

Hyperglycemia-Induced Apoptosis in Mouse Myocardium Mitochondrial Cytochrome *c*-Mediated Caspase-3 Activation Pathway

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Diabetic cardiomyopathy is related directly to hyperglycemia. Cell death such as apoptosis plays a critical role in cardiac pathogenesis. Whether hyperglycemia induces myocardial apoptosis, leading to diabetic cardiomyopathy, remains unclear. We tested the hypothesis that apoptotic cell death occurs in the diabetic myocardium through mitochondrial cytochrome *c*-mediated caspase-3 activation pathway. Diabetic mice produced by streptozotocin and H9c2 cardiac myoblast cells exposed to high levels of glucose were used. In the hearts of diabetic mice, apoptotic cell death occurred as detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. Correspondingly, caspase-3 activation as determined by enzymatic assay and mitochondrial cytochrome *c* release detected by Western blotting analysis were observed. Supplementation of insulin inhibited diabetes-induced myocardial apoptosis as well as suppressed hyperglycemia. To explore whether apoptosis in diabetic hearts is related directly to hyperglycemia, we exposed cardiac myoblast H9c2 cells to high levels of glucose (22 and 33 mmol/l) in cultures. Apoptotic cell death was detected by TUNEL assay and DAPI nuclear staining. Caspase-3 activation with a concomitant mitochondrial cytochrome *c* release was also observed. Apoptosis or activation of caspase-3 was not observed in the cultures exposed to the same concentrations of mannitol. Inhibition of caspase-3 with a specific inhibitor, Ac-DEVD-cmk, suppressed apoptosis induced by high levels of glucose. In addition, reactive oxygen species (ROS) generation was detected in the cells exposed to high levels of glucose. These results suggest that hyperglycemia directly induces apoptotic cell death in the myocardium *in vivo*. Hyperglycemia-induced myocardial apoptosis is mediated, at least in part, by activation of the cytochrome *c*-activated caspase-3 pathway, which may be triggered by ROS

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Diabetes causes various cardiovascular complications, which have become the major cause of morbidity and mortality in the diabetic population (1). Moreover, mortality from cardiac diseases is approximately two- to fourfold higher in patients with diabetes than in those who have the same magnitude of vascular diseases without diabetes, and diabetic cardiomyopathy can occur without any vascular pathogenesis (1–3). Several studies have shown that hyperglycemia as an independent risk factor directly causes cardiac damage, leading to diabetic cardiomyopathy (1–4). However, mechanisms for the pathogenesis remain unclear (1–5). Diabetic hearts, including streptozotocin (STZ)-induced diabetic animal models, display a reduction in cardiac mass over time, myocardial hypertrophy, and interstitial and perivascular fibrosis at late phase (5,6). These late-phase changes are believed to result from early responses of myocardium to suddenly increased glucose levels (1,6–8). Early responses of myocardial cells to hyperglycemia include metabolic abnormalities, subcellular defects, abnormal expression of genes (7–9), and, consequently, cardiac cell death (8,10,11).

Cell death, as a comprehensive consequence of myocardial abnormalities, is an important cause of various cardiomyopathies (10–13). In particular, cell death can cause a loss of contractile tissue, compensatory hypertrophy of myocardial cells, and reparative fibrosis (10). Diabetes-induced cell death has been observed in multiple organs *in vivo* (14–16) and in endothelial cells *in vitro* (17–19). Recent studies showed that the incidence of apoptosis increases in the heart of patients with diabetes (20) and STZ-induced diabetic animals (21,22). However, whether the increased myocardial apoptotic cell death is related directly to hyperglycemia is unclear.

Mitochondria play an important role in apoptosis under a variety of proapoptotic conditions, such as oxidative stress (23). Mitochondrial cytochrome *c* release is a key event in the activation of caspase-3, a downstream pivotal step to initiate apoptosis (24). A pharmaceutical inhibitor of caspase-3, Ac-DEVD-cmk, which specifically inhibits caspase-3 activity, has been used in a variety of experimental approaches to inhibit apoptosis (25). This inhibitor thus provides a valuable tool to dissect the caspase-3-dependent apoptotic pathway.

Among apoptotic stimuli, reactive oxygen species (ROS) have been shown to cause mitochondrial cytochrome *c*

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DAB, 3,3'-diaminobenzidine; DCFDA, carboxy-dichlorodihydrofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; FBS, fetal bovine serum; HRP, horseradish peroxidase; MTT, microculture tetrazolium; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; STZ, streptozotocin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

release and activation of caspase-3 (11,16,18,26–28). A correlation between ROS generation and the pathogenesis of various diabetic complications has been observed (29). Therefore, it is possible that ROS accumulation occurs in diabetic myocardium, in which apoptosis may take place and lead to cardiomyopathy. In the present study, we determined 1) apoptotic cell death in the myocardium of diabetic mice and in the cultured cardiac myoblast cells exposed to high levels of glucose, 2) cytochrome *c*-mediated caspase-3 activation and its essentiality in myoblast cell apoptosis induced by high levels of glucose, and 3) the involvement of ROS in the apoptotic pathway.

RESEARCH DESIGN AND METHODS

Diabetic mouse model. FVB mice, originally obtained from Harlan Bioproducts for Science (Indianapolis, IN), were housed in the University of Louisville Research Resources Center at 22°C with a 12-h light/dark cycle. They had free access to rodent diet and tap water. All animal procedures were approved by the Institutional Animal Care and Use Committee, which is certified by the American Association of Accreditation of Laboratory Animal Care. Eight-week-old male mice were given a single intraperitoneal dose of STZ (150 mg/kg body wt; Sigma, St. Louis, MO) dissolved in sodium citrate buffer (pH 4.5). Whole-blood glucose obtained from mouse tail vein was monitored using SureStep complete blood glucose monitor (LifeScan, Milpitas, CA). Mice with whole-blood glucose >12 mmol/l were considered diabetic, and mice with whole-blood glucose <8 mmol/l were used as STZ control. Mice that served as vehicle controls were given the same volume of sodium citrate. At day 3 after STZ treatment, some of the diabetic mice received an injection of insulin (2 units/kg sodium insulin; Sigma) twice daily at 12-h intervals.

Cell cultures and treatments. H9c2 cells (ATCC CLR-1446; Rockville, MD) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) from Atlanta Biologicals (Norcross, GA). When cell populations reached 40–50% confluence, the cultures were exposed to D-glucose (Sigma) in a final concentration of 22 and 33 mmol/l in cultures for in vitro treatment with high levels of glucose according to previous publications (17–19) and exposed to 5.5 mmol/l D-glucose as control. To exclude a hyperosmolar effect, we added identical concentrations of mannitol (5.5, 22, and 33 mmol/l; Sigma) in control cultures. After exposure for varying time periods, the monolayer cultures were washed with PBS and the cells were removed by trypsinization. In some experiments, the cells were grown in chamber slides, which were used for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, DAPI staining, and ROS detection.

Analysis of cell viability. Cell viability was determined by a short-term microculture tetrazolium (MTT) assay. In a 96-well microplate, 2.5×10^4 cells/well were incubated in 100 μ l of culture media and exposed to different concentrations of glucose for varying time periods. The media were removed again and replaced with 90 μ l of DMEM (containing no phenol red or FBS) and 10 μ l of MTT solution (2 mg/ml phosphate buffer) for 4 h. After the MTT-containing DMEM was removed, the remaining formazan blue crystals were dissolved in 75 μ l of 0.04 N HCl/isopropyl alcohol solution. Absorbance at 540 nm was measured using a microplate reader (model FL 311; Bio-Tek Instruments, Winooski, VT).

TUNEL assay. Heart tissues from mice were fixed in 10% formalin, embedded in paraffin, and sectioned at 5 μ m. The cells cultured on chamber slides were fixed in 1% paraformaldehyde. The slides were processed for a TUNEL assay. An ApopTag in situ detection kit from Intergen (Purchase, NY) was used according to the manufacturer's instructions. Briefly, the slides were treated with H₂O₂ and incubated with the reaction mixture containing TdT and digoxigenin-conjugated dUTP for 1 h at 37°C. Labeled DNA was visualized with peroxidase-conjugated anti-digoxigenin antibody using 3,3'-diaminobenzidine (DAB) as the chromogen. Mouse testicular tissue (14) was used as positive control. For negative control, TdT was omitted from the reaction mixture.

Caspase-3 immunohistochemical staining. Briefly, after deparaffinization and rehydration, slides of heart tissues were treated with 3% H₂O₂ in PBS for 10 min and with 5% normal sera in PBS for 20 min. Sections were incubated overnight at 4°C with polyclonal rabbit anti-active caspase-3 (PharMingen, San Diego, CA) at 1:500 dilution, then incubated for 20 min in biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA), followed by incubation with horseradish peroxidase (HRP)-streptavidin for 20 min. The antibody binding sites were visualized through reaction with DAB-H₂O₂ solution. Finally, sections were counterstained with 0.5% methyl green.

Caspase-3 enzymatic assay. Fresh heart tissues were homogenized with Telfon homogenizer in an extract buffer, which contained 25 mmol/l HEPES buffer (pH 7.4), 5 mmol/l EDTA, 2 mmol/l dithiothreitol (DTT), 0.1% CHAPS, 1.0 mmol/l phenylmethylsulfonyl fluoride (PMSF), and 10 μ g/ml aprotinin. Cell pellets were resuspended in the same extract buffer and homogenized. The homogenate was centrifuged at 20,000g for 30 min. The supernatant was diluted with an assay buffer (50 mmol/l HEPES, 10 mmol/l DTT, 1 mmol/l EDTA, 100 mmol/l NaCl, 0.1% CHAPS, and 10% glycerol [pH 7.4]) and incubated at 37°C with 200 μ mol/l caspase-3 substrate I (N-Acetyl-Asp-Glu-Val-Asp-pNA [Ac-DEVD-pNA]; CalBiochem, La Jolla, CA). Cleavage of the substrate was monitored at 405 nm using a microplate reader and recorded in 10-min intervals for 2 h. The specific activity is expressed in picomoles per minute per mg of protein.

Detection of mitochondrial cytochrome *c* release. Subcellular fractionation and Western blotting analysis were used to detect cytochrome *c* content in cytosol and mitochondria. The increase in the cytosol with a concomitant decrease in mitochondria is indicative of cytochrome *c* release from mitochondria. Briefly, a whole mouse heart was excised and washed in cold PBS and then homogenized in cold lysis buffer supplemented with 250 mmol/l sucrose and incubated on ice for 30 min. The buffer contains 20 mmol/l HEPES (pH 7.5); 150 mmol/l NaCl; 1% NP-40; 0.1% SDS; 1 mmol/l EDTA; 1 mmol/l DTT; 2 μ g each of the protease inhibitors aprotinin, leupeptin, and pepstatin A; and 0.5 μ mol/l PMSF. Cultured cells were collected by trypsin-EDTA (0.5%), followed by two washes with cold PBS and lysed in the same lysis buffer for 30 min. The homogenate was centrifuged twice at 750g at 4°C for 10 min. Aliquots of the supernatant from the second 750g were kept for assay of total protein, and the remaining was removed to fresh tubes and centrifuged at 10,000g at 4°C for 20 min. The supernatant of the 10,000g spin was removed to clean tubes and centrifuged at 100,000g for 1 h at 4°C (the supernatant of this spin is the cytosolic light membrane fraction). The 10,000g mitochondrial pellet was resuspended and lysed in lysis buffer containing 1% vol/vol Triton X-100 (this is the heavy membrane fraction). To probe possible cross-contamination, we measured a cytosolic marker enzyme, lactate dehydrogenase, and a mitochondrial marker, citrate synthase, in both fractions. Cross-contamination between the fractions was not detectable. The protein concentration was assayed with an aliquot of each, and the remaining was boiled in 2 \times SDS sample buffer. The protein samples from each fraction were separated via SDS/PAGE and subsequently transferred to nitrocellulose filters for Western blotting. Filters were probed using purified mouse anti-cytochrome *c* monoclonal antibodies (PharMingen) and subsequently exposed to secondary HRP-conjugated IgG. Antigen-antibody complexes were then visualized by enhanced chemiluminescence.

Assay for ROS accumulation in H9c2 cells. Detection of ROS accumulation in cultured cardiac myoblast H9c2 cells was done using a carboxy-dichlorodihydrofluorescein diacetate (carboxy-H₂-DCFDA; Molecular Probes, Eugene, OR), as described previously (27). Cells cultured on glass chamber slides were incubated for 1 h at 37°C with 5 μ mol/l carboxy-H₂-DCFDA dissolved in the culture media. The chamber slides were then fixed with fixative containing 2% glutaraldehyde and 2% formaldehyde dissolved in PBS and analyzed under a confocal laser microscope using a FITC barrier filter.

Statistical analysis. Data were collected from repeated experiments and are presented as mean \pm SD. One-way ANOVA and Student's *t* test were used for statistical analysis. Differences were considered to be significant at *P* < 0.05.

RESULTS

Diabetes-induced myocardial cell death in the heart.

The incidence of STZ-induced diabetes in the FVB mice by STZ was ~60% according to the criteria of blood glucose: >12 mmol/l were considered diabetic and <8 mmol/l were nondiabetic and used as STZ control. Compared with vehicle control, the STZ control group did not show reduced body weight gain, but the diabetic group showed a significant decrease in body weight gain, which was prevented by supplementation of insulin that also significantly reduced glucose levels in the diabetic mice (Table 1).

Hematoxylin and eosin (H & E) staining of the heart tissues showed that compared with vehicle control (Fig. 1A), diabetic hearts displayed structural abnormalities (Fig. 1B), but the hearts from the STZ control group did not show the same alterations (Fig. 1C). More important, structural abnormalities in the heart of diabetic mice were

TABLE 1
Glucose levels and induction of diabetes by STZ in FVB mice

Basal whole-blood glucose (mmol/dl)		Whole-blood glucose in fasting state (mmol/dl)				Body-weight gain (g)			
Fasting	Nonfasting	N	STZ	D	D+I	N	STZ	D	D+I
4.3 ± 0.2	5.4 ± 0.2	4.6 ± 0.3	4.7 ± 0.5	14.9 ± 0.8*	8.5 ± 0.1†	1.7 ± 0.3	1.2 ± 1.2	-2.4 ± 0.8*	1.7 ± 0.4†

Data are means ± SE. * $P < 0.05$ vs. N; † $P < 0.05$ vs. D.

completely prevented by insulin supplementation (Fig. 1D).

In the vehicle control mouse hearts, TUNEL-positive cells were seldom identified (Fig. 1E), but in diabetic mouse hearts, numerical TUNEL-positive cells were observed at day 3, and the number continued to increase and

reached a peak value at day 7 and maintained at the high value at day 14 after STZ treatment (Figs. 1F and 2). This is consistent with the result obtained from other animal studies (21,22). The same TUNEL analysis was applied to the heart of STZ controls at day 14 after STZ treatment and of diabetic mice supplemented with insulin for 12 days. In

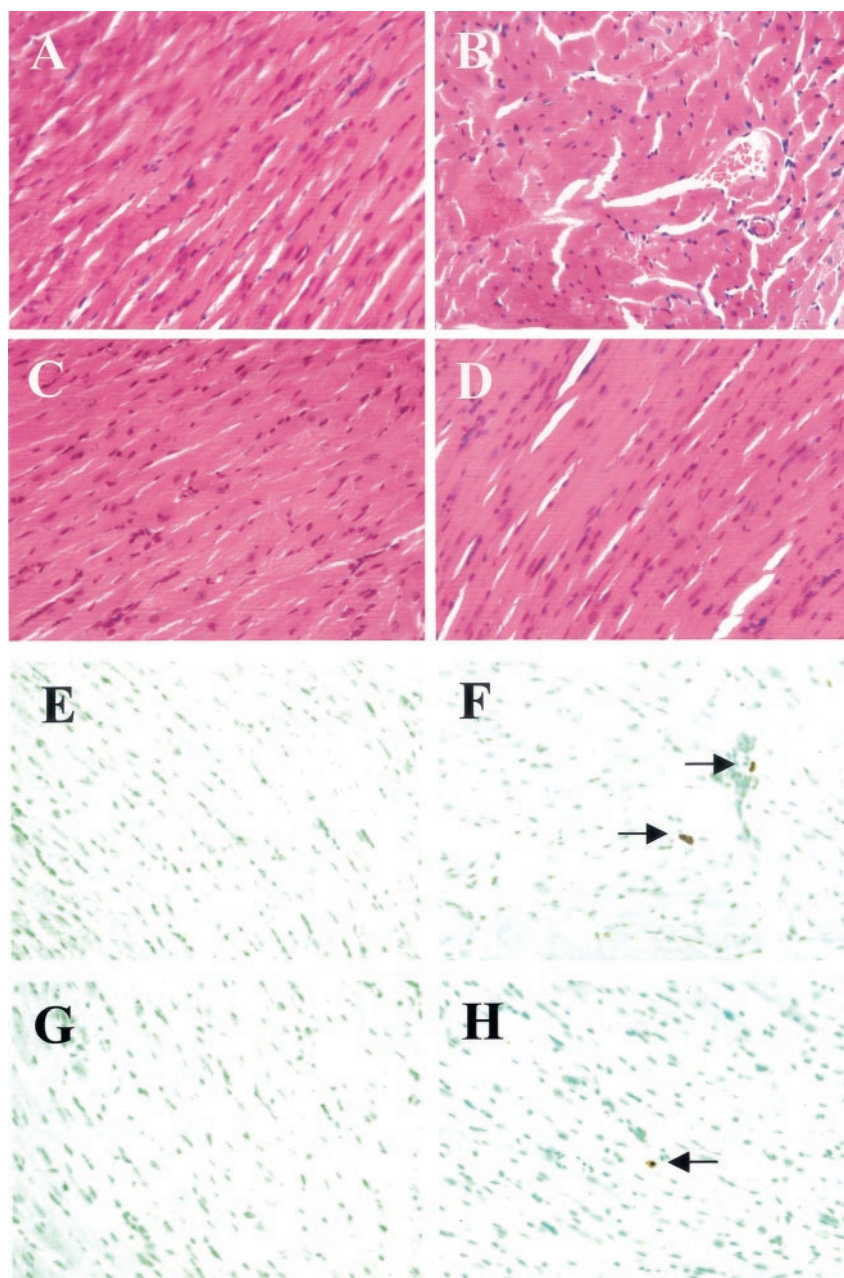


FIG. 1. Gross morphology and TUNEL detection of apoptotic cells in the heart of diabetic mice. Heart tissues from normal (saline; A and E), STZ-induced diabetes (B and F), STZ control (C and G), and insulin-supplemented diabetic (D and H) mice were sectioned at 5 μ m. These slides were processed for H & E staining (A-D) and TUNEL assay to detect apoptotic cells (E-H).

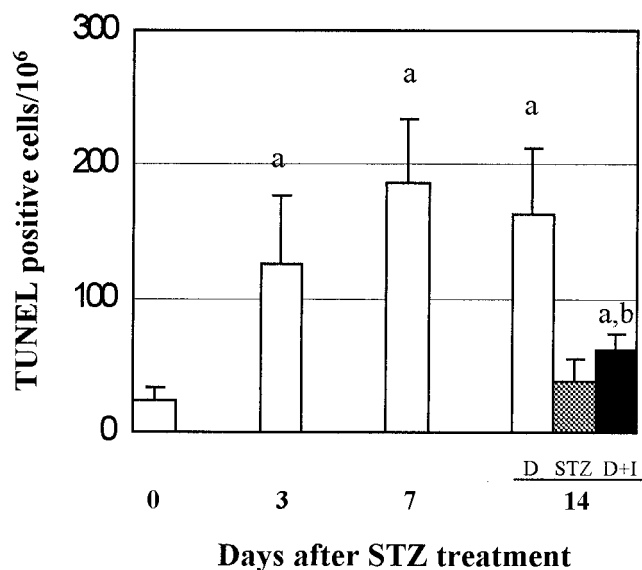


FIG. 2. Quantitative analysis of apoptotic cell death in the heart of diabetic mice. TUNEL-positive cells were quantified in the diabetic (D) mice at indicated times. TUNEL-positive cells were also quantified in the STZ control (STZ) and insulin-supplemented diabetes (D + I) groups at day 14 after STZ treatment. a, $P < 0.05$ vs. control (day 0); b, $P < 0.05$ vs. D.

the heart of STZ control mice, TUNEL-positive cells were seldom detectable (Figs. 1G and 2). Diabetic mice treated with insulin showed a significant reduction in the number of TUNEL-positive cells as compared with diabetic mice without insulin supplementation (Figs. 1H and 2).

Immunohistochemical staining for active caspase-3, another important indication of apoptotic cell death, showed an increase in the immunoreactivity with the antibody against active caspase-3 in the heart of diabetic mice as compared with vehicle controls and STZ controls (Fig. 3A). The increased caspase-3 immunoreactivity was accompanied by an increased caspase-3 activity (Fig. 3B). No caspase-3 activation was observed in the STZ control group (Fig. 3A and B). All of these results suggest that apoptosis via caspase-3 activation occurred in the myocardium of early-stage diabetic mice.

Although there are a few upstream pathways that lead to the activation of caspase-3, mitochondrial cytochrome *c* release is a critical one. Therefore, to determine whether caspase-3 activation is mediated by mitochondrial cytochrome *c* release, we used a Western blotting method to detect the cytochrome *c* translocation from mitochondria to cytosol. Results showed that increased cytosolic concentrations of cytochrome *c* were accompanied by decreased mitochondrial concentrations (Fig. 3C and D), suggesting that diabetes-induced apoptosis in the heart is likely mediated, at least in part, by the cytochrome *c*-mediated caspase-3 activation pathway.

Apoptosis in cardiac myoblast H9c2 cells induced by high levels of glucose in vitro. To explore whether the induction of apoptotic cell death in the myocardium of diabetic mice is related directly to hyperglycemia, we used cardiac myoblast H9c2 cells to determine the effect of high levels of glucose on apoptotic cell death in cultured cells. First, cell viability was determined by MTT assay. Cells were treated with glucose at a final concentration of 22, 33, and 60 mmol/l in cultures for varying time periods. Cells

treated with 5.5 mmol/l glucose were used as control. There was no significant decrease in cell viability in the cultures exposed to 33 mmol/l until 96 h postexposure (data not shown), but a significant decrease in the cultures exposed to 60 mmol/l for 72 h was observed. There was no effect in the cultures exposed to 22 mmol/l glucose for 72 h. Therefore, in the following experiments, 33 mmol/l glucose was used because it may mimic the glucose levels of the mouse models without insulin treatment in the present study and also was commonly used in previous studies (17–19). In addition, a dose of 22 mmol/l glucose was used because it may mimic the whole-blood glucose levels in patients with medication of blood glucose control and the status of the mouse diabetic model with insulin supplementation in the present study.

As compared with low levels of glucose (Fig. 4A), high levels of glucose induced a significant increase in the number of TUNEL-positive cells (Figs. 4A and 5A). An increase in the number of condensed and fragmented nuclei in the cells exposed to high levels of glucose was also observed by DAPI fluorescence staining (Fig. 4B). Quantitative analysis for the TUNEL assay (Fig. 5A) shows a time-dependent manner, i.e., an increase at 48 h and a maximum level at 72 h after exposure to 33 mmol/l glucose and at 96 h after exposure to 22 mmol/l glucose. However, cells exposed to high levels of mannitol (22 mmol/l; Fig. 5B) did not show increased numbers of apoptotic cells. The same result was obtained in the cultures exposed to 33 mmol/l mannitol for 96 h (data not shown).

Inconsistent with the in vivo data, high levels of glucose also caused caspase-3 activation (Fig. 6A) but not high levels of mannitol (Fig. 6B). This activation was also associated with mitochondrial cytochrome *c* release (Fig. 7). To determine whether the activation of caspase-3 is responsible for apoptotic cell death, we used a caspase-3-specific inhibitor, Ac-DEVD-cmk (CalbioChem), in the presence of high levels of glucose. The addition of Ac-DEVD-cmk significantly inhibited the activity of caspase-3 induced by high levels of glucose (Fig. 8A). The number of apoptotic cells detected by the TUNEL assay was significantly reduced in the cultures exposed to high levels of glucose in the presence of caspase-3 inhibitor (Fig. 8B).

ROS formation induced by high levels of glucose in cardiac H9c2 myocytes. Cells were cultured for 24 h and treated with 5.5 and 33 mmol/l glucose for the measurement of ROS production. ROS formation was detected by laser confocal microscopy detecting the fluorescence formed by the reaction of ROS with carboxy-H₂-DCFDA dissolved in the culture media. In control cultures treated with 5.5 mmol/l glucose, no significant fluorescence was detected (Fig. 9); however, ROS concentrations were significantly increased in the cells exposed to 33 mmol/l glucose. Cell density in each culture chamber was checked through phase-contrast microscopy. Cross-comparison verified equal cell density in all of the cultures used for ROS detection.

DISCUSSION

The results obtained from this study demonstrate that apoptosis occurs in diabetic myocardium and provide evidence that high levels of glucose directly cause apoptosis. Importantly, this study has identified that mitochon-

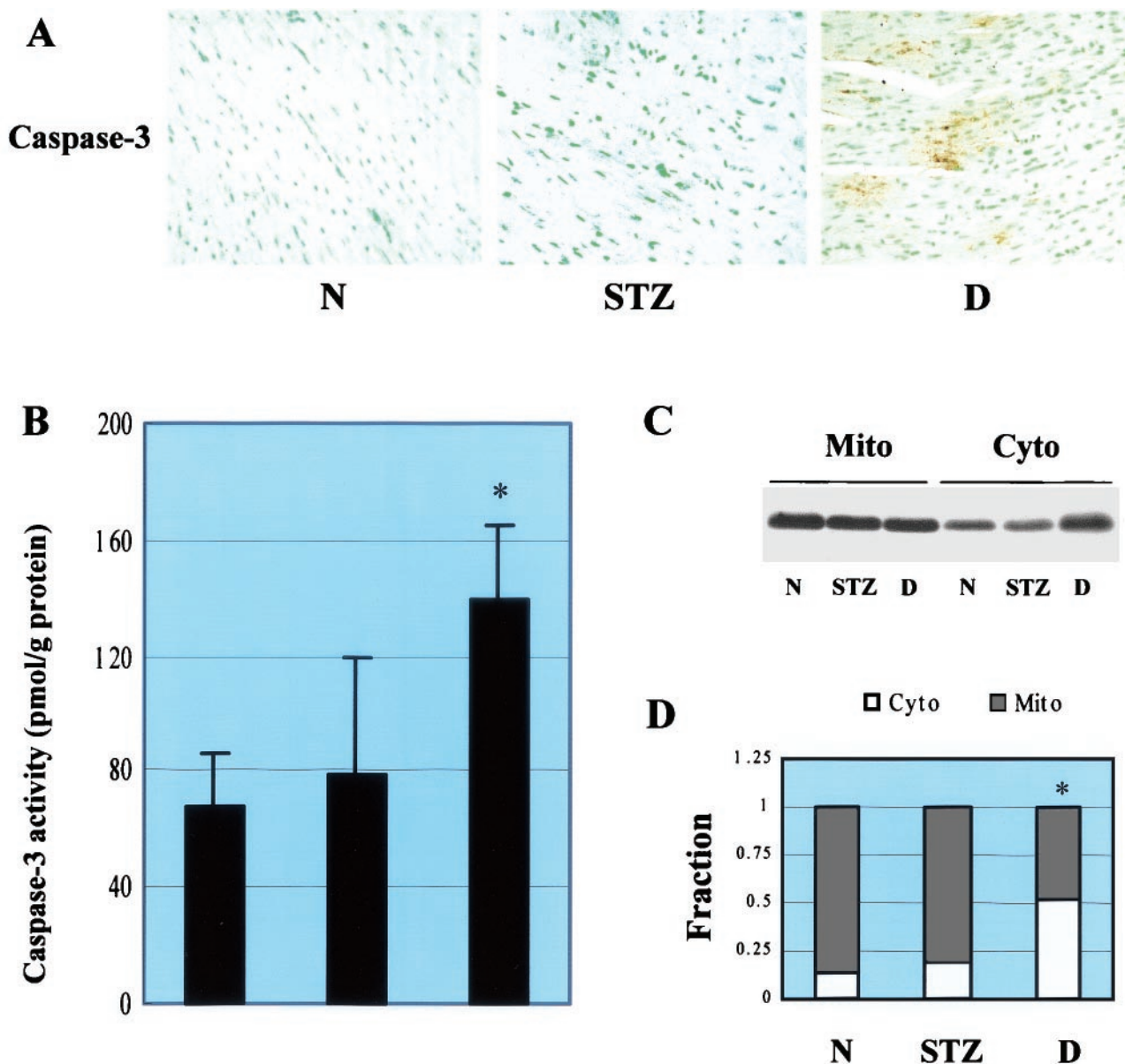


FIG. 3. Detection of caspase-3 and mitochondrial cytochrome *c* release. Activation of caspase-3 was measured by immunohistochemical staining methods (**A**) and enzymatic assay for its activity (**B**), and mitochondrial cytochrome *c* release was measured by Western blotting (**C** and **D**) from the hearts of normal (N), STZ control (STZ), and diabetic (D) mice as described in RESEARCH DESIGN AND METHODS. **D**: The quantitative analysis of cytochrome *c* release from mitochondria into cytosol. * $P < 0.05$ versus control (N).

drial cytochrome *c* release and caspase-3 activation are associated with hyperglycemia-induced myocardial apoptosis. The inhibition of apoptosis induced by high levels of glucose by Ac-DEVD-cmk indicates that the caspase-3 activation pathway is causally involved in hyperglycemia-induced myocardial apoptosis. The correlation between ROS production and mitochondrial cytochrome *c* release-mediated caspase-3 activation suggests that ROS derived from high levels of glucose may trigger the apoptotic process.

Diabetic cardiomyopathy is characterized by a reduction in cardiac mass over time, myocardial hypertrophy, and interstitial and perivascular fibrosis at late phase (1–5). Because myocytes rarely proliferate in adult cardiac muscles, the loss of cardiac muscle cells would eventually lead to compromised cardiac function. That myocyte death causes the decrease in myocardium performance

and ventricular dilation has been demonstrated (20,21). In the present study, we found that partial inhibition of increased levels of glucose by supplementation of insulin almost completely prevented myocardial morphological abnormalities and partially inhibited myocardial cell death (Fig. 1). This indicates that severe hyperglycemia that needs to reach a threshold may be necessary for the development of early morphological abnormalities, whereas apoptotic cell death may be a dose-dependent response to hyperglycemia. In addition, in the clinical practice in most patients under the control of blood glucose reduction, heart failure still occurs, suggesting that cell death may be the major cause for these effects in diabetic patients. Indeed, cell death, as a comprehensive consequence of abnormal cellular metabolism and gene expression at early stage in response to hyperglycemia, has been considered to be the important cause of cardiomyopathy

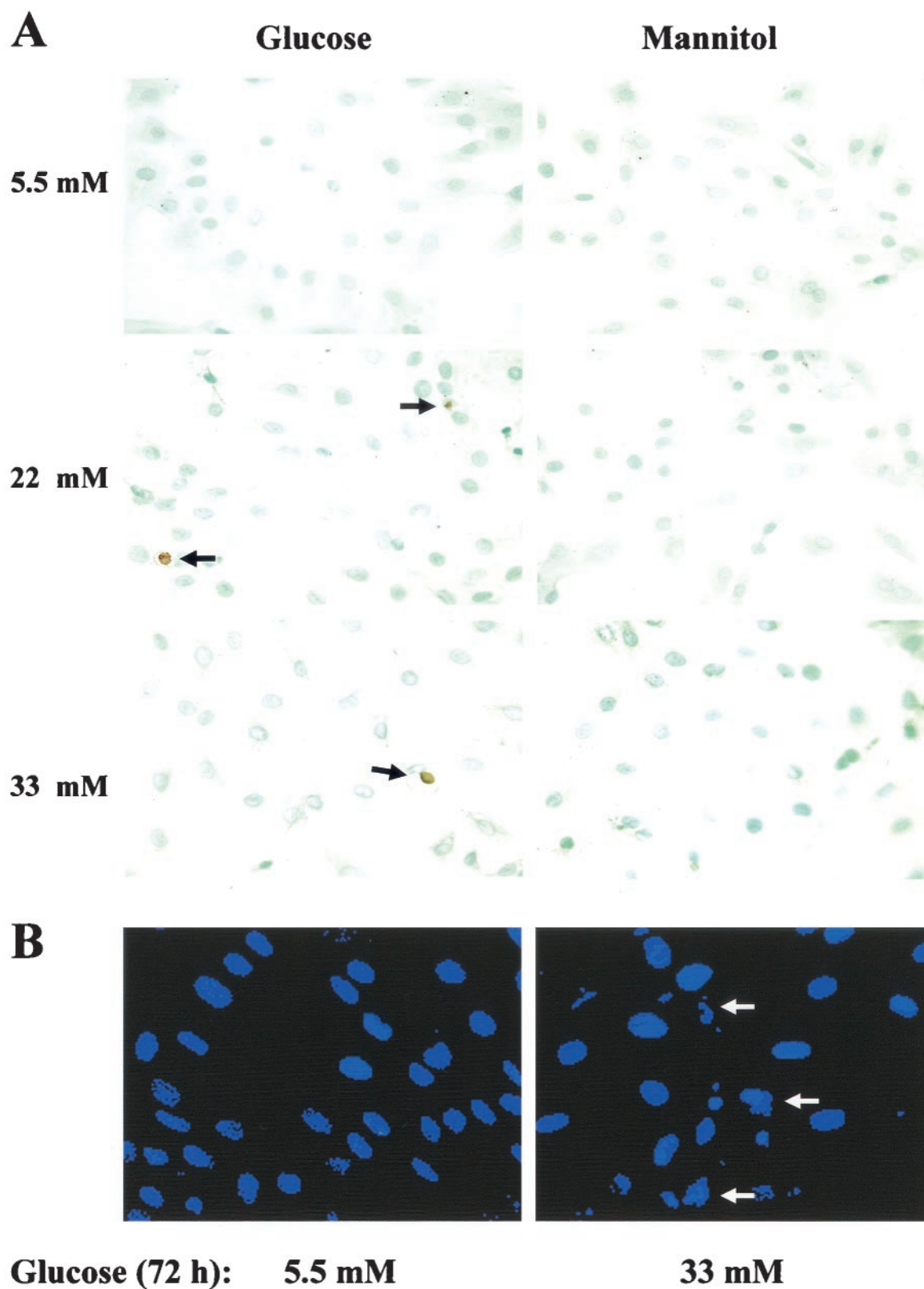


FIG. 4. Detection of apoptotic cells in the H9c2 cells treated with high levels of glucose. H9c2 cells were cultured on chambered slides for 24 h and then treated with 5.5, 22, and 33 mmol/l glucose or 5.5, 22, and 33 mmol/l mannitol for different time periods for TUNEL assay (*A*) and DAPI staining (*B*). *A*: Representative slides from cultured H9c2 exposed to low or high levels of glucose or mannitol for 96 h. Black arrows indicate apoptotic cells detected by TUNEL, and white arrows indicate the fragmented and condensed nuclei by DAPI staining.

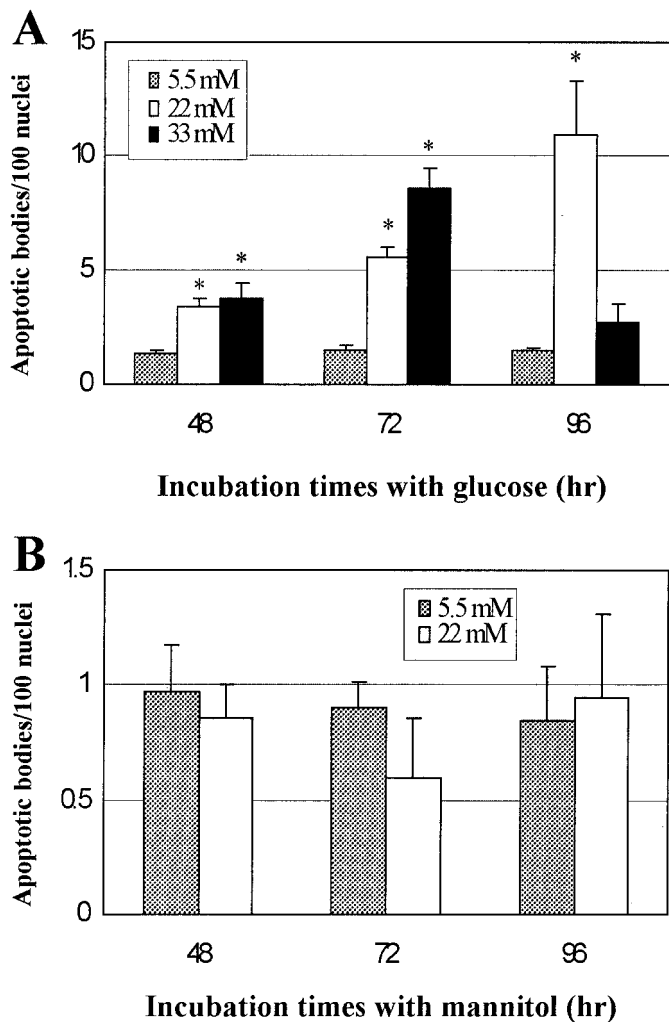


FIG. 5. Quantitative analysis of TUNEL-positive cells in H9c2 cells. Apoptotic cells were analyzed quantitatively on the basis of TUNEL-positive cells in the cultured H9c2 cells exposed to different concentrations of glucose (A) or mannitol (B). * $P < 0.05$ vs. control (5.5 mmol/l).

(10–13). Apoptosis of cardiac muscle cells and endothelial cells has been observed in the heart of patients with diabetes (20) and in STZ-induced diabetic rats (21) and mice (22; present study).

The question is whether apoptosis detected in myocytes is related directly to hyperglycemia. That there is no difference in cardiac cell apoptosis between patients with diabetes and those with diabetes and hypertension suggests that hypertension does not cause additional cardiac cell death (20). Recent studies (21) showed that apoptotic cell death in diabetic heart is independent of diabetic dehydration. Both diabetes and several restrictions in food intake decreased body weight and heart weight and led to a modest depression in cardiac function, but only diabetic hearts showed an increase in myocyte apoptosis ~8- to 13-fold at 3–10 days after STZ treatment in rats (21). These results indicate that hyperglycemia may directly cause apoptotic cell death. Using the STZ-induced diabetic mouse model, we first focused our effort on the relationship between hyperglycemia and apoptosis. In previous studies, the distinction between STZ effect and STZ-induced diabetic effect on the heart has not been eluci-

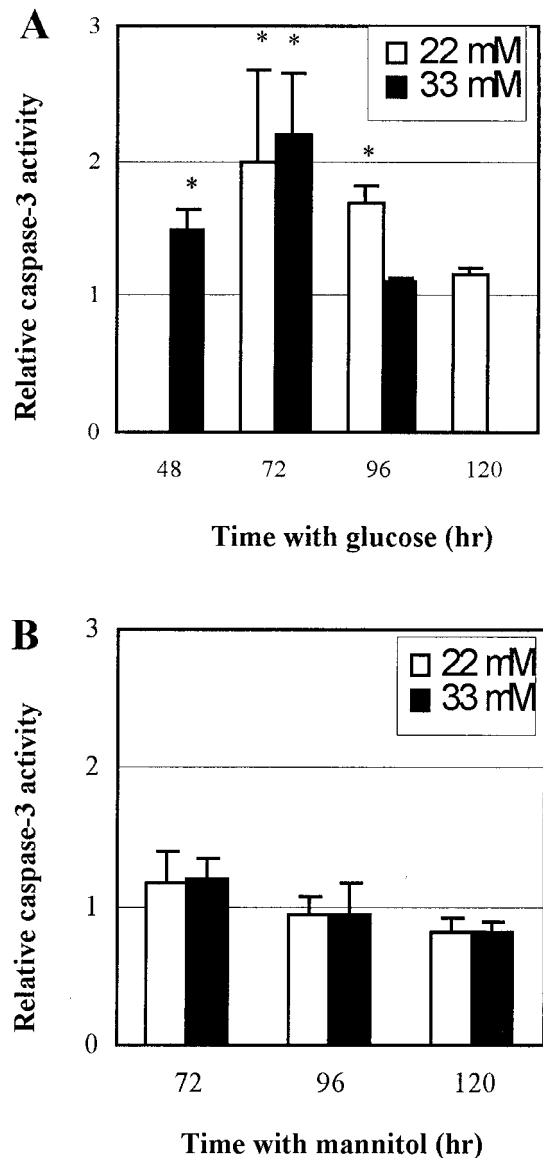


FIG. 6. Caspase-3 activation by high levels of glucose in cardiac H9c2 cells. Cells were cultured for 24 h and then exposed to 22 or 33 mmol/l glucose (A) or mannitol (B) for different times. The activation of caspase-3 was detected by enzymatic assay. Caspase-3 activity in cells exposed to 5.5 mmol/l glucose or mannitol from different experiments was normalized as 1 unit. Therefore, the activities of caspase-3 in the cells exposed to 22 and 33 mmol/l glucose or mannitol were analyzed by their proportion to 1. * $P < 0.05$ vs. control.

dated (21,22). In the present study, we observed that there was no significant increase in myocardial apoptosis in the STZ-treated mice without hyperglycemia. Importantly, in the present study, we found that diabetic mice treated with insulin at day 3 after STZ treatment displayed reduced elevation of whole-body glucose levels and inhibition of apoptotic cell death in the hearts. All of these findings suggest that the apoptosis observed in the diabetic myocardium was not caused by STZ per se. In addition, the data obtained from our in vitro experiments further demonstrate that high levels of glucose directly caused apoptosis in the cultured cardiac cells.

To demonstrate further the importance of apoptosis in the hyperglycemia-induced cardiomyopathy and to explore the possible pathway that leads to apoptosis by hyperglycemia,

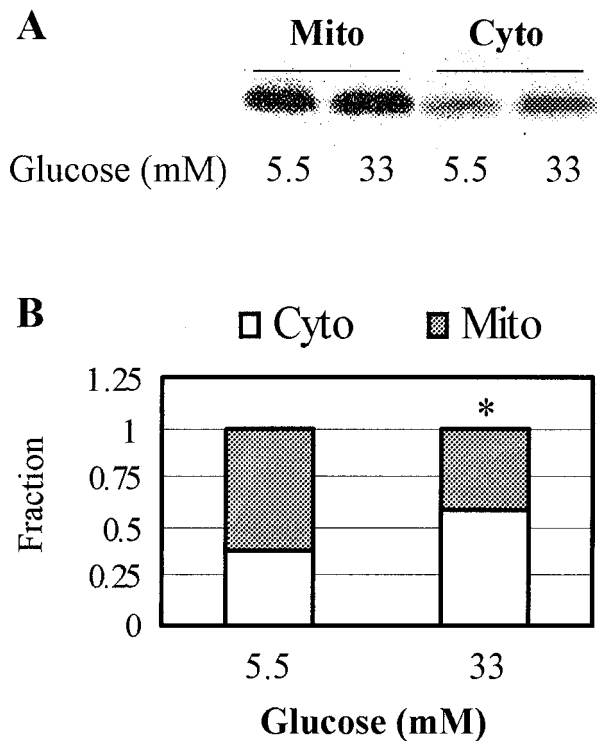


FIG. 7. Cytochrome *c* release from mitochondria to cytosol was detected in cells exposed to 5.5 or 33 mmol/l glucose by Western blotting (A), and the relative portion in cytosol was quantified (B). * $P < 0.05$ versus control (5.5 mmol/l).

we examined the caspase-3-mediated apoptotic pathway. Caspase-3 plays a pivotal role in the execution of apoptosis (24,30). Cells deficient in caspase-3 were resistant to apoptosis (31). The activation of caspase-3 alone was sufficient to cause cell death in cardiac muscle (32). By activating caspase-3, the induction of apoptotic cell death has been demonstrated in endothelial cells, neonatal or adult cardiomyocytes, and cardiac myoblast H9c2 cells by Adriamycin and ischemia/reperfusion (26–28,32–34). Hyperglycemia was also able to induce apoptotic cell death in neuron cells in vivo and in vitro and endothelial cells in vitro by activating caspase-3 (19,35). The data obtained from the present study demonstrate that activation of caspase-3 is associated with hyperglycemia-induced myocardial apoptosis. Most importantly, apoptosis and caspase-3 activation induced by high levels of glucose were suppressed by a caspase-3-specific inhibitor, indicating that caspase-3 activation is causally involved in the hyperglycemia-induced apoptotic cell death in the myocardium.

Activation of caspase-3 is mediated by multiple pathways, simply divided into mitochondria-dependent and -independent pathways (24,25,30). In the present study, the mitochondria-dependent pathway was examined. Mitochondrial cytochrome *c* release was observed in both diabetic myocardium and cultured myoblast cells. Mitochondrial dysfunction in diabetic tissues has been known to be one of the critical events associated with diabetic injury (1–4,6,16,36–38). In diabetic neurons, mitochondrial damage and translocation of cytochrome *c* from

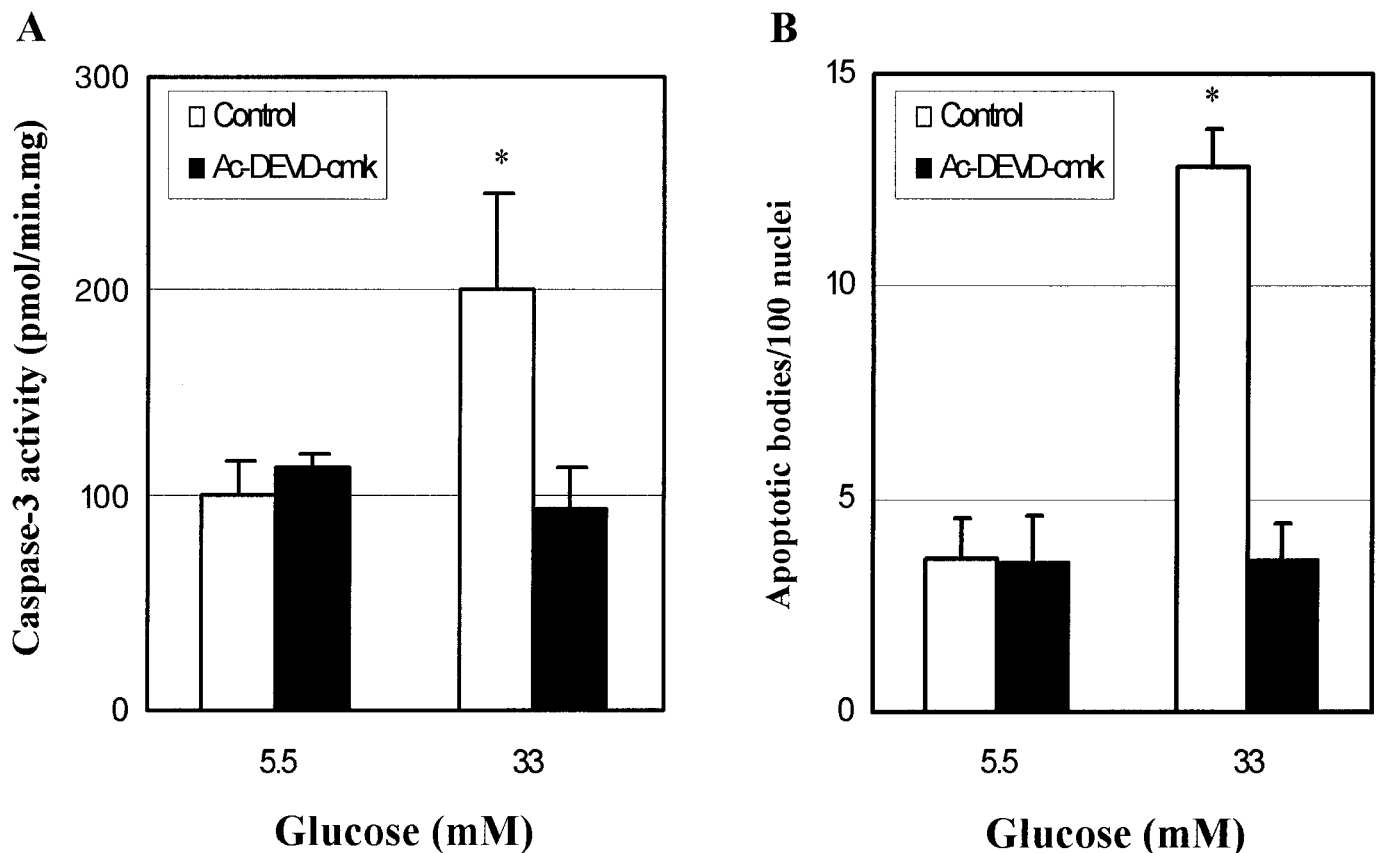


FIG. 8. Effect of caspase-3 inhibitor on caspase-3 activity and apoptotic cell death in the cardiac H9c2 cells treated with high levels of glucose. A: Inhibitory effect of Ac-DEVD-cmk on caspase-3 activity detected by enzymatic assay. B: Quantitative analysis of apoptotic cells detected by the TUNEL assay. *Significantly different from the cells treated with 5.5 mmol/l glucose.

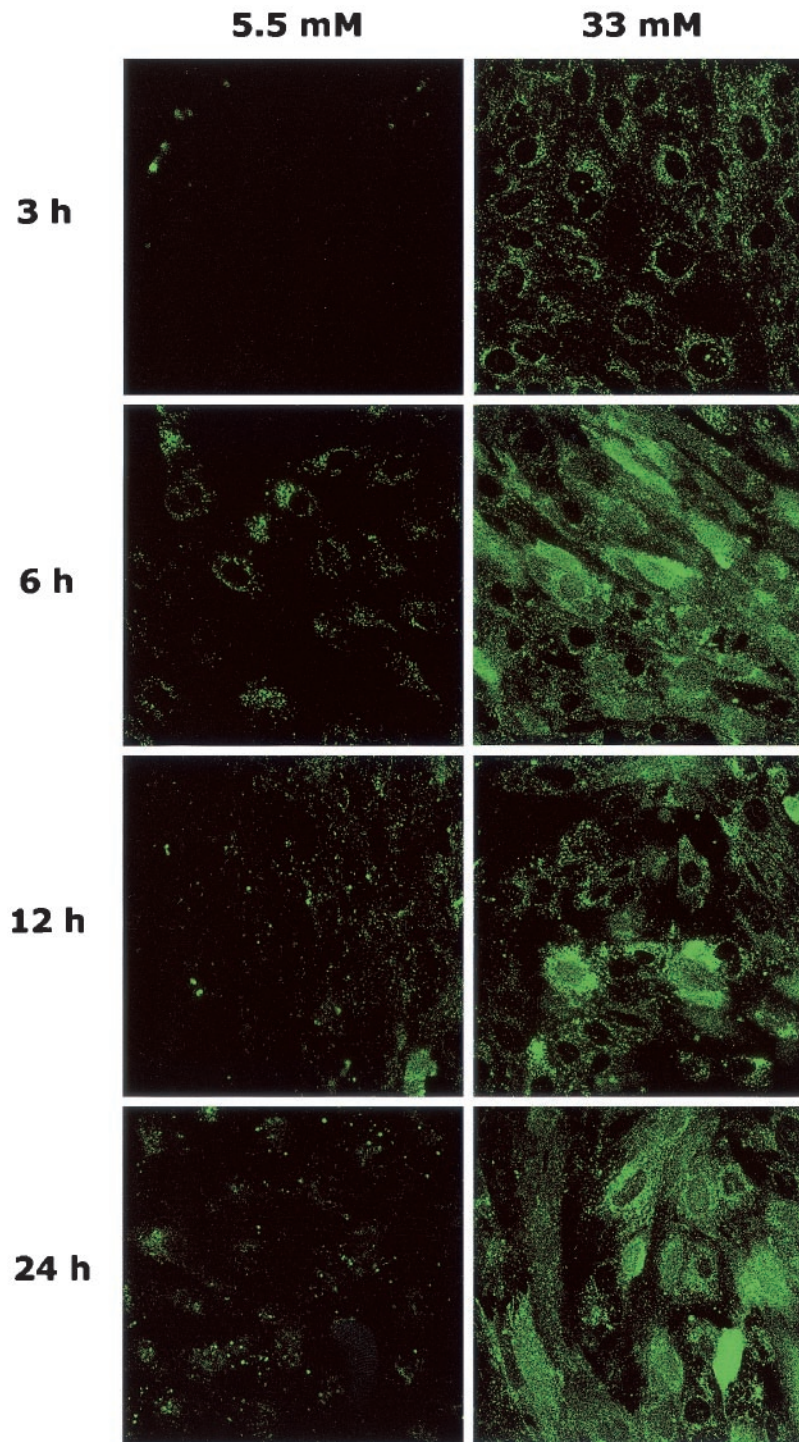


FIG. 9. Assay of ROS accumulation in cells exposed to high levels of glucose. Cells exposed to 5.5 mmol/l as control or high levels of glucose such as 22 and 33 mmol/l glucose for varying time periods as indicated were subjected to carboxyl- H_2 -DCFDA exposure for 1 h before the end of each exposure. Each culture was first checked by a phase-contrast microscope to ensure that the cell density among the cultures were comparable. The fluorescence was then detected by a confocal microscope as shown. The staining intensity thus corresponds to the extent of ROS accumulation. Original magnification $\times 63$.

mitochondria to cytosol have been observed, and both could be normalized by insulin administration (16). Taken together, these data suggest that hyperglycemia-induced myocardial cell death *in vivo* and *in vitro* most likely resulted from mitochondrial cytochrome *c*-mediated caspase-3 activation pathway.

In previous studies, we demonstrated that myocardial apoptosis induced by Adriamycin and ischemia/reperfusion

through mitochondrial cytochrome *c*-mediated caspase-3 activation pathway was triggered by ROS generation (26–28,39,40). A direct correlation of hyperglycemia and oxidative stress in diabetic injuries, in particular mitochondrial damage, has been shown (29,36,38,41). Ho et al. (19) demonstrated that exposure of endothelial cells *in vitro* to high levels of glucose caused significant ROS formation in association with caspase-3 activation and apoptosis. Both the

activation of caspase-3 and the induction of apoptotic cell death could be suppressed by addition of antioxidants in the cultures (19). ROS generation by exposure to high levels of glucose was found in the myoblast cells in the present study. A recent study also indicated that insulin-like growth factor-1 can inhibit diabetic cardiomyopathy through suppression of angiotensin II-mediated oxidative stress and myocardial cell death (22). These data suggest that ROS generated by hyperglycemia likely play, at least in part, a critical triggering role in apoptotic cell death in the diabetic myocardium.

FVB mice were used in the present study for the consideration that this strain has been used extensively to make transgenic model. In particular, cardiac-specific antioxidant overexpressing transgenic mouse models have been produced. These models include catalase- (42), metallothionein- (43), and SOD-overexpressing mice (44). These models would provide valuable tools to determine the role of oxidative stress in diabetic cardiomyopathy. Therefore, the STZ-induced FVB diabetic mouse model would be a good complement to the transgenic mouse models.

The H9c2 cell line derived from embryonic rat hearts maintains some features of cardiac myocytes and has been used extensively in in vitro studies (45–47). In the present study, we selected the H9c2 cell line instead of neonatal cardiomyocytes as used in our previous studies (25–28) for the following reasons: 1) the glucose transporting system in H9c2 cells has been investigated and does not differ from that in rat or mouse hearts (45,46); 2) as compared with neonatal cardiomyocyte culture, culturing of H9c2 cells is much easier and the phenotypes of the cultures are repeatable, but primary neonatal cardiomyocyte cultures are affected by individual differences among the litters of mice; however, additional study to understand the effect of hyperglycemia directly on myocardial cells in vivo is necessary. Nevertheless, the present study does provide evidence that hyperglycemia induces cardiac cell apoptosis in vivo, and ROS generation by high levels of glucose likely triggers the mitochondrial cytochrome *c*-mediated caspase-3 activation pathway.

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