High Glucose Induces Human Endothelial Cell Apoptosis Through a Phosphoinositide 3-Kinase–Regulated Cyclooxygenase-2 Pathway

Meei Ling Sheu, Feng Ming Ho, Rong Sen Yang, Kuo Fang Chao, Wan Wan Lin, Shoei Yn Lin-Shiau, Shing-Hwa Liu

Objectives—Diabetes mellitus causes endothelial dysfunction. The precise molecular mechanisms by which hyperglycemia causes apoptosis in endothelial cells are not yet well understood. The aim of this study was to explore the role of cyclooxygenase-2 (COX-2) and the possible involvement of phosphoinositide 3-kinase (PI3K) signaling in high glucose (HG)–induced apoptosis in human umbilical vein endothelial cells (HUVECs).

Methods and Results—For detection of apoptosis, the morphological Hoechst staining and Annexin V/propidium iodide staining were used. Glucose upregulated COX-2 protein expression, which was associated with the induction of prostaglandin (PG) E₂ (PGE₂), caspase-3 activity, and apoptosis. Unexpectedly, we found that PI3K inhibitors could suppress COX-2 expression, PGE₂ production, caspase-3 activity, and the subsequent apoptosis under HG condition. Glucose-induced activation of PI3K resulted in the downstream effector Akt phosphorylation. PI3K inhibitors effectively attenuated the intracellular reactive oxygen species (ROS) generation and nuclear factor κB (NF-κB) activation. Blocking the PI3K and Akt activities with the dominant-negative vectors greatly diminished the HG-triggered NF-κB activation and COX-2 expression and apoptosis.

Conclusions—These results suggest that HG, via PI3K/Akt signaling, induces NF-κB–related upregulation of COX-2, which in turn triggers the caspase-3 activity that facilitates HUVEC apoptosis. Also, HG may cause ROS generation in HUVECs through a PI3K/Akt–dependent pathway. (Arterioscler Thromb Vasc Biol. 2005;25:539-545.)

Key Words: glucose ■ endothelium ■ cells ■ apoptosis

Diabetes mellitus can cause a wide variety of vascular complications and cardiovascular dysfunction.† The mechanisms of hyperglycemia-related tissue damage and clinical complications still remain unclear. Apoptosis is particularly prominent in models of hyperglycemia injury, affecting a significant proportion of vascular endothelium in the tissue damage.2–4 Studies have indicated that oxidative stress can induce apoptosis, which may be regulated by different signaling pathways.5,6 Involvement of reactive oxygen species (ROS) in the induction of apoptosis by high glucose (HG) has been demonstrated in cultured human endothelial cells.7 The formation of oxygen-derived radicals might lead to an activation of transcription factors such as nuclear factor κB (NF-κB) transferring the activation signal into the nucleus and leading to changes in gene expression necessary for induction of apoptosis. We found previously a c-Jun amino-terminal kinase–dependent pathway was involved in the ROS-related HG-induced apoptosis in human endothelial cells.8 It has also been demonstrated that oxidative stress induced apoptosis in human aortic endothelial cells through the downregulation of bcl-2, translocation of bax, and upregulation of p53, probably through NF-κB activation.9 Nevertheless, the precise molecular mechanisms by which HG causes apoptosis in endothelial cells are not yet well understood.

Prostaglandins (PGs) generated by cyclooxygenase (COX) have been implicated in hyperglycemia-induced endothelial dysfunction.10–13 A recent study has shown that HG induced oxidative stress and upregulation of COX-2, resulting in reduced NO availability and altered prostanoid profile in human endothelial cells.14 Production of PG E₂ (PGE₂), via COX-2 induction, has been demonstrated to be related to the induction of cell death in human osteoarthritis chondrocytes by NO.15 However, the role of COX-2 in HG-caused cell death and the molecular mechanisms regulating COX-2 expression in endothelial cells remain to be clarified. Moreover, it has been suggested that phosphoinositide 3-kinase (PI3K) might play a central role in a diverse range of cellular

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From the Institutes of Toxicology (M.L.S., K.F.C., S.Y.L.S., S.H.L.) and Pharmacology (S.Y.L.S., W.W.L.), and the Department of Orthopedics (R.S.Y.), College of Medicine, National Taiwan University, Taipei; and the Department of Internal Medicine (F.M.H.), Tao-Yuan General Hospital, Taoyan, Taiwan.
Correspondence to Shing-Hwa Liu, PhD, Institute of Toxicology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Rd, Taipei, 10043, Taiwan. E-mail shliu@ha.mc.ntu.edu.tw
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responses, including cell growth, survival, and malignant transformation.\textsuperscript{16,17} Recent studies have shown that activation of PI3K and Akt might play a role in regulating COX-2 expression in some cultured cells.\textsuperscript{18–20} Together, in the current study, we explored the role of COX-2 and the possible involvement of PI3K signaling in HG-induced apoptosis in human endothelial cells. We also examined the role of the PI3K pathway in ROS generation, which might regulate the HG-induced apoptosis in endothelial cells.

**Methods**

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs) were obtained by collagenase treatment of umbilical cord veins as described previously.\textsuperscript{2,3} Cells were cultured on gelatin-coated dishes and propagated in M199 medium supplemented with 20% bovine calf serum ( Gibco), 50 \( \mu \)g/mL endothelial cell growth factor, 90 \( \mu \)g/mL heparin, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin in humidified air, 5\% CO\textsubscript{2} at 37°C. HUVECs of the third to fifth passages were used. In some experiments, transfection in HUVECs was performed using the Lipofectin reagent ( BD CLONTECH) according to manufacturer recommendations with 2 \( \mu \)g of total DNA (or vector) per sample. The efficiency of transfection (~80\%) was determined by using an equal amount of a plasmid encoding the green fluorescent protein under the cytomegalovirus promoter. Plasmids containing the dominant-negative (DN)-p85 (\( \delta \)-p85) or DN-Akt (Akt K179A)\textsuperscript{2,3} were kindly provided by Drs R.H. Chen and M.L. Kuo (Institutes of Molecular Medicine and Toxicology, National Taiwan University); DN-inhibitor eBoz (eBoz)\textsuperscript{26} was provided by Dr W.W. Lin. (Institutes of Pharmacology, National Taiwan University).

**Apoptosis and Caspase-3/Cleaved Caspase 3 Activity**

HUVECs were collected and fixed in methanol/acetone solution for 5 minutes and washed with PBS. Fixed cells were then stained with 0.1 ng/mL Hoechst 33258 (Boehringer Mannheim) for 10 minutes in the dark to counterstain nuclei. Cells were observed and photographed under a Nikon fluorescence microscope. In some experiments, the Annexin V/propidium iodide (PI; BD CLONTECH) was used to quantify numbers of apoptotic cells. Cells were washed twice with PBS and stained with Annexin V and PI for 20 minutes at room temperature. The level of apoptosis was determined by measuring the fluorescence of the cells by flow cytometry analysis.

Also, we measured caspase-3 activity to quantify the apoptotic ratio. Caspase-3 activity was measured as described previously.\textsuperscript{2,3} Cell lysates were incubated at 37\°C with 10 \( \mu \)mol/L N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC), a caspase-3/Cleaved Caspase 3 (CPP32) substrate. The fluorescence of the cleaved substrate was measured by a spectrophotometer with an excitation wavelength at 380 nm and an emission wavelength at 460 nm.

**Cell Viability**

Viability was measured using the Cell Titer 96\textsuperscript{TM} AQueous cell viability assay kit (Promega). This assay is based on the cellular conversion of the colorimetric reagent MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt), in the presence of electron-coupling reagent phenazine methosulfate, into soluble formazan by dehydrogenase enzymes found only in metabolically active, living cells. Formazan formation is found only in metabolically active, living cells. Formazan formation was measured on the basis of increased absorbance at 490 nm.

**PI3K Activity**

PI3K activity was assayed as described previously.\textsuperscript{26} Cell extracts were incubated with 2 \( \mu \)g of anti-p85 antibody overnight at 4\°C. The immunocomplex was precipitated with protein A-Sepharose for 1 hour at 4\°C and washed 3\times with lysis buffer, twice with LiCl buffer, and twice with Tris-NaCl-EDTA (TNE) buffer (10 mmol/L Tris, 100 mmol/L NaCl, and 1 mmol/L EDTA). The immunocomplex was preincubated with 20 mmol/L Heps, containing 2 mg/mL PI on ice for 10 minutes. Kinase reaction was performed by adding reaction buffer (10 \( \mu \)Ci of \( \gamma \)-\textsuperscript{32}P-ATP, 20 mmol/L Heps, pH 7.4, 20 \( \mu \)mol/L ATP, and 5 mmol/L MgCl\textsubscript{2}) for 15 minutes. The radiolabeled lipids were separated by thin-layer chromatography and visualized by phosphorimaging.

**Immunoblotting**

Protein levels of Akt, phospho-Akt, and COX-2 were analyzed by Western blot as described previously.\textsuperscript{26} Cells lysates were prepared, electrotransferred, and then immunoblotted with anti-Akt, anti-phospho-Akt (New England BioLabs), and anti-COX-2 antibodies (Santa Cruz Biotechnology). Detection was performed with Western blotting reagent ECL (Amersham), and chemiluminescence was exposed by the filters of Kodak X-Omat films.

**Electrophoretic Mobility Shift Assay**

Electrophoretic mobility shift assay (EMSA) was performed as described previously.\textsuperscript{27} The following oligonucleotide with the NF-\( \kappa \)B consensus binding sequence was used: 5'-GATCCAAGGGGACCTTTCCATGGAATCAGGTCATCTTCCATG-3' (Life Technologies). The consensus oligonucleotide probes were end-labeled with \( \gamma \)-\textsuperscript{32}P-ATP according to the manufacturer description. Protein–DNA complexes were separated by electrophoresis on a nondenaturing 6% polyacrylamide gel and visualized by autoradiography. For competition experiments, a 100-fold molar excess of the unlabeled oligonucleotides was added 15 minutes before incubation of nuclear extracts with the end-labeled oligonucleotides.

**ROS Production**

Intracellular ROS generation was monitored by flow cytometry using paraoxon-sensitive fluorescent probe 2',7'-dichlorofluorescin diacetate (DCFH-DA) is converted by intracellular esterases to DCFH, which is oxidized into the highly fluorescent dichlorofluorescin (DCF) in the presence of a proper oxidant, and then analyzed by flow cytometry.

**PGE\textsubscript{2} Production**

PGE\textsubscript{2} levels in cell culture supernatants were determined using the PGE\textsubscript{2} enzyme immunoassay kit (Cayman Chemical).

**Statistical Analyses**

Results are expressed as mean±SEM and data analyzed by Student’s \( t \) test. For multiple comparisons, results were analyzed by ANOVA followed by Fisher’s test. \( P<0.05 \) was considered statistically significant.

**Results**

**COX-2 Protein Expression and Involvement in HG-Induced Apoptosis**

HUVECs exposed to HG (30 mmol/L) for 8 to 12 hours increased COX-2 protein expression (4.2-fold induction; Figure 1Aa), whereas no changes were observed for COX-1 protein level (data not shown). HG-induced increase of COX-2 expression was associated with a higher release of PGE\textsubscript{2}, which could be inhibited by selective COX-2 inhibitor NS398 (10 and 20 \( \mu \)mol/L; Figure 1Ba). We also found that combining HG with PGE\textsubscript{2} (1 \( \mu \)mol/L) showed an additive effect on HUVEC death (Figure 2A). Moreover, we examined whether COX-2–mediated PGE\textsubscript{2} generation was involved in the HG-induced apoptosis. The inhibitory effect of NS398 on HG-induced apoptosis was demonstrated in caspase-3 activity assay (Figure 2B) and morphological characteristic Hoechst-33258 staining (Figure 2C). The HG-induced cell death could be reversed by NS398 (Figure 3A).
Mannitol (30 mmol/L), used as an osmotic control, did not affect COX-2 expression or apoptosis and cell viability (Figures 1A, 2C, and 3A).

PI3K Activity and Involvement in HG-Induced COX-2 Protein Expression and Apoptosis

HUVECs were cultured with HG for 12 to 72 hours in the presence or absence of PI3K inhibitors (LY294002 and wortmannin). HG-induced increased COX-2 protein expression and its related PGE2 release and cell death could be inhibited by LY294002 (10 or 20 μmol/L) or wortmannin (100 nmol/L; Figure 1Ab and 1Bb; Figure 3A). Moreover, the inhibitory effect of PI3K inhibitors on HG-induced apoptosis was demonstrated in caspase-3 activity assay (Figure 3B), morphological characteristic Hoechst 33258 staining (Figure 3C), and Annexin V/PI assay (Figure 4Ca).

Furthermore, the authentic PI3K activity of HUVECs after treatment with HG was determined. Immunoprecipitates with the anti-p85 antibody revealed a substantial increase in PI3K activity 5 to 10 minutes after HG exposure, which could be inhibited by LY294002 (10 μmol/L; Figure 4A). To further evaluate the involvement of Akt in HG-triggered responses, Akt activity was examined by determining the serine-phosphorylated status of Akt using an anti–phospho-Akt antibody. In a time-dependent manner, Akt activation was shown 10 to 60 minutes after HG treatment (Figure 4B). LY294002 markedly reduced this HG-induced response (Figure 4B).

The next aim of the investigation was to ascertain whether PI3K/Akt activity inhibition might affect the HG-induced COX-2 protein expression and apoptosis in HUVECs. To address this issue, cells were transfected with a dominant-negative form of the regulatory subunit (p85) of PI3K (DN-p85) or a dominant-negative form of Akt (DN-Akt). Transfection results revealed that the DN-p85 or DN-Akt transfection significantly inhibited HG-induced COX-2 protein expression (Figure 1Ac) and decreased cell viability (Figure 4Cb) and increased caspase-3 activity (Figure 4D) and apoptosis (Annexin/PI staining; Figure 5A). The control pcDNA3 transfection did not affect the HG-induced responses (data not shown).
Effects of PI3K Inhibitors on HG-Induced NF-κB Activation and ROS Generation

As shown in Figure 5B, stimulation of HUVECs with HG for 4 hours showed a marked increase of NF-κB activation, as determined by EMSA. LY294002 (LY; 20 μmol/L) or COX-2 inhibitor NS398 (20 μmol/L), and then cell viability was detected. HUVECs pretreated with HG for 48 hours showed increase of caspase-3/CPP32 activity. LY294002 (LY; 20 μmol/L) prevented the effect of HG. All data are presented as mean±SEM from 5 independent experiments performed in duplicate. *P<0.05 vs control; **P<0.05 vs HG alone. C. Apoptosis induced by HG was determined by fluorescent dye Hoechst 33258 method. Ca, Control; Cb, HG exposure for 48 hours; Cc, HG+LY294002 (LY); Cd, HG+wortmannin (WM/100 mmol/L). Results shown are representative of at least 4 independent experiments.

Discussion

In the present study, we demonstrate for the first time that HG causes PI3K/Akt–dependent upregulation of inducible COX-2 expression, as well as an increase of PGE2 production and increased caspase-3 activity and induction of apoptosis in HUVECs. These findings are supported by functional studies of HUVECs showing that the activity of PI3K and Akt is triggered by HG, and the inhibition of PI3K/Akt activity with LY294002 or wortmannin, or by expressing the dominant-negative p85 or Akt is capable of preventing the HG-caused COX-2–mediated PGE2 production, and subsequently leads to the inhibition of caspase-3 activity and apoptosis. Moreover, HG induces a PI3K/Akt–regulated ROS generation.

Hence, activation of the PI3K/Akt pathway may represent a proximal node in the intracellular signaling leading to hyperglycemia-induced endothelial cell apoptosis.

In a number of cell and animal models, induction of COX-2 has been shown to promote cell growth, inhibit apoptosis, and enhance cell motility and adhesion. Paradox-
ically, PGE₂ production via the induction of COX-2 expression has been demonstrated to relate to the induction of cell death in some cultured cells such as human articular chondrocytes and human synovial fibroblasts. Similarly, in the present study, we found that HG induced COX-2 protein expression in HUVECs, which was associated with a higher release of PGE₂. Selective COX-2 inhibitor NS398 inhibited PGE₂ production and blocked increased caspase-3 activity and apoptosis induced by HG. Combining HG with PGE₂ showed an additive effect on cell death. Moreover, NF-κB was known as a transcriptional regulator of expression of COX-2 gene. NF-κB has also been demonstrated as a mediator in HG (hyperglycemia) or oxidative stress-triggered apoptosis of endothelial cells. Activation of NF-κB promoted p53 upregulation, which might suppress the level of bcl-2, resulting in endothelial cell apoptosis. We also observed that HG could trigger the NF-κB activation in HUVECs, and dominant-negative IκB transfection could inhibit HG-induced COX-2 protein expression and apoptosis. Therefore, these findings suggest an involvement of COX-2–mediated PGE₂ production in HG-induced HUVEC apoptosis through activation of NF-κB. On the other hand, in the clinical setting, hyperglycemia was usually accompanied by increased free fatty acid levels, which might also have detrimental effects on endothelial cells. It has been suggested that the alterations in fatty acid metabolism, impaired Akt activation by insulin, and increased caspase-3 activity preceded visible evidence of apoptosis in HUVECs incubated in a hyperglycemic medium. It has also been identified that saturated fatty acids induced the expression of COX-2 in monocyte/macrophage cells. Hence, the involvement of free fatty acid in the hyperglycemia-induced COX-2 expression and apoptosis in HUVECs may need to clarify in the future.

PI3K signal plays an important regulatory role in a diverse range of cellular responses, including cell growth, survival, and malignant transformation. It has been shown that PI3K activation has an antiapoptotic effect in endothelial cells and elsewhere. Unexpectedly, we observed that the treatment of HG in HUVECs leads to activation of PI3K and Akt, which subsequently leads to

Figure 5. The HG-induced apoptosis in HUVECs requires activation of PI3K. HUVECs were transiently transfected with DN-p85, DN-Akt, or DN-IκBα. A, Cells were analyzed for apoptosis by Annexin V/PI staining. The percentage of cells found in each quadrant of the dot plot is depicted. Results shown are representative of at least 4 independent experiments. B, Nuclear extracts were subjected to an EMSA. Cells were treated with HG in the presence or absence of LY294002 (LY; 20 μmol/L) for 4 hours. Quantification of the NF-κB binding activity was performed by densitometric analysis. *P<0.05 vs control; †P<0.05 vs HG alone.

Figure 6. HUVECs were treated with HG in the presence or absence of transfection of DN-IκBα. A, Expression of COX-2 protein in HUVECs exposed to HG or PMA for 12 hours. Quantification of the COX-2 expression was performed by densitometric analysis. B, Apoptosis induced by HG was determined by Annexin V/PI staining. Data represent percentage of Annexin V–positive cells. C, HUVECs were treated with HG for 72 hours, and cell viability was detected by MTS assay. D, HG-induced intracellular DCF-sensitive ROS generation. HUVECs were exposed to HG for 36 to 48 hours in presence or absence of LY294002, antioxidants (ascorbic acid [vitamin C]; 100 μmol/L), diphenyleneiodonium (DPI; 20 μmol/L), or rotenone (ROT; 20 μmol/L). All data are presented as mean±SEM from 4 independent experiments. *P<0.05 vs control; †P<0.05 vs HG or PMA alone.
activation of NF-κB and COX-2 expression, and then induces apoptosis. At present, the physiological role of the activation of PI3K and Akt during HG-induced HUVEC death is not clear. In accordance with our present findings, such an apoptosis-promoting effect of PI3K has been reported in other cell systems. For example, a rapid and transient activation of PI3K was critical for Fas or C6-ceramide–induced apoptosis in Jurkat cells;37 a significant increase of PI3K activity was observed during Fas-induced human neutrophil apoptosis and suggested that Fas-induced PI3K activity was a proapoptotic signal;38 and activation of PI3K/Akt was induced during pyrrolidine dithiocarbamate–induced neuronal cell death.39 Interferon-α–induced apoptosis in tumor cells was mediated through the PI3K/mammalian target of rapamycin signaling pathway.40 Recent studies have also shown that activation of PI3K might play a role in regulating the COX-2 expression in some cultured cells.18–20 Therefore, the current results indicate that PI3K/Akt may be involved in the HG-induced HUVEC apoptosis, and PI3K/Akt is present upstream of NF-κB activation and COX-2 expression in the HG-triggered signaling pathways. In conclusion, our study demonstrates that activation of a PI3K/Akt–signaling pathway is required for HG-induced activation of NF-κB and COX-2–mediated PGE2 production and subsequent caspase-3 activation and facilitation of apoptosis in HUVECs. Also, HG triggers ROS generation through a PI3K-dependent pathway. These findings may be relevant in understanding the possible intracellular signaling associated with diabetic hyperglycemia–induced detrimental effects on endothelial cells and provide novel therapeutic strategies to prevent the development and progression of vascular complications in diabetes mellitus.

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