High-Glucose Induced Protective Effect against Hypoxic Injury Is Associated with Maintenance of Mitochondrial Membrane Potential

Min Hwa KIM, Yi-Sook JUNG*, Chang-Hyun MOON*, Soo Hwan LEE*,
Eun Joo BAIK*, and Chang-Kiu MOON

Laboratory of Hygienic Chemistry, College of Pharmacy, Seoul National University, Seoul,151–742 Korea; and *Department of Physiology, School of Medicine, Ajou University, Suwon, 442–749 Korea

Abstract: Our previous report has showed that the treatment of 48 h with 22 mM glucose prevents hypoxia-induced cardiac cell death. In the present study, we investigated whether high glucose affects the mitochondrial death pathway during hypoxia, and if it does, what relates to the high glucose induced cardioprotection. Heart-derived H9c2 cells were incubated in low (5.5 mM) or high (22 mM) glucose medium for 48 h, then transferred to a normoxic or hypoxic condition. The hypoxia-induced reduction of mitochondrial redox potential, assessed by MTT assay, was inhibited in high glucose treated cells. The mitochondrial membrane potential was significantly decreased by hypoxia in low glucose treated cells, but not in high glucose treated cells. The hypoxia-induced cytoplasmic accumulation of cytochrome c, released from the mitochondria, was blocked by a treatment of high glucose. High glucose did not induce the expression of an anti-apoptotic protein Bcl-2, nor did it reduce a proapoptotic protein Bax, but it did inhibit a hypoxia-induced downregulation of Bcl-2. The cellular ATP contents were not changed by the treatment of high glucose for 48 h, and the hypoxia-induced decline of intracellular ATP level was observed in high glucose treated cells and in low glucose. A glycolytic inhibitor, 2-deoxyglucose, did not reverse the high glucose induced reduction of LDH release. The elevation of $[ROS]_i$ induced by hypoxia was inhibited in high glucose treated cells. These results suggest that high glucose induced cardioprotection may be accounted for in part by the preservation of MMP and the maintenance of a basal level of $[ROS]_i$ during hypoxia. [The Japanese Journal of Physiology 53: 451–459, 2003]

Key words: high glucose, cardioprotection, MMP, cytochrome c, Bcl-2, ROS.

Mitochondria have recently received a lot of attention for their roles in the determination of life and death in cardiac myocytes and a variety of other cells [1–4]. The function of mitochondria is impaired by various stimuli, including ischemia, resulting in an increase of calcium in cytosol and/or in mitochondria and subsequent cell death [1, 2]. This mitochondrial death pathway has been demonstrated to involve mitochondrial permeability transition (MPT), thereby causing a disruption of mitochondrial membrane potential (MMP, $\Delta\Psi_m$), ROS production, and release of apoptogenic factors such as cytochrome c and Apaf-1 (apoptosis protease activating factor-1) [3, 5, 6]. These factors further induce the activation of downstream caspases, which causes nuclear condensation, cytoplasmic fragmentation leading to ultimate cell death [6, 7]. Bcl-2 has been known to play an anti-apoptotic role by preventing cytochrome c release

from mitochondria through the stabilization of membrane integrity and the inhibition of the opening of the MPT pore (or megachannel), whereas proapoptotic member Bax promotes cytochrome c release through the MPT pore opening [8, 9].

Although the incidence of cardiac complications and mortality after myocardial infarction is higher in diabetic patients than in nondiabetics [10], there is substantial evidence about the beneficial effect of diabetes against ischemic injury. In hyperglycemic animal models, an improvement in functional recovery after ischemia and resistance against ischemic injury has been reported [11–14].

Our previous report has demonstrated that high glucose treatment could produce cytoprotective effect against hypoxic injury in heart-derived H9c2 cells via PKC activation [15]. Recently, Seo *et al.* [4] has reported that high glucose prevents rat cortical neuron from hypoxia-induced cell death through the enhancement of mitochondrial membrane potential. Hitherto, however, there is no knowledge about the effect of high glucose on the mitochondrial death pathway in cardiac myocytes. Thus, in this study we investigated whether high glucose induced cardioprotection against hypoxia involves the improvement of the mitochondrial dysfunction in terms of the loss of $\Delta\Psi_m$, cytochrome c release and ROS generation.

METHODS

Cell culture. H9c2 cells were purchased from the American Type Culture Collection (ATCC, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5.5 mM glucose supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. For experiments, cells were further maintained for 48 h in DMEM containing low glucose (5.5 mM) or high glucose (22 mM). The duration of 48 h was chosen from the result that the protective effect of 22 mM glucose was shown at the treatment duration of at least 24 h and the maximum protective effect was shown at 48 h (data not shown). For hypoxic challenges, H9c2 cells were transferred into an anoxic chamber (<1% oxygen, ThermoForma, USA) maintained at 37°C with a humidified atmosphere of 5% CO_2, 10% H_2, and 85% N_2. In the anoxic chamber, a culture medium was replaced with serum-free, glucose-free DMEM saturated with N_2 gas for 1 h prior to use. A normoxic incubation of cells in serum-free DMEM was conducted in a 5% CO_2 incubator at 37°C.

MTT assay. The mitochondrial dehydrogenase activity that cleaves 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used to determine mitochondrial redox potential in a quantitative colorimetric assay [16]. H9c2 cells were incubated with 100 μg/ml MTT in PBS for 2 h at 37°C. Formazan product was dissolved in solubilization solution containing 5% acetic acid, 5% 1 N-HCl, 50% N,N-dimethylformamide, and 20% SDS (pH 4.7), and absorbance was recorded at 570 nm.

Measurement of mitochondrial membrane potential. H9c2 cells grown on a glass-bottom dish were loaded with 5 μM rhodamine 123 (Molecular Probes, Eugene, OR, USA), a fluorescent dye indicating mitochondrial membrane potential, in HEPES-buffered control salt solution (HCSS) containing 120 mM NaCl, 5 mM KCl, 1.6 mM $MgCl_2$, 2.3 mM $CaCl_2$, 15 mM glucose, 20 mM HEPES, and 10 mM NaOH. The cells were incubated for 10 min at 37°C and washed three times with HCSS, and the fluorescence signal (Ex=480 nm; Em=520 nm) of rhodamine 123 was analyzed on the stage of a Nikon Diaphot inverted microscope equipped with a 100 W xenon lamp. The background fluorescence signal of rhodamine 123 was determined on a glass-bottom dish without cells and subtracted from rhodamine 123 signals obtained in H9c2 cells. All images were analyzed by using a Quanticell 700 system (Applied Imaging).

Cytochrome c release. H9c2 cells were washed twice with ice-cold PBS and collected by centrifugation at 200×*g* for 10 min at 4°C. The pellets were then responded in extraction buffer containing 220 mM mannitol, 68 mM sucrose, 20 mM HEPES (pH 7.4), 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT, as well as protease inhibitors. The cells were homogenized for 40 strokes in a glass homogenizer. The homogenates were then centrifuged at 14,000×*g* for 30 s to remove pellet nuclei, and the supernatants were centrifuged once more at 200,000×*g* for 30 min. The resulting supernatants as cytosolic fraction and pellets as mitochondrial fraction were subjected to electrophoresis on 12.5% acrylamide gels, then transferred to a PVDF membrane (Millipore, Bedford, MD, USA). After being blocked in 5% nonfat dry milk for 90 min, the blots were incubated overnight with antibody to cytochrome c (Santa Cruz). Following washout three times with Tris-buffered saline containing 0.05% tween 20, the membrane was incubated with antirabbit immunoglobulin goat antibody conjugated with alkaline phosphatase (1 : 3,000 dilution, Santa Cruz) for 4 h, and washed out two times with TBS-T and the last time with alkaline phosphate buffer (pH 9.5). The cytochrome c band was visualized by using the NBT/BCIP method.

The contamination of the cytosolic fraction with mitochondria was checked with the mitochondrial protein COX IV.

Bcl-2 and Bax expression. The cell culture medium was aspirated, and the cells were washed twice with ice-cold PBS. Added to the cells was 200 μl/well of hypotonic HEPES buffer (10 mM HEPES, 5 mM MgCl$_2$, 40 mM KCl, 2 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, pH 7.4). After 30 min, the cells were aspirated repeatedly with a 25 gage needle and centrifuged at 200×g for 10 min to remove nuclei. The supernatants were further centrifuged at 10,000×g for 15 min to obtain heavy membrane fraction containing mitochondria. They were collected and subjected to electrophoresis on 12.5% acrylamide gels, then transferred to PVDF membrane (Millipore). After being blocked in 5% nonfat dry milk for 90 min, the blots were incubated 2 h with antibody to Bcl-2 and Bax (1 : 100 dilution, Santa Cruze). Following washout three times with TBS containing 0.05% tween 20, the membrane was incubated with antirabbit immunoglobulin goat antibody conjugated with alkaline phosphatase (1 : 3,000 dilution, Santa Cruze) for 4 h, and washed out two times with TBS-T and the last time with alkaline phosphate buffer (pH 9.5). Bcl-2 and Bax were visualized by using NBT/BCIP.

Measurement of intracellular ATP content. The intracellular ATP content of cells was measured with the ATP luminescent assay kit (Molecular Probes). The cells were lysed directly in lysis buffer, and the lysate was assayed according to the manufacturer's instructions. Light emitted was measured with a luminometer (TD-30/20, TURNER DESIGNS). The ATP content was calculated by comparison with a standard curve derived from known concentrations of ATP, ranging from 0.01 to 10 pmol.

LDH release. Overall cell injury was assessed by measuring the amount of lactate dehydrogenase (LDH) released into the medium 10 h after hypoxia as previously described [4]. The percent of LDH release was calculated from the maximum LDH release (100%) induced by adding 0.1% triton X-100.

Measurement of intracellular ROS generation. H9c2 cells grown on a glass-bottom dish were loaded with 5 μM dihydrodichlorofluorescein diacetate (DHDCF-DA; Molecular Probes) plus 2% Pluronic F-127 in HEPES-buffered control salt solution (HCSS). The cells were incubated for 20 min at 37°C and washed three times with HCSS, and the fluorescence signal of DCF (Ex=490 nm; Em=510 nm), an oxidation product of DHDCF-DA by free radicals, was analyzed on the stage of a Nikon Diaphot inverted microscope equipped with a 100 W xenon lamp. To minimize background signal caused by the direct oxidation of DHDCF-DA by illumination at 490 nm, the intracellular levels of ROS were analyzed within 3 sec after illumination by using a Quanticell 700 system (Applied Imaging).

Statistic. All data were compared by using Student's t-test for unpaired observations between two groups. The p value of <0.05 was considered significant.

RESULTS

Effect of high glucose on mitochondrial redox potential during hypoxia

MTT reductase activity represents mitochondrial redox potential and cellular viability. Mitochondrial redox potential in low glucose treated cells fell to below 90% of normoxia control level within 2 h of hypoxia (Fig. 1). It remained in a steady state at 85% for the next 4 h, then further decreased. But in high glucose treated cells, mitochondrial redox potential was significantly higher than in low glucose control cells and did not decrease within 10 h of hypoxia.

Impairment in mitochondrial redox activity (Fig. 1) occurred before any sign of cell death was observed (data not shown), indicating that the decrease in metabolism and the consequent fall in energy production was not a result of the loss of live cells, but instead was due to a metabolic impairment within a population that was still viable.
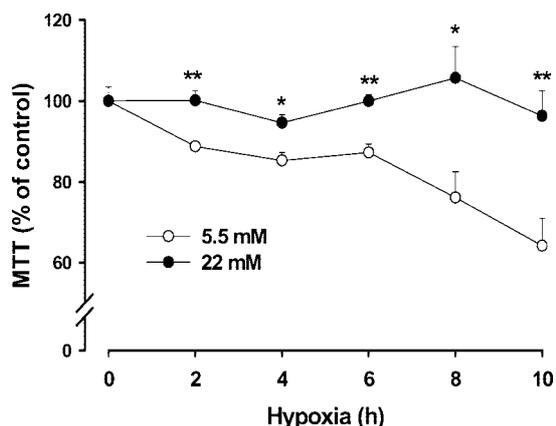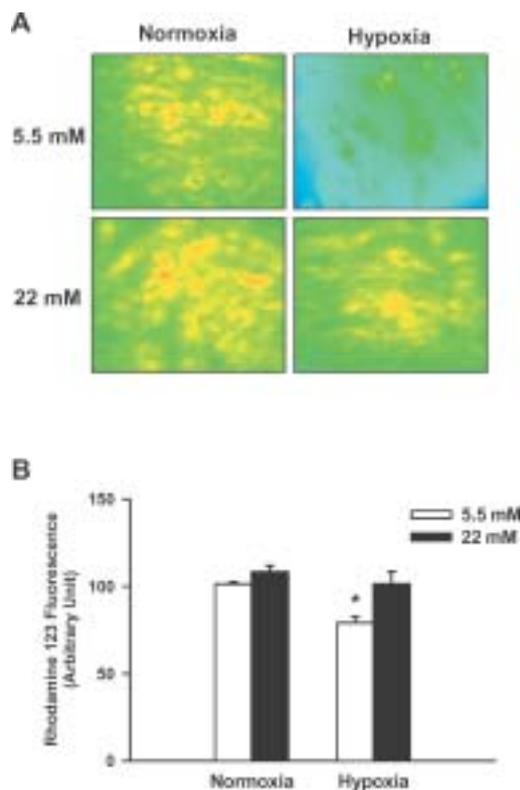

Fig. 1. Effect of high glucose on mitochondrial redox potential during hypoxia. The cells were incubated in low (5.5 mM) or high (22 mM) glucose medium for 48 h, then transferred to hypoxic condition. Mitochondrial dehydrogenase activity was assayed by a reduction of MTT at 0, 2, 4, 6, 8, and 10 h after exposure to hypoxia. Open circle, 5.5 mM glucose; closed circle, 22 mM glucose. Data represent mean±SEM (n=6). Significant difference from relevant control group (treated with 5.5 mM glucose) at * $p<0.05$, ** $p<0.01$, using Student's t-test.

## Effect of high glucose on mitochondrial membrane potential during hypoxia

Because the loss of mitochondrial membrane potential can be sufficient to activate cardiac cell death [3, 5, 17], we hypothesized that the cytoprotective effect of high glucose against hypoxic injury might be related to the effect of high glucose on the level of mitochondrial membrane potential. MMP was examined by using the positively charged and lipophilic rhodamine 123 that permeates into the negatively charged mitochondria and therefore reflects the mitochondrial membrane potential [18]. To measure the fluorescence signal of rhodamine 123 on the stage of an inverted microscope without the interference of plastic culture ware, we should culture cells by using a glass-bottom dish and on the dish; the cells will be easily detached under hypoxic condition. Therefore we could not measure the precise MMP beyond 4 h. On the other hand, a loss of MMP lagged slightly behind changes in MTT reductase activity, and intracellular ATP content was reported previously [3]. The fluorescence signal of rhodamine 123 in cells cultured in high glucose medium (108.3 ± 3.6) was not significantly different at normoxic condition compared to cells cultured in low glucose medium (101.3 ± 1.3) (Fig. 2A, B). After 4 h of hypoxia, the fluorescence signal in cells cultured in low glucose media was significantly decreased (cold color, 79.0 ± 3.8). However, the fluorescence signal in cells cultured in high glucose medium was not altered (101.3 ± 7.0). These results demonstrate that mitochondrial membrane potential in high glucose treated cells is preserved during hypoxia.

Effect of high glucose on hypoxia-induced cytochrome c release from mitochondria

During the apoptotic process, mitochondria undergo major changes in membrane integrity that concern both the inner and the outer membranes, leading to a disruption of the inner membrane potential and the release of intermembrane proteins through the outer membrane. Cytochrome c, a mitochondrial intermembrane protein, release into the cytosol at the late phase of apoptotic cell death after mitochondrial membrane function is lost [19]. We therefore investigated the effects of high glucose on the hypoxia-induced cytochrome c release into cytosol. Cytochrome c was detected in low glucose treated cytosolic fraction after 6 h of hypoxia, but not in high glucose treated cytosolic fraction (Fig. 3). Hypoxia-induced cytochrome c release was completely blocked by the treatment of high glucose.

Fig. 2. Effect of high glucose on mitochondrial membrane potential during hypoxia. The cells were incubated in low (5.5 mM) or high (22 mM) glucose medium for 48 h, then transferred to normoxic or hypoxic condition for 4 h. **A**: Fluorescence photomicrographs (stained with rhodamine 123) of cells after incubation in normoxic or hypoxic condition. The more warm the color, the higher MMP. **B**: Mitochondrial membrane potential was analyzed by measuring the fluorescence intensity of rhodamine 123. Open bar, 5.5 mM glucose; closed bar, 22 mM glucose. Data represent mean$\pm$SEM ($n=4$). Significant difference from 5.5 mM glucose treated cells in normoxia at * $p<0.05$, using Student's *t*-test.
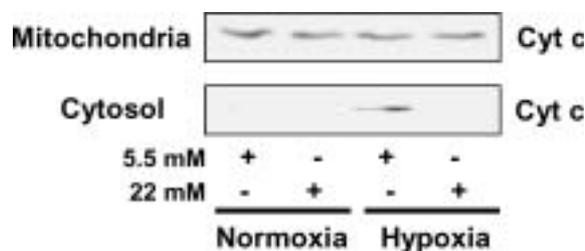
Fig. 3. Subcellular localization of cytochrome c in low or high glucose treated myocytes following exposure to normoxic or hypoxic condition. The cells were incubated in low (5.5 mM) or high (22 mM) glucose medium for 48 h, then transferred to normoxic or hypoxic condition for 6 h. The cytosolic and mitochondrial fractions of cells were separated. Western blotting for cytochrome c was performed as described in method. Similar results were obtained in three additional independent cell preparations.

Effect of high glucose on hypoxia-induced decline of Bcl-2

The Bcl-2 family is well known as a key regulator of the apoptotic response through the modulation of cytochrome c release from mitochondria and intracellular ROS generation [8, 9, 20, 21]. We examined the response of antiapoptotic Bcl-2 and proapoptotic Bax to high glucose leading to cardioprotection against hypoxia. The expression levels of Bcl-2 and Bax were not altered by high glucose treatment for 48 h (Fig. 4). After 6 h of hypoxia, the level of Bax was not altered, but Bcl-2 expression was downregulated. Hypoxia-induced downregulation of Bcl-2 was blocked in high glucose treated cells.

Effect of high glucose on ATP levels before and during hypoxia

To test the possibility that the ATP production through the utilization of high glucose is responsible for high glucose induced protection, we measured the ATP contents of cells that were treated with low or high glucose for 48 h and cells that were exposed to hypoxia after treatment of low or high glucose for 48 h.
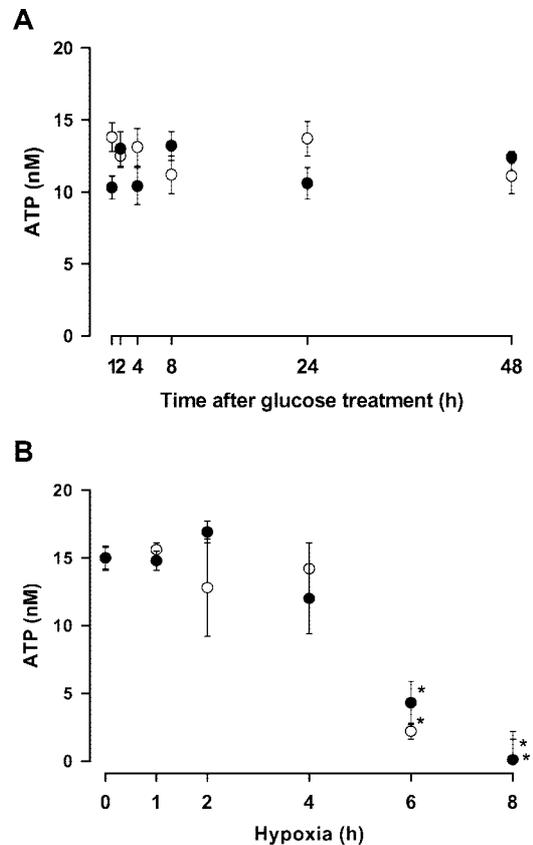
The intracellular ATP contents during low or high glucose treatment were not significantly changed (Fig. 5A). During hypoxia, the cellular ATP levels were decreased within 6 h (5.5 mM: $15 \pm 0.8 \rightarrow 2.2 \pm 0.6$, 22 mM: $15 \pm 0.9 \rightarrow 4.3 \pm 1.6$), which ultimately declined to <1% of control cells up to 8 h both in low and high glucose treated cells (5.5 mM: 0.1 ± 1.5, 22 mM: 0.1 ± 2.1) (Fig. 5B). These results reflect that high glucose does not act as an energy source and the absence of the glycolytic production of ATP.

Effect of glycolytic inhibitor, 2-deoxyglucose on the high glucose induced protective effect against hypoxic cell death

To confirm that high glucose induced protection was not due to ATP production through the glycolysis, the cells were incubated in low or high glucose medium with or without 2-deoxyglucose (1, 3, 5 mM), which is a glycolytic inhibitor, then transferred to hypoxic condition. 2-Deoxyglucose accumulates as 2-deoxyglucose-6-PO_4, which cannot be further metabolized by phosphohexose isomerase in the glycolytic pathway. Hypoxia-induced LDH release was dramatically inhibited in high glucose treated cells (5.5 mM: 65.3 ± 2.8; 22 mM: 28.1 ± 1.2, $p < 0.01$) (Fig. 6). 2-Deoxyglucose did not reverse the high glucose-induced reduction of LDH release (2-DOG 1 mM: 28.6 ± 1.6, 3 mM: 21.7 ± 1.8, 5 mM: 29.4 ± 1.7).
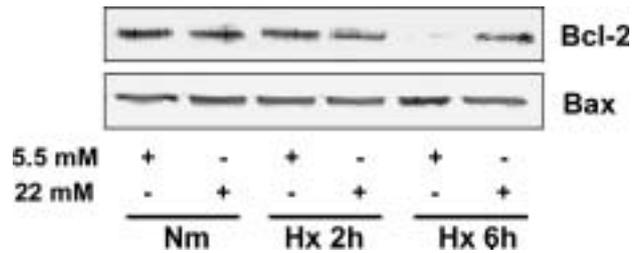
Fig. 4. Effect of high glucose on Bcl-2, Bax expression. The cells were incubated in low (5.5 mM) or high (22 mM) glucose medium for 48 h, then transferred to normoxic or hypoxic condition for 6 h. Western blotting for Bcl-2 and Bax were performed as described in method. Similar results were obtained in three additional independent cell preparations.

Fig. 5. Effect of high glucose on intracellular ATP content. The cells were incubated in low (5.5 mM) or high (22 mM) glucose medium for 48 h. **A**: ATP concentrations at 1, 2, 4, 8, 24, and 48 h after incubation were determined. **B**: After incubation in low or high glucose medium for 48 h, the cells were transferred to hypoxic condition. ATP concentrations at 1, 2, 4, 6, and 8 h after hypoxia were determined. Open circle, 5.5 mM glucose; closed circle, 22 mM glucose. Data represent mean±SEM ($n=6$). Significant difference from control at * $p < 0.05$, using Student's t-test. Similar results were obtained in two additional independent myocyte preparations.
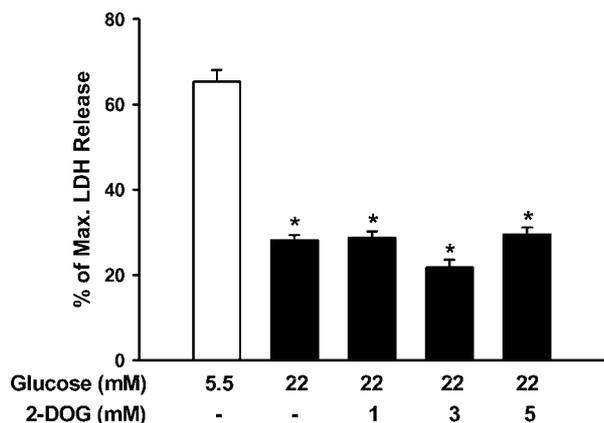
Fig. 6.   **Effect of glycolytic inhibitor on high glucose-induced cardioprotection.** The cells were incubated in low (5.5 mM) or high (22 mM) glucose medium with or without glycolytic inhibitor, 2-deoxyglucose (2-DOG, 1, 3, 5 mM), for 48 h, then transferred to hypoxic condition for 10 h. The activity of lactate dehydrogenase released into medium was measured as described in method. Open bar, 5.5 mM glucose; closed bar, 22 mM glucose. Data represent mean±SEM (*n*=6). Significant difference from 5.5 mM glucose control at * $p<0.01$, using Student's *t*-test.
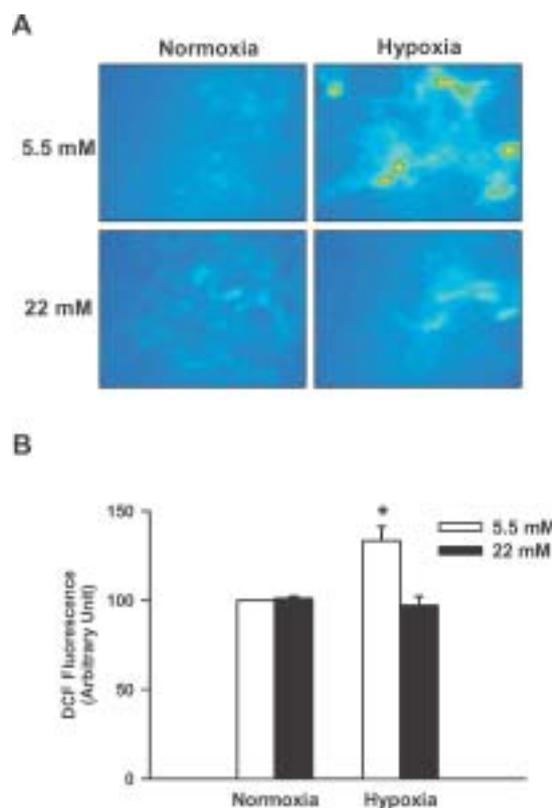
Effect of high glucose on ROS generation during hypoxia

ROS includes superoxide anion, hydrogen peroxide, and hydroxyl radical and is known to play a central role in apoptotic death and in necrotic death [5]. During ischemia, increased ROS triggers mitochondrial dysfunction leading to cardiac cell death [17]. Therefore we examined the effect of high glucose on [ROS]i during hypoxia. The baseline levels of [ROS]i were similar between the low and high glucose treated cells at normoxia (100 ± 0.1, 101.1 ± 1.1, respectively). After 4 h of hypoxia, [ROS]i was dramatically increased in low glucose treated control cells (warm color, 133.3 ± 8.1). Whereas [ROS]i in high glucose treated cells was not altered (97.1 ± 5.0) (Fig. 7A, B). For the same reason described above, we measured [ROS]i at 4 h after hypoxia. These results represent that high glucose absolutely inhibits the ROS generation induced by hypoxia in H9c2 cells.

DISCUSSION

Following our previous report that the treatment of 22 mM high glucose for 48 h conferred the H9c2 cells resistance to the hypoxic injury [14], this study has demonstrated that the mechanism of the high glucose induced cardioprotection involves the preservation of mitochondrial membrane potential and the inhibition of ROS generation during hypoxia.

Mitochondria are recognized as central regulators

Fig. 7.   **Effect of high glucose on generation of reactive oxygen species during hypoxia.** The cells were incubated in low (5.5 mM) or high (22 mM) glucose medium for 48 h, then transferred to normoxic or hypoxic condition for 4 h. **A**: Fluorescence photomicrographs (stained with DHDCF-DA) of cells after incubation in normoxic or hypoxic condition. The more warm color, the higher concentration of ROS. **B**: ROS generation was analyzed by measuring the fluorescence intensity of oxidized DHDCF-DA. Open bar, 5.5 mM glucose; closed bar, 22 mM glucose. Data represent mean±SEM (*n*=4). Significant difference from 5.5 mM glucose-treated cells in normoxia at * $p<0.05$, using Student's *t*-test.

of life and death under various stresses in a variety of cells [1, 2, 22]. Cardiac mitochondria are highly vulnerable to injury induced by ischemia, which precipitates myocardial dysfunction in several disease conditions. In heart, mitochondrial dysfunction induced by hypoxia or other stimuli has been demonstrated to produce mitochondrial permeability transition (MPT), the disruption of mitochondrial membrane potential (MMP), and subsequent cytochrome c release, leading to lethal cell injury [3, 5, 23]. Therefore preserving the functional and structural integrity of mitochondria is considered a prerequisite for successful cardioprotection [24]. Indeed, several strategies that modulate mitochondrial function and ATP production have been considered [25, 26] to reduce myocardial injury and to improve cardiac function. We previously demonstrated that hyperglycemic rat heart [27] and high glu-

cose treated cardiac cells [15] were less susceptible for ischemic injury. A recent report has demonstrated that the enhanced $\Delta\Psi_m$ is responsible for the high glucose induced protective effect in neurons [4]. Since the investigation about the effect of high glucose on the mitochondrial dysfunction during hypoxia has not been done in cardiac cells, this study was performed by using heart-derived H9c2 cells to investigate whether high glucose can affect hypoxia-induced mitochondrial dysfunction, in consideration of the mitochondrial membrane potential, cytochrome c release, Bcl-2/Bax expression, and ROS generation.

In this study, we found that high glucose significantly improved the hypoxia-induced decrease in the redox potential of mitochondria (Fig. 1), as assessed by the conversion of the tetrazolium dye MTT to its reduced form, a reaction mediated by mitochondrial reductase [16]. Furthermore, our results showed that H9c2 cells exhibited a reduction of mitochondrial membrane potential after 4 h of hypoxia, and that the loss of mitochondrial membrane potential by hypoxia was reversed by pretreatment with high glucose (Fig. 2). Consistent with our results, other reports (or, another report) also proved that cardiac myocytes from hyperglycemic hearts were less prone to ischemia/reperfusion-induced mitochondrial dysfunctions, such as a depolarization of $\Delta\Psi_m$ [28].

It has been reported that cytochrome c release results from changes in mitochondrial membrane permeability after a loss of membrane potential ($\Delta\Psi_m$). Although cytochrome c release can precede and occur independently of decreased $\Delta\Psi_m$, a dissipation of $\Delta\Psi_m$ has often been sufficient for cytochrome c release to occur [29]. We therefore examined the effect of high glucose on cytochrome c release to cytosolic fraction and found that the hypoxia-induced appearance of cytochrome c in the cytosolic fraction of control cells was completely reduced in the cytosol of high glucose treated cells (Fig. 3). Our results showing the good correlation between the effects of glucose on mitochondrial membrane potential and cytochrome c release further support the hypothesis that mitochondrial membrane potential controls the permeability of membrane and regulates cytochrome c release [30].

Among intracellular apoptosis regulating proteins that have been reported, the Bcl-2 family plays a pivotal role in regulating the responses of cells to a wide variety of apoptotic signals [8, 9]. Bcl-2 has been known to play antiapoptotic role by preventing cytochrome c release from mitochondria through the stabilization of membrane integrity and the inhibition of the opening of the MPT pore (or megachannel),

whereas proapoptotic member Bax promotes cytochrome c release through the MPT pore opening [8, 9]. According to Schaffer [31], Bcl-2 overexpression has blocked cardiac cell death induced by chemical hypoxia. Recently, the balance between Bcl-2 and Bax has been regarded more important than Bcl-2 or Bax by itself. In ischemic preconditioning, the level of Bcl-2 expression was not altered, but proapoptotic protein Bax was downregulated [32]. During hypoxia in this study, the level of Bax was not altered, but Bcl-2 expression was downregulated (Fig. 4). Although our study did not show the effect of high glucose itself on Bcl-2 upregulation or Bax downregulation, a hypoxia-induced reduction of Bcl-2 was prevented by high glucose treatment (Fig. 4) without affecting Bax expression. Therefore high glucose-induced inhibition of Bcl-2 reduction during hypoxia may be attributed to the stabilization of mitochondrial membrane potential and the prevention of cytochrome c release.

On the basis of the previous reports that ATP depletion during hypoxia causes mitochondrial dysfunction followed by cell death [33], and that hyperglycemia produces an increase in myocardial glucose uptake [34], we hypothesized that high glucose induced maintenance of mitochondrial function may be ascribed to the preservation of ATP production through increased glucose uptake and glycolysis. To examine this hypothesis, we investigated the effect of high glucose on ATP levels in intact cells before and after hypoxia. It is interesting that we found ATP levels in high glucose-treated H9c2 cells remaining similar to those observed in control cells, and that the remarkable decline of ATP levels during hypoxia shown in control cells was not altered by pretreatment with high glucose throughout the course of hypoxia (Fig. 5). Consistent with our results, another study has demonstrated that the level of ATP was not increased, but remained unaltered under the condition of substantial increase in intracellular glucose concentration [35]. We further found that the glycolytic inhibitor, 2-deoxyglucose, did not reverse the high glucose induced protective effect against hypoxia-induced cell death (Fig. 6). From these results, it is suggested that the high glucose induced protective effect may not be attributed to an increase in ATP production through glycolysis, but to a blockade of death signaling a pathway somewhere near or upstream of the mitochondria. Our results further support the others that the feedback regulation of hexokinase, a rate-limiting enzyme in the glycolytic pathway, by glucose-6-phosphate, a product of hexokinase, may have an influence on the limitation of the overall glycolysis and ATP production in high glucose treated cells [35]. On the other

hand, our findings are quite inconsistent with those of Malhotra *et al.* [7], who found that a 30 min treatment with high glucose protects cardiac cells from hypoxic injury through an increased glycolysis of extracellular glucose. The differences between the duration of glucose treatment (48 h vs. 30 min) and the cell type (H9c2 cells vs. primary cultures) may explain this discrepancy in terms of cellular energetics.

Mitochondria has long been known to play a role in the generation and processing of [ROS]$_i$, and that mitochondrial dysfunction can result in accumulation of [ROS]$_i$ leading to cell death [5, 36]. In contrast, there is also increasing evidence that ROS is a major contributing factor on the mitochondrial dysfunction through mitochondrial permeability transition [1, 22], suggesting the a causative relationship between ROS generation and mitochondrial dysfunction is still controversial [22]. Indeed, it has been demonstrated that during hypoxia, ROS has been explosively produced at the respiratory chain of the mitochondrial membrane, causing the loss of mitochondrial function in cardiomyocytes [36], and that antioxidant could protect the mitochondrial function against ischemia-reperfusion [37]. Recently, it has been postulated that ROS may play a dual role in apoptosis, either as activators of permeability transition or as a consequence of this transition, depending on the death stimulus [1]. In the present study, ROS generation was elevated in a hypoxic condition in H9c2 cells, and this elevation was blocked by high glucose treatment (Fig. 7).

In summary, we found a good correlation between glucose-induced inhibitory effects on the ROS generation and on the mitochondrial dysfunction during hypoxia, though this study did not address the causative relationship between these effects of high glucose. From our results, it is suggested that the improvement of mitochondrial dysfunction and the inhibition of ROS generation during hypoxia may be, at least in part, involved in the high glucose induced cardioprotection against hypoxic injury in heart-derived H9c2 cells.

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