High Glucose–Induced Apoptosis in Human Endothelial Cells Is Mediated by Sequential Activations of c-Jun NH₂-Terminal Kinase and Caspase-3

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Background—Diabetes mellitus causes multiple cardiovascular complications. High glucose can induce reactive oxygen species and apoptosis in endothelial cells. Little is known about the molecular mechanisms in high glucose–induced endothelial cell apoptosis.

Methods and Results—We elucidated the signaling pathway of high glucose–induced apoptosis in human umbilical vein endothelial cells (HUVECs). HUVECs were treated with media containing 5.5, 19, or 33 mmol/L of glucose in the presence or absence of an antioxidant, ascorbic acid. The level of intracellular H₂O₂ was measured by flow cytometry. For detection of apoptosis, the cell death detection ELISA assay and the morphological Hoechst staining were used. High glucose was capable of inducing the activity of c-Jun NH₂-terminal kinase (JNK) but not extracellular signal–regulated kinase 1/2 or p38 mitogen-activated protein kinase during the treatment periods, as evidenced by immunocomplex kinase assay. Moreover, we found that the interleukin 1β–converting enzyme (ICE)/CED-3 family protease (caspase-3) became activated in high glucose–induced apoptosis. Caspase-3/CPP32–specific inhibitor, Ac-DEVD-CHO, could inhibit high glucose–induced apoptosis. Furthermore, we found that JNK1 specific antisense oligonucleotide could suppress caspase-3 activity but not affect H₂O₂ generation and could block apoptosis induced by high glucose. Also, H₂O₂ generation, JNK activity, caspase-3 activity, and the subsequent apoptosis induced by high glucose could be suppressed by ascorbic acid.

Conclusions—The present study indicates that reactive oxygen species induced by high glucose may be involved in JNK activation, which in turn triggers the caspase-3 that facilitates the apoptosis in HUVECs. (Circulation. 2000;101:2618-2624.)

Key Words: glucose ■ endothelium ■ cells ■ apoptosis ■ JNK ■ ICE ■ caspase

Diabetes mellitus can cause various vascular complications. In the Diabetes Control and Complications Trial, close correlations have been observed between blood glucose and late diabetes–associated complications, including vascular diseases. However, the mechanisms of hyperglycemia–related tissue damage and clinical complications remained unclear.

Vascular endothelium plays important roles in maintaining vascular tone and function, in part by the synthesis and release of vasoactive substances, including nitric oxide. Endothelial dysfunction contributes to the pathogenesis of vascular diseases in diabetics. The mechanisms of endothelial dysfunction in diabetes mellitus are not clear, but one possibility is increased inactivation of endothelium-derived nitric oxide by reactive oxygen species (ROS), acting as signaling intermediates. ROS has been demonstrated to be capable of producing tissue damage associated with diabetes. In diabetic animals, accelerated disappearance of capillary endothelium, morphological and functional alterations of endothelial cells, and weakening of intercellular junctions have been described. In vitro, high ambient glucose was demonstrated to affect endothelial and other vascular cells at the cellular level, delay endothelial cell replication, and cause excessive cell death. Studies have indicated that oxidative stress can induce apoptosis, which may be regulated by different signaling pathways. Evidence has been demonstrated that ROS can activate c-Jun NH₂-terminal kinases (JNKs)/stress-activated protein kinases (SAPK), which can regulate apoptosis in certain cells. Overexpression of activated JNK1 causes cell death in transfected Jurkat cells, whereas the expression of a dominant-negative mutant of JNK1 prevents ultraviolet C– and γ-irradiation–induced cell death. Some well-known chemotherapeutic agents, such as Adriamycin and vinblastine, can also activate JNK and...
trigger subsequent apoptosis programs in different cell lines.\(^6\) Recently, the relationship between SAPK/JNK and interleukin 1β–converting enzyme (ICE)/CED-3 family proteases in apoptotic cell death has been investigated.\(^6,21\) However, it is not known whether SAPK/JNK is involved in high-glucose–induced apoptosis in human endothelial cells.

ICE and related cysteine proteases, such as CED-3, CPP32/Yama, Ich-2/ICErel-II/IX, or Mch2, are thought to be downstream executors of apoptosis.\(^22,23\) Evidence indicates that ICE (caspase-1) seems not to be required for most apoptosis in vivo.\(^24,25\) whereas many other members of the caspase family might be involved in apoptosis. Among them, ICE/CED-3–like protease (caspase-3/CPP32) has been considered to be a central component of the proteolytic cascade and plays a key role during apoptosis.\(^26,27\) However, the activation of caspase-3 in endothelial cells during the high glucose–induced apoptotic process is still not determined. Such information is important for understanding molecular mechanisms of high-glucose–induced endothelial cell apoptosis.

In the present study, we investigated the role of ROS and the possible involvement of the signal pathway of JNK and the downstream executor cysteine protease in high-glucose–induced apoptosis in human endothelial cells.

Methods

Cell Culture and High-Glucose Experiments

Human umbilical vein endothelial cells (HUVECs) were cultured as previously described.\(^28\) Cells were seeded at a density of \(1 \times 10^5\) per 75-cm\(^2\) flask in medium 199 (Gibco), supplemented with 20 mmol/L HEPES, 100 \(\mu\)g/mL endothelial cell growth substance (Collaborative Research Inc), and 20% FCS (Gibco). The cultures were maintained at 37°C with a gas mixture of 5% CO\(_2\)–95% air. Subcultures were performed with trypsin-EDTA. All media were supplemented with 5 U/mL heparin, 100 \(\mu\)U/mL penicillin, and 0.1 mg/mL streptomycin. Medium was refreshed every third day. The endothelial cells were identified by the presence of factor VIII–related antigen (Histostet Kit, Immunolok) and a typical “cobblestone” appearance. Endothelial cells of the third to fifth passages in the actively growing condition were used for experiments. In some modification. In brief, after treatment with high glucose and/or ascorbic acid, cells were washed twice with ice-cold PBS and lysed in kinase buffer containing 20 mmol/L HEPES (pH 7.4), 50 mmol/L \(\beta\)-glycerophosphate, 1% Triton X-100, 10% glycerol, 2 mmol/L EGTA, 1 mmol/L DTT, 10 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 1 \(\mu\)g/mL aprotinin, 1 \(\mu\)g/mL leupeptin, and 1 mmol/L PMSF. The soluble extracts were prepared by centrifugation at 14 500 rpm for 15 minutes at 4°C. After normalization of protein concentration, equal amounts of protein were incubated with protein A-Sepharose and anti-JNK1 (1 \(\mu\)g; C17, Santa Cruz Biotechnology), anti-ERK1 (1 \(\mu\)g; C16, Santa Cruz Biotechnology), or anti-p38 (1 \(\mu\)g; N20, Santa Cruz Biotechnology) for 3 hours at 4°C. The immune complexes were then resuspended in 20 \(\mu\)L of kinase assay buffer containing 5 \(\mu\)Ci of [\(\alpha\]-\(\beta\)P]ATP, 30 \(\mu\)mol/L cold ATP, and 2 \(\mu\)g of substrate and incubated for 20 minutes at 30°C. Reactions were terminated by the addition of SDS sample buffer and boiling for 5 minutes. The proteins were resolved by SDS-PAGE and visualized by autoradiography. GST–c-Jun (1/79) was used as a substrate for JNK1, myelin basic protein for ERK1, and ATF-2 for p38.

Western Blot Analysis

Protein levels of JNK1, ERK1, p38, and caspases (caspase-3, -6, -7, -8, -10) were analyzed by Western blot as described previously.\(^29\) Briefly, cell lysates were prepared, electrotransferred, and then immunoblotted with anti-JNK1, anti-ERK1, anti-p38, and caspase-3 subfamily (caspase-3, -6, -7, -8, -10) polyclonal antibody (Santa Cruz Biotechnology). Detection was performed with Western blotting reagent ECL (Amersham), and chemiluminescence was exposed by the filters of Kodak X-Omat films.

Inhibition of JNK1 by Antisense Oligonucleotides

JNK1 sense (5'–ATCATGAGCAGAAGCAAGCAGAC–3') and antisense (5'–GTCACGCTTGCTTCTGCTCATGAT–3') oligonucleotides were synthesized under phosphorothioate-modified conditions and purified by high-performance liquid chromatography (Greiner Japan). These sequences represent the amino acid codons –1 to +7 of JNK1. The oligonucleotides were dissolved in 30 mmol/L HEPES (pH 7.0) and added to culture media. After treatment with the oligonucleotides for 48 hours, cells were analyzed for intracellular hydrogen peroxide production and kinase activity and protein level.

Assay of Caspase-3/CPP32 Enzyme Activity

Caspase-3 activity was measured by the method of Enari et al,\(^32\) with some modification. In brief, after cells (1\(\times\)10\(^5\)) were treated as indicated, cytosolic extracts were prepared by repeated cycles of freezing and thawing in 300 \(\mu\)L of extraction buffer (12.5 mmol/L Tris [pH 7.0], 1 mmol/L DTT, 0.125 mmol/L EDTA, 5% glycerol, 1 mmol/L PMSF, 1 \(\mu\)g/mL leupeptin, and 1 \(\mu\)g/mL aprotinin). Cell lysates (100 \(\mu\)g) were diluted with the buffer (50 mmol/L Tris [pH 7.0], 1 mmol/L EDTA, and 10 mmol/L EGTA) and incubated at 37°C with 10 \(\mu\)mol/L Ac-DEVD-AMC, a caspase-3/CPP32 substrate. The fluorescence of the cleaved substrate was measured by a spectrophotometer (Hitachi F-3000) with an excitation wavelength at 380 nm and an emission wavelength at 460 nm.

Statistical Analysis

Cell counts for endothelial cells under various conditions represent mean ± SD. All statistical data were obtained by ANOVA followed by Student’s t test. Statistical significance was assigned at the level of \(P<0.05\).
Results

ROS Generation and Involvement in High Glucose–Induced Apoptosis

To investigate whether H$_2$O$_2$ was generated in high glucose–induced apoptosis, flow cytometry was used. Treatment with high glucose on HUVECs was found to increase DCFH fluorescence in a time- and dose-dependent manner (Figure 1). Ascorbic acid (100 μmol/L) treatment completely suppressed the increase of DCFH fluorescence induced by high glucose. Moreover, we examined whether ROS generation was involved in the high glucose–induced apoptosis in human endothelial cells. The inhibitory effect of ascorbic acid on high glucose–induced apoptosis was demonstrated in morphological characteristic staining (Figure 2A). High glucose–induced apoptosis showed a time-dependent manner that could be effectively inhibited by ascorbic acid (Figure 2B). Apoptosis and the level of DCFH fluorescence were not affected by mannitol (33 mmol/L) treatment, excluding the involvement of osmotic effects of high glucose concentrations (data not shown).

High Glucose–Elicited JNK Activity

To study the apoptosis-related signaling pathways activated in HUVECs treated with high glucose (33 mmol/L), we examined JNK, ERK1/2, and p38 kinase activities by immunocomplex kinase assay. In a time-course study, JNK activity was increased at 24 hours and sustained up to 48 hours (Figure 3A, top). Western blot analysis showed that the level of JNK protein was not changed during high-glucose treatment, suggesting that enhancement of JNK activation was not due to the expression of JNK protein (Figure 3A, bottom). The activity and protein expression of ERK1/2 and p38-MAPK were not changed during the treatment periods (Figure 3, B and C). After 48 hours of treatment with high glucose in the presence of ascorbic acid (100 μmol/L), the increase of JNK1 activity could be reversed (Figure 4, top). Ascorbic acid alone did not alter the JNK1 activity. Taken together, these data suggest that JNK, but not ERK1/2 or p38, is persistently activated during high glucose–induced apoptosis.

High Glucose–Elicited Caspase-3/CPP32 Activity

Because caspase-3 plays an important role in various drug-induced forms of apoptosis, we investigated whether caspase-3 was involved in the high glucose–induced apoptosis. High glucose could induce an increase of caspase-3 activity with time up to 48 hours, as determined by use of a fluorogenic tetrapeptide substrate, Ac-DEVD-AMC (Figure 5A). High glucose–

![Figure 1](image1.png)

**Figure 1.** High glucose–induced intracellular H$_2$O$_2$ formation in HUVECs. HUVECs were exposed to high glucose (19 or 33 mmol/L) for 24 to 48 hours in presence or absence of ascorbic acid (Vit. C; 100 μmol/L). Control level of glucose was 5.5 mmol/L. Intracellular H$_2$O$_2$ production was determined by fluorescence of DCFH-DA as described in Methods. Data are mean±SD. *P<0.05 vs control group. **P<0.05 vs group with high glucose treatment.

![Figure 2](image2.png)

**Figure 2.** High glucose–induced apoptosis in HUVECs. A, Apoptosis induced by high glucose (33 mmol/L) was determined by fluorescent dye Hoechst 33258 method. a, Control; b, high glucose exposure for 48 hours; c, ascorbic acid alone; d, high glucose + ascorbic acid. B, HUVECs were treated with high glucose (33 mmol/L) for 24 to 48 hours in presence or absence of ascorbic acid (Vit. C; 100 μmol/L). Cell death was detected by ELISA as described in Methods. Data are mean±SD. *P<0.05 vs control. **P<0.05 vs group with high glucose treatment.
induced caspase-3 activity was also suppressed by ascorbic acid (Figure 5B). Ascorbic acid alone did not affect the caspase-3 activity. Furthermore, in testing whether other active caspases, such as caspase-6, -7, -8, and -10, were also involved, the Western blot analysis was performed. The caspase-3 cleavage product was seen 48 hours after treatment with high glucose, but not other members of the caspase-3 subfamily (caspase-6, -7, -8, -10) (Figure 6).

**Blockade of JNK and Caspase-3/CPP32 Activity on High Glucose–Induced Apoptosis**

To further investigate the role of JNK activity and caspase-3/CPP32 in high glucose–induced apoptosis, we designed JNK antisense and sense oligonucleotides for experiments. The apoptosis induced by high glucose could be prevented by pretreatment with JNK1 antisense oligonucleotide (25 μmol/L) for 12 hours but not by its sense oligonucleotide (25 μmol/L) (Figure 7A). The sense and antisense oligonucleotides of JNK1 were marginally nontoxic to the endothelial cells. The apoptosis induced by high glucose could also be effectively inhibited by pretreatment for 30 minutes with caspase-3/CPP32–specific inhibitor, Ac-DEVD-CHO (100 μmol/L), which was nontoxic to the endothelial cells (Figure 7B). To show the efficacy and specificity of the JNK1 antisense oligonucleotide, we used a kinase activity assay and Western blot analysis, which showed that the JNK1 antisense oligonucleotide could specifically suppress the expression of endogenous JNK1 activity and protein level after 48 hours of treatment with high glucose (33 mmol/L) (Figure 7C), but did not affect ERKs or p38-MAPK (data not shown). The sense oligonucleotides have no effect.

**Figure 3.** Effect of high glucose on activation of JNK, ERK1/2, and p38 in HUVECs. Activities and protein levels of JNK (A), ERK1/2 (B), and p38 kinase (C) were measured after high glucose treatment at various time intervals. Kinase activity assays were performed by immunocomplex assay (top), and protein levels were determined by Western blot (bottom), as described in Methods. GST-c-jun, myelin basic protein (MBP), and ATF-2 were used as substrates for JNK1, ERK1/2, and p38, respectively.

**Figure 4.** Effect of ascorbic acid on high glucose–induced JNK activity. HUVECs were treated with high glucose (33 mmol/L) for 48 hours in presence or absence of ascorbic acid (Vit. C, 100 μmol/L). Kinase activity assays were performed by immunocomplex assay (top), and protein levels were determined by Western blot (bottom), as described in Methods.

**Figure 5.** Effect of high glucose on caspase-3/CPP32 activity. A, HUVECs treated with high concentration of glucose (33 mmol/L) for 12 to 48 hours showed increase of activity of caspase-3/CPP32 in a time-dependent manner, as determined by spectrofluorometry. B, Ascorbic acid (Vit. C; 100 μmol/L) can effectively inhibit caspase-3/CPP32 activity after exposure to high glucose (33 mmol/L). Data are mean±SD. *P<0.05 vs control. **P<0.05 vs group with high glucose treatment.

**Figure 7.** Effect of JNK antisense oligonucleotide on high glucose–induced apoptosis. JNK1 activity assay (A) and Western blot analysis (B) were performed 48 hours after high glucose treatment with JNK1 antisense (Vit.C, 25 μmol/L) or sense (Vit.C, 25 μmol/L) oligonucleotides.
Relationship Between JNK and Caspase-3/CPP32 During the Apoptotic Process

When HUVECs were pretreated with JNK1 antisense oligonucleotide, high glucose (48 hours of treatment)–induced DCFH fluorescence was not altered (Figure 8A). However, the caspase-3/CPP32 activity induced by high glucose could be blocked by JNK1 antisense oligonucleotide (Figure 8B). JNK1 sense oligonucleotide did not influence the effects of high glucose.

Discussion

It has been reported that high glucose increased superoxide anion generation in human aortic endothelial cells. In cultured HUVECs, high glucose has been found to trigger apoptosis. The association between ROS generation and induction of apoptosis by high glucose in HUVECs seems possible but is not yet documented. Moreover, the molecular
mechanisms of high glucose–induced apoptosis in human endothelial cells need further investigation.

ROS production has been demonstrated to cause apoptotic cell death.13–16 Accumulating evidence indicates that the generation of ROS may play an important role in the development of diabetic vascular complications.6,35 The epidemiological studies have demonstrated that antioxidant agents can reduce the risk of coronary artery disease and improve the endothelial function.36–38 These observations suggest that antioxidant may be useful in the prevention of endothelial cell injury induced by ROS. In this study, we demonstrated that increased DCFH fluorescence, which was used for intracellular H2O2 detection,31,39 and apoptosis were induced by high glucose in HUVECs, which were suppressed by the antioxidant agent ascorbic acid. These results suggest that ROS may be involved in high glucose–induced apoptosis in human endothelial cells.

Many cellular mediators of the apoptotic process, such as SAPK/JNK and ICE/CED-3 family proteases, have been demonstrated.22,23,40 Overexpression of SAPK/JNK or activation of its upstream kinases in cells induces apoptosis, and blocking the activation of SAPK/JNK protects against apoptosis in PC-12 cells.41 Recently, a variety of stresses, such as mechanical stretching42 and angiotensin II treatment,43 have been reported to activate SAPK/JNK in cultured cardiomyocytes. However, the role of SAPK/JNK in high glucose–induced apoptosis in human endothelial cells has not yet been investigated. In this study, we found that high glucose could induce activation of SAPK/JNK (but not the other MAPK subfamilies ERK1/2 and p38), congruent with the increase in cell death, and vitamin C inhibited these responses. In addition, treatment of HUVECs with JNK1-specific antisense oligonucleotides could effectively abolish high glucose–induced apoptosis. Therefore, these results suggest that JNK1 but not other MAPK subfamilies, ie, ERK1/2 and p38, contributes to ROS-mediated high glucose–induced apoptosis in HUVECs.

ICE/CED-3 family proteases can activate themselves in vitro, and some can activate other family members, which in turn cleave various substrate proteins that account for many of the biochemical and morphological changes that occur during apoptosis.23,26,27 Among them, caspase-3/CPP32 has been considered a central component of the proteolytic cascade during apoptosis.26,27 We examined the substrate specificity of proteolytic activity and identified caspase-3 activity in the extracts from high glucose–activated human endothelial cells. Treatment of HUVECs with high glucose resulted in a significant increase in proteolytic activity toward Ac-DEVD-AMC, which shows the relative specificity for caspase-3/CPP32.27 Both the activation of caspase-3/CPP32 and apoptosis induced by high glucose could be inhibited by Ac-DEVD-CHO, the caspase-3/CPP32–specific inhibitor, and the antioxidant vitamin C. Moreover, to verify whether only caspase-3/CPP32 is activated in high glucose–induced apoptosis, we examined the role of other members of the caspase-3 subfamily, such as caspase-6, -7, -8, and -10, in apoptosis. The results showed that caspase-3 was the predominant one activated. Therefore, although the role of the other members of the caspase family in high glucose–induced apoptosis still needs to be clarified, our current data suggest that caspase-3 may be a predominant target involved in the ROS-mediated high glucose–induced apoptosis in human endothelial cells.

Some studies have demonstrated that JNK induction appears to be upstream of ICE/CED-3 proteases in apoptosis induced by UV-C and γ-radiation18 and anticancer drugs,20 whereas others documented the activation of SAPK/JNK downstream of ICE/CED-3 proteases in the CD95 (APO-1/Fas) pathway.44 The relationship between SAPK/JNK and ICE/CED-3 protease pathways in apoptosis still remains to be clarified. Thus, understanding the molecular sequence of ROS, SAPK/JNK, and the proteoregulating apoptotic process would be very important. In our results, JNK activity is elevated by 24 hours, but DCFH fluorescence is not increased until 36 hours after the treatment of high glucose in HUVECs. Nevertheless, the increase of DCFH

Figure 8. Effects of JNK1-specific sense and antisense oligonucleotides on high glucose–induced H2O2 generation and caspase-3/CPP32 activity in HUVECs. H2O2 generation (A) and activity of caspase-3/CPP32 (B) induced by high glucose (33 mmol/L) in presence or absence of JNK-specific antisense or sense oligonucleotide phosphorothioates (25 μmol/L) pretreated for 12 hours before addition of high glucose for another 48 hours. Data are mean±SD. *P<0.05 vs control.
fluorescence and JNK activation induced by high glucose can be effectively suppressed by ascorbic acid (Figures 1 and 4). This leads us to propose that reactive species (i.e., O$_2^·$, H$_2$O$_2$, and other radicals) may participate in this signal event. Future studies may be necessary to clarify the role of various reactive species. Moreover, the JNK1 antisense oligonucleotide is capable of inhibiting the activation of caspase-3 but not the DCFH fluorescence in HUVECs treated with high glucose (Figure 8). These findings imply that ROS induced by high glucose may be involved in SAPK/JNK activation, which in turn triggers caspase-3 activation in human endothelial cells.

In conclusion, in this study, we delineated the possible signaling pathway of high glucose–induced apoptosis, in which ROS are involved in JNK activation, which leads to triggering of caspase-3 and facilitation of apoptosis in human endothelial cells. These findings further imply that vitamin C and/or subsequent blockers of this signaling pathway may have potential in reducing diabetic high glucose–induced detrimental effects on human endothelial cells.

References

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Circulation. 2000;101:2618-2624
doi: 10.1161/01.CIR.101.22.2618
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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