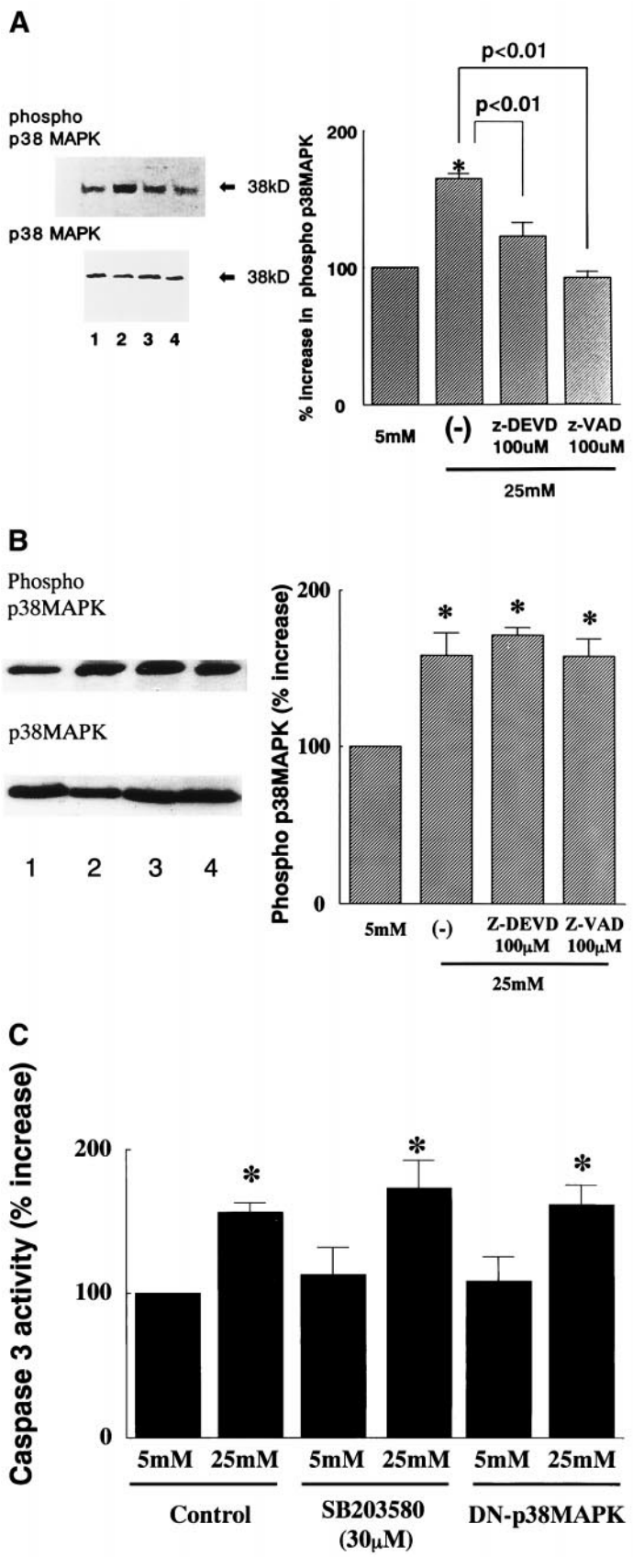

FIG. 6. ***A*** **and** ***B*****: Effect of caspase inhibitors on phosphorylation of p38 MAPK in endothelial cells treated with high D-glucose (25 mmol/l) at (*A*) 12 h and (*B*) 30 min after treatment. The left panel shows a typical Western blot of p38 MAPK (lower lanes) and phosphorylated p38 MAPK (upper lanes) in endothelial cells treated with caspase inhibitors. The right panel shows the percent change in phosphorylated p38 MAPK in endothelial cells treated with caspase inhibitors; $n = 6$ per group. Values were calculated from six independent experiments. *C*: Effect of SB 203580 and transfection of kinase-inactive p38 MAPK cDNA on caspase-3 activation at 6 h after high D-glucose (25 mmol/l) treatment; $n = 6$ per group. Values were calculated from six independent experiments. *A*– *C*: 5 mM = endothelial cells maintained under normal glucose conditions (5 mmol/l); 25 mM = endothelial cells maintained under high D-glucose conditions (25 mmol/l); 100 μM z-DEVD = endothelial cells maintained under high D-glucose conditions (25 mmol/l) with 100 μmol/l z-DEVD; 100 μM z-VAD = endothelial cells maintained under high D-glucose conditions (25 mmol/l) with 100 μmol/l z-VAD; control = endothelial cells transfected with pcDNA3 (control vector); SB 203580 = endothelial cells transfected with pcDNA3 (control vector) and treated with SB 203580 (30 μmol/l); dn-P38 MAPK = endothelial cells transfected with kinase-inactive. *$P < 0.01$ vs. 5 mmol/l D-glucose.**

activation of p38 MAPK may play a pivotal role in the control of cell death (51,52), the cross-talk between p38 MAPK and caspase is not still clear. The present study demonstrated that phosphorylation of p38 MAPK induced by high D-glucose-mediated cell death and apoptosis. Interestingly, the sustained phosphorylation of p38 MAPK was observed after high D-glucose stimulation, whereas caspase inhibitors completely attenuated the sustained phosphorylation of p38 MAPK at 12 h, but not 30 min, after high D-glucose treatment. From these results, sustained phosphorylation of p38 MAPK is located downstream of activation of caspase proteases. Similar results about the cross-talk between p38 MAPK and the caspase protease pathway were recently reported by Berra et al. (52). More importantly, the inhibitor of p38 MAPK and the overexpression of kinase-inactive p38 MAPK attenuated endothelial cell death despite evidence of caspase activation.

These MAPK family members are in turn activated by upstream MAP/Efr kinases (MEKs) such as SAPK/ERK kinase-1 (SEK1, also known as MKK4) and MKK3/MKK6, which is upstream of JNK/SAPK and p38 MAPK. These MEKs are in turn activated by MEKKs, of which MEKK1, -2, -3, and -5 have been characterized to date (53,54). Although the identity of the MEKK upstream of the p38 MAPK pathway is unknown, previous studies provide evidence for the role of MEKK1 as an upstream activator of both p46 and p54 SAPK as well as p38 MAPK (55,56). On the other hand, MEKK1 is cleaved at Asp^{874} by caspases, and the cleaved kinase domain of MEKK1 itself stimulates caspase activity leading to apoptosis (41,42). Therefore, we focused on MEKK1 to further examine the cross-talk between p38 MAPK and the bax-caspase proteases pathway. Treatment of human aortic endothelial cells with high D-glucose induced MEKK1 cleavage, whereas the addition of z-VAD as well as z-DEVD significantly attenuated the cleavage. Importantly, kinase-inactive MEKK1 also blocked the phosphorylation of p38 MAPK induced by high D-glucose. Taken together, the present data clearly demonstrated that phosphorylation of p38 MAPK mediated by MEKK1 downstream of bax-caspase proteases determines the cell death and apoptosis induced by high D-glucose (Fig. 8). Cross-talk between the signal transduction system and the caspase pathway may determine the cellular fate, although the present study using in vitro condition may or may not mimic what happens in the in vivo diabetic state.

Overall, we demonstrated that high D-glucose, but not mannitol or L-glucose, induced aortic endothelial cell death through the induction of apoptosis. In addition, this

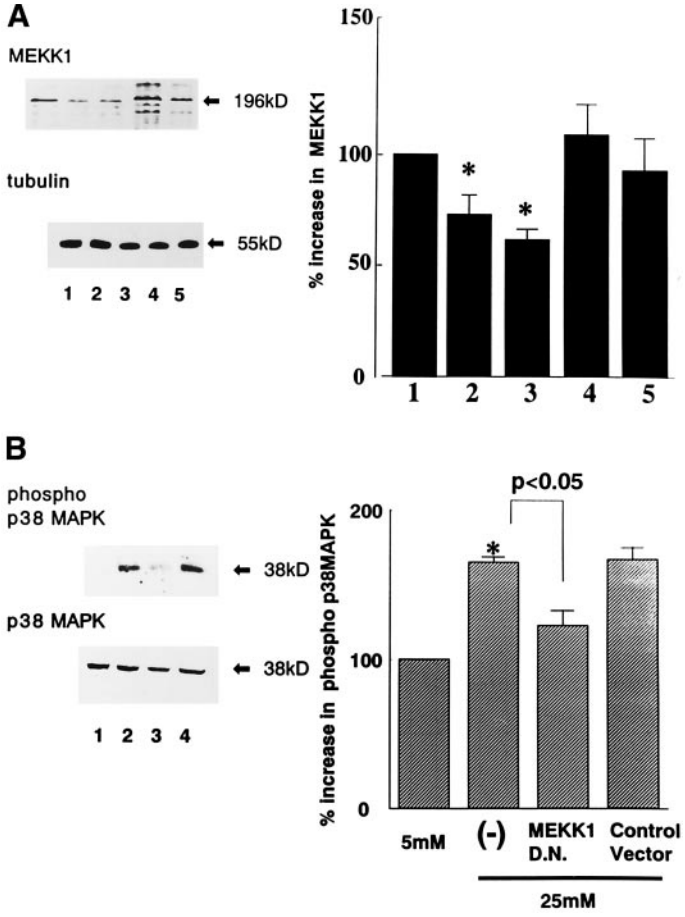

FIG. 7. *A*. Effect of caspase inhibitors on the cleavage of MEKK1 in endothelial cells with high D-glucose treatment. The left panel shows a typical Western blot of MEKK1 (upper lanes) and tubulin (lower lanes) in endothelial cells treated with caspase inhibitors. The right panel shows the percent change in the level of MEKK1 in endothelial cells treated with caspase inhibitors; $n = 6$ per group calculated from six independent experiments. Lane 1, prestimulation; lane 2; endothelial cells maintained under high D-glucose conditions (25 mmol/l) at 12 h; lane 3, endothelial cells maintained under high D-glucose conditions (25 mmol/l) at 24 h; lane 4, endothelial cells maintained under high D-glucose conditions (25 mmol/l) with 100 μmol/l z-VAD at 12 h; lane 5, endothelial cells maintained under high D-glucose conditions (25 mmol/l) with 100 μmol/l z-DEVD at 12 h. *$P < 0.05$ vs. prestimulation. *B*: Effect of transfection of dominant-negative MEKK1 plasmid on phosphorylation of p38 MAPK in endothelial cells with high D-glucose treatment at 12 h. The left panel shows a typical Western blot of p38 MAPK (lower lanes) and phosphorylated p38 MAPK (upper lanes) in endothelial cells transfected with dominant-negative MEKK1 plasmid. The right panel shows the percent change in phosphorylated p38 MAPK in endothelial cells transfected with dominant-negative MEKK1 plasmid; $n = 6$ per group calculated from six independent experiments. Lane 1, 5 mM = endothelial cells maintained under normal glucose conditions (5 mmol/l); lane 2, (-) = untransfected endothelial cells maintained under high D-glucose conditions (25 mmol/l); lane 3, MEKK1 D.N. = endothelial cells maintained under high D-glucose conditions (25 mmol/l) transfected with dominant-negative MEKK1 plasmid; lane 4, Control Vector = endothelial cells maintained under high D-glucose conditions (25 mmol/l) with control vector lacking dominant-negative MEKK1 plasmid. *$P < 0.01$ vs. D-glucose 5 mmol/l.

study demonstrated that endothelial cell death and apoptosis induced by high D-glucose may be mediated by *1*) caspase protease activation mediated by an appropriate increase and translocation of bax (modulating the activity of proapoptotic molecules) and *2*) the phosphorylation of p38 MAPK mediated by MEKK1 downstream of caspase protease activity. These results demonstrate the molecular mechanism of high D-glucose–induced cell death, suggesting the possible mechanisms of glucose toxicity.

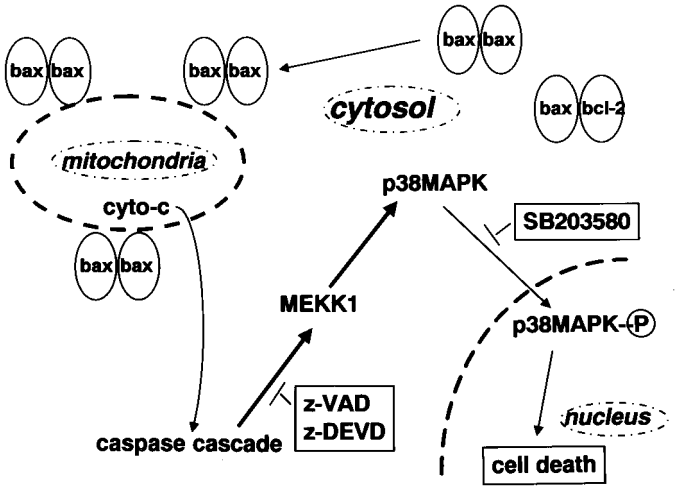

FIG. 8. Potential mechanisms of endothelial apoptosis induced by high D-glucose. High D-glucose treatment increased bax protein without affecting bcl-2 protein and also stimulated the translocation of bax to the mitochondrial heavy membrane. These changes in bax released cytochrome c from mitochondria, resulting in activation of the caspase cascade. Activation of the caspase cascade cleaved MEKK1, leading to the phosphorylation of p38 MAPK. Finally, phosphorylated p38 MAPK led to apoptosis.

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