Abbreviations used:

COX-2, Cyclooxygenase-2;

DCF, dichlorofluorescein;

DCFH-DA, 2′,7′-dichlorofluorescein diacetate;

DHE, dihydroethidium

DM, diabetes mellitus;

DPI, diphenylene iodonium

EMSA, electrophoretic mobility shift assay;

HG, high glucose;

IkB, inhibitor k B;

IKK, inhibitor IkB kinase;

JNK, c-Jun N-terminal kinase;

MAPKs,; mitogen-activated protein-kinases;

MTT, 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium-bromide;

NAC, N-acetyl cysteine;

NADPH, nicotinamide adenine dinucleotide phosphate;

NF-κB, nuclear factor-κB;

NG, normal glucose;

ROS, reactive oxygen species;

SAPKs, stress-activated protein kinases;

STZ, streptozotocin;

TUNEL, Terminal Deoxynucleotide Transferase-mediated dUTP Nick End Labeling.

NADPH oxidase-derived superoxide anion-induced apoptosis is mediated via the JNK-dependent activation of NF-κB in cardiomyocytes exposed to high glucose

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Abstract

Hyperglycemia-induced generation of reactive oxygen species (ROS) can lead to cardiomyocyte apoptosis and cardiac dysfunction. However, the mechanism by which high glucose causes cardiomyocyte apoptosis is not clear. In this study, we investigated the signaling pathways involved in NADPH oxidase-derived ROS-induced apoptosis in cardiomyocytes under hyperglycemic conditions. H9c2 cells were treated with 5.5 or 33 mM glucose for 36 hr. We found that 33 mM glucose resulted in a time-dependent increase in ROS generation as well as a time-dependent increase in protein expression of $p22^{phox}$, $p47^{phox}$, $gp91^{phox}$, phosphorylated IκB, c-Jun N-terminal kinase (JNK) and p38, as well as the nuclear translocation of NF-κB. Treatment with apocynin or diphenylene iodonium (DPI), NADPH oxidase inhibitors, resulted in reduced expression of $p22^{phox}$, $p47^{phox}$, $gp91^{phox}$, phosphorylated IκB, c-Jun N-terminal kinase (JNK) and p38. In addition, treatment with JNK and NF-κB siRNAs blocked the activity of caspase-3. Furthermore, treatment with JNK, but not p38, siRNA inhibited the glucose-induced activation of NF-κB. Similar results were obtained in neonatal cardiomyocytes exposed to high glucose concentrations. Therefore, we propose that NADPH oxidase-derived ROS-induced apoptosis is mediated via the JNK-dependent activation of NF-κB in cardiomyocytes exposed to high glucose.

Key words:

Hyperglycemia; reactive oxygen species (ROS); NADPH oxidase; c-Jun N-terminal kinase (JNK); nuclear factor *k* B (NF-*k*B); cardiomyocyte apoptosis.

Introduction

Patients with diabetes mellitus (DM) are at increased risk of cardiovascular diseases. Hyperglycemia, the major feature of diabetic cardiomyopathy, induces the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-mediated generation of reactive oxygen species (ROS). In addition, ROS are major initiators of myocardial injury. Studies have shown that abnormally high blood glucose levels can result in cardiac cell damage and apoptosis (Cai et al., 2002; Dyntar et al., 2001). However, the mechanisms by which hyperglycemic conditions can lead to ROS-induced cardiomyocyte apoptosis are not well understood.

NADPH oxidase, a ubiquitously distributed multisubunit enzyme complex, is a major source of ROS. This enzyme complex generates large amounts of $O_2^{\bullet -}$ on the outside of the cell membrane through one electron reduction of oxygen, using NADPH as an electron donor (Babior, 1999; Lambeth, 2004). NADPH-derived ROS have been demonstrated to modulate redox-sensitive signaling pathways in endothelial cells and cardiomlyocytes (Ushio-Fukai, 2006; Ushio-Fukai, 2009). In addition, accumulating evidence suggests that increased oxidative stress resulting mainly from NADPH oxidase-generated ROS in cardiac cells contributes to the development of cardiac diseases (Cave et al., 2006; Guzik et al., 2000).

The detrimental effects of oxidative stress on the diabetic heart include abnormal gene expression, altered signal transduction, and activation of pathways leading to cardiomyocyte apoptosis (Kuo et al., 2009; Ou et al.2010; Singal et al., 2001). Oxidative stress triggers cellular responses by enhancing protein phosphorylation of stress-activated protein kinases (SAPKs), namely c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein-kinase. JNK and p38 are activated by different extracellular stimuli, and their activation has been shown to play a role in left

ventricular and vascular dysfunction (Li et al., 2005; Liang and Molkentin, 2003). Studies have shown that activation of JNK and p38 in perfused rat heart is mediated by oxidative stress and that excess ROS in cardiomyocytes treated with adriamycin, a common chemotherapeutic agent known to generate significant amounts of ROS, leads to p38/JNK-mediated apoptosis (Ohno et al., 1998; Yue et al., 1998). Studies have also shown that ROS-dependent JNK and caspase-3 activation mediates apoptosis in human umbilical vein endothelial cells (Ho et al., 2000) and pancreatic β cells (Hou et al., 2008) exposed to high concentrations of glucose. Liu et al. reported that activation of NF-κB following exposure to high levels of glucose enhances JNK signaling cascades involved in vascular cell apoptosis (Ho et al., 2006). However, whether JNK and p38 are involved in hyperglycemia-induced apoptosis of cardiomyocytes is unknown.

Nuclear factor-κB (NF-κB) is a sequence-specific transcription factor that controls a variety of pathological processes in the myocardium including cardiomyocyte inflammation, ischemia/reperfusion injury, hypertrophy, and apoptosis. NF-κB signaling is initiated through the phosphorylation of Inhibitor-κB (IκB) at Ser-32 and Ser-36, or Tyr-42, and further degradation is catalyzed by the inhibitor I*k*B kinases (IKKs). The loss of the inhibitor unmasks a nuclear localization sequence within the p65/p50 subunits of NF-κB, thereby enhancing their translocation to the nucleus where they bind specific NF-κB response elements in the promoter region of target genes involved in inflammatory responses and apoptosis. A growing body of evidence indicates that NF-*k*B signaling is involved in hyperglycemia-induced cardiac cell damage (Chen et al., 2009; Sheu et al., 2008). However, whether cross-talk between NF-κB and SAPKs, namely p38 and JNK, is involved in ROS-induced apoptosis of cardiomyocytes exposed to hyperglycemic conditions is not clear.

In the present study, we investigated the signaling pathways involved in NADPH oxidase-derived ROS-induced apoptosis in cardiomyoblast H9c2 cells and primary cardiomyocytes under hyperglycemic conditions. Our results indicate that NADPH oxidase-derived ROS-induced apoptosis in cardiomyocytes exposed to high glucose is mediated via the JNK-dependent activation of NF-κB. These signaling pathways, therefore, may be potential therapeutic targets for the treatment of diabetic cardiomyopathy.

Materials and Methods

Cell culture

H9c2 cell lines were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, 100units/ml penicillin, 100µg/ml streptomycin, and 1mM pyruvate in humidified air (5% CO_2) at 37 ºC. During the treatment period, H9c2 cells were cultured in normal glucose (5.5 mM) medium with minimal essential medium for 12 h, followed by the exposure of high glucose (33 mM) for 36 h. The specificity of the inhibitors used for ROS and NADPH oxidase in this study were ~~N-acetyl cysteine (NAC) (500 µM)~~ diphenylene iodonium (DPI) (20µM) and apocynin (100 µM), respectively.

Reactive oxygen species production

H9c2 cells were cultured in 35-mm dishes and then exposed to NG and HG for 24–48 h or ~~treated with various of glucose conditions (5.5-55 mM) for 36 h~~. time periods as indicated. Intracellular generation of ROS was examined by flow cytometry using peroxide-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes). DCFH-DA is formed by intracellular esterases to DCFH, which can be oxidized into the fluorescent dichlorofluorescein (DCF) by a proper oxidant, and then quantified by flow cytometry. In addition, generation of reactive free radicals of superoxide ($O_2^{\bullet -}$) was examined using chemical probes of dihydroethidium (DHE, Molecular Probes). DHE, a nonfluorescent membrane-permeable probe, interacts with $O_2^{\bullet -}$, causing the liberation of membrane-impermeable ethidium cations. Experiments for the examination of $O_2^{\bullet -}$

were repeated for 3 times with duplicate wells for each treatment. The culture slides were photographed immediately after mounted with coverslips by UV light microscopic observations. For every well, 8 fields were randomly selected to photograph and integrated optical density (IOD)s of the images were determined.

Gp91ds-tat

Gp91ds-tat is a peptide from NOX2 cytosolic domain B, which interferes with the association of cytosolic oxidase subunit $p47^{phox}$ for membrane assembly. The peptide is designed to link a 9-amino acid peptide as a TAT sequence for translocating to make it cell-permeable. The NADPH oxidase peptide inhibitor coupled to TAT peptide has been shown to inhibit ROS production (Rey et al., 2001). Gp91ds-tat was dissolved in 0.01 mM acetic acid in saline. Cardiac cells were incubated with gp91ds-tat (20μM) for 1 hr before high glucose incubation.

Western blot analysis

Protein levels were estimated by Western blot as described previously (Ou et al., 2010). Cultured H9c2 cells were scraped and washed once with PBS, then cell suspension was spun down, and cell pellets were lysed for 30 min in lysis buffer (50 mM Tris (pH 7.5), 0.5M NaCl, 1.0 mM EDTA (pH 7.5), 10% glycerol, 1 mM BME, 1% IGEPAL-630 and proteinase inhibitor) and spun down 12,000 rpm for 20 min, the supernatant was collected. Proteins were separated in 12% gradient SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific protein binding was stopped in blocking buffer (5% milk, 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween-20) and blotted with specific antibodies against α-tubulin, JNK, c-Jun, caspase 3, NF*k*B, HDAC1, p22phox, I*k*Bα, IKK, ERK, p38, COX-2 (Santa Cruz

Biotechnology), phospho-IKK (Ser176) phospho-JNK (Thr183), phospho-c-Jun (Ser63), phospho-ERK (Ser63), phospho-p38 (Ser63) (Santa Cruz Biotechnology) and phospho-I*k*Bα (Ser32) (Cell Signaling) in the blocking buffer at 4℃ overnight. After incubation with secondary antibody for 2 h, densitometric analysis of immunoblots was performed using Fuji LAS 3000 imaging system. α-tubulin was used as a loading control.

MTT assay

H9c2 cells were inoculated into 24-well plate and cultured in HG medium for 0-48 h. Then, the medium was removed and MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium-bromide] solution (0.5mg/ml) was added to each well. The cells were incubated in a 5% CO_2 incubator at 37℃ for an additional 4 h. MTT solution was replaced by isopropanol to dissolve blue formazan crystals, and absorbance was measured at 570 nm by using a microplate reader.

Transfection of luciferase or siRNA assay

Transient transfections were carried out by the proprietary cationic polymer reagent (Fermentas) (TurboFect™ *in vitro* Transfection Reagent) following the manufacturer's instruction. In some experiments 2×10^4 cells were plated onto 24-well plates and grown overnight. Vectors, including the reporter vectors, and the internal Renilla luciferase control vector (0.1 μg) were cotransfected as indicated in the figure legends. All assays for firefly and Renilla luciferase activity were performed using one reaction plate sequentially (Promega). Briefly, at 24 h posttransfection and stimulation, the cells were washed with phosphate buffered saline and lysed with Passive Lysis Buffer. After a freeze/thaw cycle, samples were mixed with Luciferase

Assay Reagent II, and the firefly luminescence was measured with a Luminometer. Next, samples were mixed with the Stop & Glo reagent, and the Renilla luciferase activity was measured as an internal control to normalize the luciferase activity values. Double-stranded si-RNA sequences targeting ERK, JNK, p38 and NFκB mRNAs were obtained from Santa Cruz Biotechnology. The non-specific si-RNA (scramble) consisted of a nontargeting double-stranded RNA. Cells were cultured in 60-mm well plates in medium. Transfection of si-RNA was carried out with transfection reagent. Specific silencing was confirmed by immunoblotting with cellular extracts after transfection.

Immunostaining.

Cells were grown in 6-cm dish and subjected to glucose exposure with U0126 (ERK inhibitor, 10μM), SP600125 (JNK inhibitor, 10μM), SB203580 (p38 inhibitor, 10μM) or QNZ (NFκB inhibitor, 10 μM) Then, the cells were washed five times with ice-cold PBS and fixed with 4% paraformaldehyde at room temperature for 30 min. Subsequently, cells were washed five times with ice-cold PBS and permeabilized with 0.5% Triton X-100 for 10 min at 4°C. Nonspecific binding of the fixed cells was blocked with PBS containing 2% bovine serum albumin at 37°C for 30 min, followed by incubation with primary NFκB p65 antibody overnight at 4 °C. After washing, cells were incubated with anti-rabbit FITC- conjugated antibody at 37°C for 1 h. Cells were stained with cy5-conjugated antibody alone as a negative control. After staining with antibody, the fluorescence was visualized using a fluorescence microscope coupled with an image analysis system.

Cardiomyocyte Culture

Neonatal cardiomyocyte were isolated and cultured using the commercial Neonatal Cardiomyocyte Isolation System Kit (Cellutron Life Technology, Highland Park, New Jersey, USA) as described previously (Liu et al., 2009). Briefly, hearts from one- to two-day-old Sprague-Dawley rats were removed, the ventricles were pooled, and the ventricular cells were dispersed by digestion solution at 37°C. Ventricular cardiomyocyte were isolated and grown in DMEM containing 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. After 3-4 days, cells were incubated in serum-free essential medium overnight before treatment with indicated agents.

DAPI staining and TUNEL assays

Neonatal cardiomyocytes were prepared and maintained in HG condition for 36 h with the transfections of JNK and NFκB si-RNAs (10μM) with or without apocynin (0.1 mM) or DPI (20μM). DAPI staining and TUNEL assays were performed as described previously (Liu et al., 2009). Then, H9c2 cells grown on 6 mm plate were fixed with 4% paraformaldehyde solution for 30 min at room temperature. After a rinse with PBS, cells were treated with permeation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min at 4ºC. Following washing with PBS, samples were first incubated with Terminal Deoxynucleotide Transferase-mediated dUTP Nick End Labeling (TUNEL) reagent containing terminal deoxynucleotidyl transferase and fluorescent isothiocyanate-dUTP. The cells were also stained with 1 μg/ml DAPI for 30 min to detect cell nucleus by UV light microscopic observations (blue). Samples were analyzed in a drop of PBS under a fluorescence and UV light microscope. Using an excitation wavelength in the range of 450–500 nm and detection in the range of

515–565 nm (green), the number of TUNEL-positive cardiac myocytes and apoptotic bodies was determined by counting 3×10^5 cardiac myocytes. All morphmetric measurements were performed by at least three independent individuals in a blinded manner.

Statistical analysis

The data are presented as mean ± SEM from three independent experiments. The significant difference versus the controls in each experiment was assessed by using analysis of variance and Student t test with $p < 0.01$ and $p < 0.05$ were considered significant.

Results

Sustained incubation of H9c2 cells in high glucose conditions increases ROS production via NADPH oxidase activation

We examined the cellular ROS levels in cardiomyocytes that had been incubated in sustained high glucose conditions. Cells were initially maintained in media containing 1 g/L (5.5 mM) glucose as the normal glucose concentration. For high glucose incubation, cells were transferred to media containing 33 mM glucose for 36 hr. ROS levels were measured by a 2', 7'-dichlorofluorescein diacetate (DCFH-DA) assay. ROS levels were approximately 3-fold higher in cells exposed to 33 mM glucose for 36 h and 48 h than in cells exposed to 5.5 mM glucose (Fig. 1A). In addition, ~~the intensity of DCF fluorescence was significantly greater among cells exposed to 33- and 55 mM glucose than in cells exposed to 5.5 mM glucose. At 36 hours of incubation, ROS levels were approximately two- to three-fold higher in cells incubated with 33 mM glucose than in cells incubated with 5.5 mM glucose (Fig. 1B).~~ To further study the formation of reactive free radicals of superoxide ($O_2^{\bullet -}$), the main product of NADPH oxidase, The ROS levels were measured using chemical probes of dihydroethidium (DHE, Molecular Probes). The ROS levels were increased in a time-dependent manner to 36 hr (Fig. 1B). We also found that high glucose concentrations resulted in a time-dependent increase in protein expression of $p22^{phox}$, $p47^{phox}$ and $gp91^{phox}$ in the membrane fraction of H9c2 cells (Fig. 1C and 1D).

High glucose concentrations result in cell death

A MTT assay was performed to investigate whether high concentrations of glucose inhibit the growth of cardiomyocytes. We found that high glucose (33 mM) had a time-dependent cytotoxic effect on H9c2 cells (Fig. 2A). Because caspase-3 plays an important role in the development of apoptosis, we examined the expression of activated caspase-3 in H9c2 cells exposed to high glucose. We found that high glucose (33 mM) induced cleavage of caspase-3 (Fig. 2B) and the activation of caspase-3 in a time-dependent manner (0-48 h) (Fig. 2C). Therefore, high glucose treatment in the following series of experiments comprised a concentration of 33 mM for a duration of 36 h.

High glucose induces the expression of MAPK family proteins and NF-*κ*B signaling in H9c2 cells

To investigate the apoptosis-related signaling pathways that are activated in H9c2 cells treated with 33 mM glucose, we used Western blot to examine the protein expression of MAPK family proteins in cells exposed to 33 mM glucose for different time periods (0–48 h). The results of our time-course study showed that the protein levels of JNK and c-Jun were elevated after high glucose treatment (Fig. 3A). High glucose also induced the phosphorylation of p38, but had no effect on ERK phosphorylation (Fig. 3B). Studies have shown that the NF-*κ*B pathway is involved in ROS-induced apoptosis and that NF-*κ*B plays a key role in cardiac dysfunction in patients with diabetes (Mariappan et al., 2010; Min et al., 2009). We, therefore, evaluated the activation of NF-*κ*B and expression of its downstream protein cyclooxygenase-2 (COX-2) in H9c2 cells after exposure to 33 mM glucose for different time periods (0–48 h). As seen in Fig. 3C, there was an increase in IKK and I*κ*B phosphorylation coupled with elevated NF-*κ*B phosphorylation and COX-2

protein expression. To confirm that the activation of NF-κB was induced by exposure to a high concentration of glucose, H9c2 cells were transfected with construct containing the NF-κB-responsive luciferase reporter gene (NF-κB-Luc) and the luciferase activity was measured at different time periods (0-48 h). We found that the luciferase activity was 2.5-fold higher in H9c2 cells that had been exposed to 33 mM glucose than in control cells, indicating that exposure to high concentrations of glucose stimulates the activation of NF-κB (Fig. 3D).

NADPH-oxidase-mediated phosphorylation of JNK and NFκB is involved in hyperglycemia-induced apoptosis

~~To further identify the signal transduction pathways involved in hyperglycemia-induced cardiomyocyte apoptosis, H9c2 cells were incubated with 33-mM glucose in the presence of the ERK1/2 inhibitor U0126 (10 μM), the p38 MAPK-inhibitor SB203580 (10 μM), and the JNK-inhibitor SP600125 (10 μM) for 36h. The results of the immunoblot assay revealed that only the JNK-inhibitor SP600125-significantly attenuated the glucose-induced cleavage of caspase-3 (Fig. 4A). These findings suggest that JNK may be the key regulatory mediator of hyperglycemia-induced myocardial apoptosis.~~ To better understand the pathways governing hyperglycemia-induced apoptosis, we incubated cells with JNK and NF-κB siRNAs (10 nM) for 24h and then exposed cells to a 33-mM concentration of glucose in the presence or absence of NADPH oxidase inhibitors, apocynin (100μM) or DPI (20μM) for 36h. The results showed that treatment with JNK and NF-κB siRNAs as well as apocynin markedly attenuated the high glucose-induced activation of caspase-3 (Fig. 4A). In addition, apocynin, but not NF-κB siRNA, inhibited the phosphorylation of JNK and apocynin and JNK siRNA inhibited the phosphorylation

of the p65 subunit. Furthermore, the levels of phosphorylated JNK and NF-κB in cells exposed to JNK and NF-κB siRNAs were similar to those in the control group, demonstrating that those siRNAs successfully inhibited the phosphorylation of their respective proteins (Fig 4B&C). These observations suggest that high glucose-induced apoptosis of cardiomyocytes is mediated by NADPH oxidase activation, JNK signaling, and the nuclear activation of NF-κB.

High glucose-induced NF-κB activation is dependent on NADPH oxidase and JNK

NF-κB activation has been suggested to play an important role in the induction of apoptosis in a variety of cell types exposed to high concentrations of glucose (Shou et al., 2002; Wang et al., 2002). It has also been shown that NADPH oxidase-regulated NF-κB signaling is involved in hyperglycemia-induced apoptosis in human endothelial cells (Sheu et al., 2008). To clarify the role that NF-κB plays in high glucose-treated cardiomyoblasts, we incubated cells with U0126, an ERK1/2 inhibitor, SB203580, a p38 MAPK inhibitor, SP600125, a JNK inhibitor, and QNZ, an NF-κB inhibitor and then evaluated the degree of nuclear translocation of NF-κB using an immunofluorescense assay. SP600125 and QNZ (10μM), but not U0126 or SB253580, significantly blocked the high glucose-induced nuclear translocation of NF-κB (Fig. 5A). To further examine the activation of NF-κB by high glucose, we incubated cells in the presence of ERK, JNK, p38, and NF-κB siRNAs (10 nM) and then performed an electrophoretic mobility shift assay (EMSA) and a luciferase assay. EMSA revealed that the high glucose-induced increase in NF-κB binding to the DNA promoter was significantly inhibited by JNK siRNA and apocynin, but not by ERK or p38 siRNAs (Fig. 5B). The luciferase assay showed that the high glucose-induced

NF-κB-dependent luciferase reporter activity was significantly inhibited by JNK siRNA and apocynin, but not by ERK or p38 siRNAs (Fig. 5C). Taken together, these results indicate that high concentrations of glucose induce NFκB nuclear activation via a NADPH oxidase/JNK signaling pathway.

High glucose-induced neonatal cardiomyocyte apoptosis is mediated through NADPH oxidase-derived ROS-related JNK- and NF-κB signaling

We further confirmed the involvement of ROS-related signaling in high glucose-induced apoptosis of primary cardiomyocytes. Cells were initially maintained in media containing 1 g/L (5.5 mM) glucose. For high glucose incubation, cells were transferred to media containing 33 mM glucose for 36 h. Cells were then transfected with JNK and NF-κB siRNAs (10 nM) for 24h and then treated with or without NADPH oxidase inhibitors (apocynin, 100μM or DPI, 20μM) for 36h. The results showed that caspase-3 activity was significantly suppressed by apocynin and by JNK and NF-κB siRNAs (Fig. 6A). We then performed a TUNEL assay to investigate the cytotoxic effects induced by high glucose in neonatal cardiomyocytes. After incubation with 33 mM glucose for 36 h, there was a significant increase in the number of apoptotic bodies; however, treatment with apocynin and DPI as well as JNK and NF-κB siRNAs led to a significant reduction in the number of TUNNEL-positive cells (Fig. 6B). These results indicate that high glucose-induced cardiomyocyte apoptosis is mediated through ROS-related JNK/NF-κB signaling.

Discussion

Diabetes mellitus affects more than 50% of adults in the USA (Must et al., 1999). Although the mechanisms by which high glucose levels induce apoptosis in kidney cells, endothelial cells, pancreatic β-cells, and retinal cells have been investigated by many scientists, few studies have investigated the relationship between hyperglycemia and diabetic cardiomyopathy. In this study we studied the relationship between hyperglycemia-induced apoptosis and NADPH oxidase-generated ROS signaling. We found that high glucose-induced NADPH oxidase-derived superoxide production leads to cardiomyocyte apoptosis by upregulating the JNK signaling pathway and the subsequent phosphorylation of NF-*κ*B. Similar results were obtained in neonatal cardiomyocytes exposed to high glucose concentrations (Fig. 7). Our findings suggest that the inhibition of NADPH oxidase and its downstream oxidative signaling pathways might be potential therapeutic targets for the treatment of diabetic cardiomyopathy.

Excessive ROS generated by NADPH oxidase has been linked to the pathogenesis of diabetic complications (Kislinger et al., 1999; Li and Shah, 2003; Matsunaga-Irie et al., 2004; Thallas-Bonke et al., 2008). Apocynin (4-hydroxy-3-methoxyacetophenone) is a naturally occurring methoxy-substituted catechol found in the medicinal herb *Picoria kurroa*. It inhibits NADPH oxidase activity by blocking the migration of $p47^{phox}$ to the cell membrane, thereby preventing it from associating with the NADPH oxidase complex (Hart and Simons, 1992; Meyer and Schmitt, 2000). Animal studies have demonstrated that administration of apocynin protects against the development of some diseases by inhibiting the production of $O_2^{\bullet -}$ (Asaba et al., 2005; Nam et al., 2009; Shen et al., 2009). In the

present study, we found an increase in cellular generation of superoxide following high glucose treatment, and that administration of apocynin inhibited high glucose-induced apoptosis in H9c2 cells and in neonatal cardiomyocytes, indicating that NADPH oxidase-derived ROS generation is involved in the apoptosis of cells exposed to high levels of glucose. In addition, our finding that JNK and NF-*k*B were inactivated following apocynin treatment of cells exposed to high glucose indicates that NADPH oxidase-produced ROS is the preliminary step in the high glucose-induced apoptotic cascade. It has recently indicated that apocynin is not an inhibitor of NADPH oxidase, but acts as an antioxidant (Heumuller et al., 2008). Therefore, we used the gp91ds-tat as the specific inhibitor of NADPH oxidase. The similar results were observed (supplementary data) as the usage of apocynin. On this data basis, ~~Therefore,~~ our results suggest that blockade of NADPH oxidase is a valid intervention for combating established diabetic cardiomyopathy.

JNKs (c-Jun N-terminal kinases) are members of the family of mitogen-activated protein (MAP) kinases. JNKs regulate multiple activities in development and cell function by phosphorylating the activator protein-1 (AP-1) complex, including c-Jun. Activation of JNK signaling and its downstream effectors is associated with the development of cell death (Davis, 2000; Weston and Davis, 2002); however, the mechanism is controversial and might be stimulus-specific, or tissue-specific, or both (Liu and Lin, 2005). For example, JNK activation mediates caspase-3- or p53-dependent apoptosis of leukemic cells exposed to NH_2Cl, a neutrophil-derived oxidant (Ogino et al., 2009), and cantharidin, an antitumor drug (Huh et al., 2004). The activation of JNK signaling in response to hyperglycemia-induced oxidative stress has been implicated in the development of pancreatic beta-cell dysfunction (Kaneto et al., 2007; Kaneto et al., 2005).

In human endothelial cells (HUVEC), high glucose-induced cell apoptosis has been shown to be mediated through NF-κB and the activation of JNK signaling (Ho et al., 2006; Ho et al., 2000). In contrast to HUVECs, however, cardiomyocytes possess L-type calcium channels, which regulate cell contraction, hypertrophy, and contractile dysfunction in response to hyperglycemic conditions. The mechanism through which JNK is involved in high glucose-induced apoptosis of cardiac cells needs to be elucidated. In this study, we found that high glucose led to an increase in NADPH oxidase-generated ROS, which in turn activated JNK, but not ERK or p38, resulting in an increase in the nuclear activation of NF-κB and the subsequent apoptosis of cardiomyocytes. Although the high glucose-induced protein expression of p38 was attenuated by apocynin, indicating that p38 is also downstream of NADPH oxidase-derived ROS, the failure of SB253580, a p38 inhibitor, to inhibit NF-κB nuclear translocation and apoptosis following high glucose treatment in cells suggests that high glucose-induced expression of p38 is not associated with JNK/NF-κB-mediated apoptosis. In addition, treatment with JNK and NF-κB siRNAs effectively abolished NF-κB nuclear activation and high glucose-induced apoptosis. These results, therefore, suggest that activation of JNK, but not ERK 1/2 or p38, due to NADPH oxidase-derived ROS stimulation leads to NFκB-mediated cell apoptosis. Therefore, JNK activation, which occurs downstream of high glucose-induced ROS, might also be a potential target for the treatment of diabetic cardiomyopathy.

In addition to controlling cellular apoptosis, JNK also plays important roles in other pathways involved in the pathogenesis of diabetes. For example, studies have established that activation of JNK-1, but not JNK-2, can lead to insulin resistance by increasing IRS-1 phosphorylation at serine 307, thereby impairing insulin signaling in obese patients with type II diabetes mellitus (Hirosumi et al., 2002; Hotamisligil,

2005; Weston and Davis, 2007; Yang and Trevillyan, 2008). In addition, deletion of the JNK-1 gene protects against insulin resistance by decreasing IRS-1 serine phosphorylation in JNK-1 knockout mice (Hirosumi et al., 2002). Therefore, JNK inhibitors might not only inhibit high-glucose induced cardiac cell apoptosis but also improve insulin sensitivity in target organs, making them potential agents for the treatment of a variety of diabetic complications.

In our series of experiments we have shown the efficacy of therapies that inhibit NADPH oxidase-dependent ROS production and its downstream signaling events, such as JNK activation that occur in response to hyperglycemic conditions. On the basis of these findings, the action of apocynin is predicted not to affect the high concentration of blood glucose *in vivo*; however, the important downstream pathways implicated in the pathogenesis of diabetic cardiomyopathy, that is, ROS production, JNK phosphorylation, NF-*k*B nuclear activation, and apoptotic body accumulation, were normalized by apocynin treatment of cells under hyperglycemic conditions in our *in vitro* study. JNK, therefore, may be a potential target for the treatment of diabetic cardiomyopathy.

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Appendix Supplementary data

Supplementary data associated with this study can be found at the end of the figures.

Competing interests

The authors declare that they have no competing interests.

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Figure Legends

Figure 1. HG-induced NADPH oxidase-related oxidative stress in H9c2 cardiomyoblasts. (A) ROS production in H9c2 cells exposed to NG and HG for 24–48 h. (B) H9c2 cells treated with ~~various concentrations of glucose (5.5-55 mM) for 36 h.~~ high concentration of glucose for different time periods as indicated. Intracellular $O_2^{\bullet -}$ generation was detected by the procedures described in the Materials and Methods section. Data are presented as mean ± SEM from three independent experiments. **P*<0.05, ***P*<0.01 vs NG group. (C) The expression levels of $p22^{phox}$ and $p47^{phox}$ protein in H9c2 cells exposed to HG for 0–48 h were analyzed by Western blot. Equal loading was assessed with an anti-α-tubulin antibody. This result is representative of at least three independent experiments. (D) The protein levels of $gp91^{phox}$ in the membrane fractions of H9c2 cells exposed to high glucose for 0-48 h were analyzed by Western blot. Equal loading was assessed with an anti-foltilin antibody.

Figure 2. Inhitition of cell growth in H9c2 cardiomyoblasts exposed to HG. (A) Cells were cultured in HG medium for the hours indicated. Cell viability was determined by a MTT assay. The results are expressed as mean ± SEM from 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs NG. (B) H9c2 cells were exposed to various concentrations of glucose (5.5-33mM) for 36 h, or (C) exposed to HG for 0-48 h. Caspase-3 activity was determined by Western blot. Equal loading was assessed with an anti-α-tubulin antibody. The fold number represents the quantitative results compared to those of NG.

Figure 3. Effects of HG on the activation of MAPK family proteins and the NF-*κ*B signaling pathway in H9c2 cells. H9c2 cells were treated with HG for various time periods (0-48 h), and then were harvested and lysed. Total protein of cell extracts was separated by 12% SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies against (A) p-JNK, JNK, p-c-Jun, c-Jun. (B) p-ERK, ERK, p-p38, p-38. (C) p-IKK, IKK, p-I*κ*Bα, I*κ*Bα, p- NF*κ*B, NF*κ*B, and COX-2. Equal loading was assessed with an anti-α-tubulin antibody. The fold number represents the quantitative results compared to those of NG. (D) H9c2 cells were treated with HG for various time periods (0-48 h), and the NF-*κ*B luciferase reporter assay was performed as described in the Materials and Methods section. The results are expressed as mean ± SEM from 3 independent experiments. *p<0.05 vs control (0 h).

Figure 4. The involvement of caspase-3 activation induced by JNK/ NF-*κ*B signaling in HG-exposed H9c2 cells. ~~(A) H9c2 cells were treated with HG in the absence or presence of U0126 (ERK inhibitor, 10μM), SP600125 (JNK inhibitor,~~

~~10μM) or SB203580 (p38 inhibitor, 10μM) for 36 h. Active caspase-3 levels were determined by an immunoblotting assay.~~ (A) After H9c2 cells had been transfected with small interfering RNAs (siRNA) of JNK-1, NF-*κ*B (10 nM) for 24 h, followed by treatment with HG for 36 h with or without apocynin (100 μM) or diphenylene iodonium (DPI, 20μM), the levels of active caspase-3 were analyzed by Western blot. (B) and (C) After the same treatment procedures as (A), the levels of proteins indicated were analyzed by Western blot. The scramble represents the non-specific siRNA. Data are presented as mean ± SEM from three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs control. # $p < 0.05$ vs HG alone. Equal loading was assessed with an anti-α-tubulin antibody. ~~The fold number represents the quantitative results compared to those of NG.~~

Figure 5. HG-induced NF-*k*B nuclear activation is mediated by JNK, but not ERK or p38. (A) Cells were incubated with U0126, SP600125, SB203580, or the NF-kB inhibitor, QNZ (10 μM) and cultured with HG for 36 h. Cells were then fixed, and the immunofluorescence staining with antibody against p65 was performed and visualized under a fluorescence microscope coupled with an image analysis system. After H9c2 cells had been transfected with 10μM siRNAs of ERK, p38, JNK and NF-*κ*B, they were treated with HG for 36 h with or without apocynin (100 μM). Additionally, after H9c2 cells had been transfected with siRNAs of ERK, p38, JNK and NF-*κ*B (10μM) for 24 h, followed by treatment with high glucose for 36 h with or without apocynin (0.1 mM), (B) the electrophoretic mobility shift assay, and (C) the NF-*k*B luciferase reporter assay were performed as described in the Materials and Methods section. The scramble represents the non-specific siRNA. The results are expressed as mean ± SEM from 3 independent experiments. **$p<0.01$ vs NG, ##

$p<0.01$ vs HG.

Figure 6. HG-induced apoptosis is mediated via JNK/NF-κB pathways in neonatal cardiomyocytes. Neonatal cardiomyocytes were prepared as described in the Materials and Methods, and maintained in HG for 36 h following transfection of JNK and NF-κB si-RNAs (10μM) with or without apocynin (0.1 mM) or diphenylene iodonium (DPI, 20μM). (A) Caspase-3 protein level was determined by Western blot. The fold number represents the quantitative results compared to those of NG. (B) HG-induced cell death was evaluated by DAPI staining and TUNEL assay. Blue spots represent cell nuclei and green spots represent apoptotic bodies. The scramble represents the non-specific siRNA. Data are presented as mean ± SEM from three independent experiments. * $p < 0.05$ vs control. # $p < 0.05$ vs HG alone.

Fig 7. Proposed mechanism for diabetes-induced cardiac apoptosis. Hyperglycemia enhances NADPH oxidase-derived superoxide generation, which promotes JNK phosphorylation to further activate NFkB nuclear activation, and consequent activation of cardiac cell apoptosis. Inhibition of $O_2^{\bullet -}$ formation either by inhibition of apocynin, which prohibits the translocation of p47 phox to associate with cell membrane, or by inhibition of NAC, blocks hyperglycemia-augmented ROS-stimulated downstream signalings. Overall, we propose that NADPH oxidase-derived ROS-induced apoptosis is mediated via the JNK-dependent activation of NF-κB in cardiomyocytes exposed to high glucose.