Protective Effects of the Potassium Channel Opener-Diazoxide against Injury in Neonatal Rat Ventricular Myocytes

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Abstract. The mitochondrial ATP-regulated potassium channel is present in the inner membrane of heart mitochondria. Similarly to plasma membrane K_{ATP}, the mitochondrial channel is inhibited by antidiabetic sulfonylureas and activated by potassium channel openers, such as diazoxide. In the present work, the cytoprotective properties of diazoxide on the H9c2 cardiac myoblast cell line and neonatal rat ventricular cardiomyocytes were analysed. It was observed that 100 μmol/l diazoxide protected neonatal rat ventricular cardiomyocytes, but not H9c2 myoblasts, against injury induced by hydrogen peroxide or simulated ischemia. Moreover, diazoxide prevented hydrogen peroxide-induced mitochondrial potential depolarisation in neonatal rat ventricular cardiomyocytes. Diazoxide, at the same time, did not affect the expression level of the anti-apoptotic protein bcl-2 in these cells. The protective effects of diazoxide were suppressed by 5-hydroxydecanoic acid, a potassium channel blocker. These observations suggest that activation of the mitochondrial ATP-regulated potassium channel plays an important role in protection of neonatal cardiomyocytes against injury.

Key words: Mitochondrial K_{ATP} channel — Cardiac cells — Oxidative stress — Ischemic injury — Apoptosis

Abbreviations: 5-HD, 5-hydroxydecanoic acid; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; FBS, foetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HS, horse serum; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; KCOs, potassium channel openers; LDH, lactate dehydrogenase; mitoK_{ATP} channel, mitochondrial ATP-regulated potassium channel; $\Delta\Psi_m$, mitochondrial potential.

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Introduction

The mitochondrial ATP-regulated potassium channel (mitoK$_{ATP}$ channel) was identified by the patch-clamp technique in the inner membrane of liver mitochondria (Inoue et al. 1991). Later, a similar channel was described in heart (Paucek et al. 1992), brain (Bajgar et al. 2001; Debska et al. 2001) and skeletal muscle mitochondria (Debska et al. 2002). Similarly to the plasma membrane ATP-regulated potassium channel (K$_{ATP}$ channel), the mitochondrial channel is inhibited by antidiabetic sulfonylureas such as glibenclamide, and activated by potassium channel openers (KCOs) (for a review, see Szewczyk and Wojtczak 2002). Diazoxide seems to be an especially potent activator of the mitoK$_{ATP}$ channel (Garlid et al. 1996). The primary function of this channel is to allow K^+ transport into the mitochondrial matrix. This phenomenon could be involved in maintaining mitochondrial volume homeostasis.

The molecular identity of the mitoK$_{ATP}$ channel is unknown. Probably, it is composed of two types of subunits: the pore forming subunit (Kir family) and the sulfonylurea receptor. Several observations based on using antibodies suggest that the mitoK$_{ATP}$ channel belongs to the inward rectifier K^+ channel family, Kir6.x (Suzuki et al. 1997; Zhou et al. 1999). Similarly, the molecular properties of the mitochondrial sulfonylurea receptor are not clear. The use of ^{125}I-glibenclamide led to labeling of a 28 kDa protein in heart mitochondria (Szewczyk et al. 1997, 1999). Recently, with the use of the fluorescent probe BODIPY-glibenclamide, a 64 kDa protein has been labeled in brain mitochondria (Bajgar et al. 2001).

The cardiac mitoK$_{ATP}$ channel plays an important role in protecting cardiomyocytes during ischemia/reperfusion (for a review, see Szewczyk and Marban 1999; Grover and Garlid 2000; O'Rourke 2000). Activation of the channel by diazoxide seems to mimic ischemic preconditioning in heart muscle (Garlid et al. 1997; Liu et al. 1999). The cardioprotective action of mitochondrial KCOs probably involves multiple mechanisms: including an effect on ischemia-induced cellular apoptosis.

In the present work, the effects of the KCO diazoxide on the H9c2 cardiac myoblast cell line and on neonatal rat cardiomyocytes were analyzed. It was shown that diazoxide caused protection of cardiac ventricular myocytes (but not of H9c2 cells) against simulated ischemia/reperfusion injury. These effects have been antagonized by the mitoK$_{ATP}$ channel inhibitor 5-hydroxydecanoic acid (5-HD). Further, we have investigated the effect of oxidative stress induced by hydrogen peroxide on the mitochondrial membrane potential in cardiac ventricular myocytes. We have observed that diazoxide protects cardiac mitochondria in ventricular myocytes against depolarisation induced by oxidative stress. Diazoxide-induced protection was not accompanied by changes in the expression level of the anti-apoptotic protein bcl-2 in ventricular myocytes.

Materials and Methods

Materials

All reagents for cell culture were purchased from Life Technologies, Inc. (Gibco-BRL, UK) and from Sigma-Aldrich Co. Ltd. (UK). 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was from Molecular Probes (USA), Percoll was from Amersham Pharmacia Biotech (UK). All other reagents, if not indicated otherwise, were obtained from Sigma-Aldrich Co. Ltd. (UK).

Cell culture

The embryonic rat heart-derived myogenic cell line H9c2 was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were routinely cultured in high glucose (4.5 g/l) Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 4 mmol/l L-glutamine. The cells were incubated in an atmosphere containing 5% CO_2/95% air at 37℃.

Primary culture of neonatal rat cardiomyocytes

Cultured cardiac myocytes were prepared from ventricles of 1–3 day-old rats as described previously (Chiesi at al. 1994). In brief: ventricles were minced and subjected to five 10-min collagenase/pancreatine digestion cycles in a balanced salt solution at 37℃. Digests were then centrifuged ($200 \times g$, 3 min) and the cells were loaded on a discontinuous Percoll gradient and centrifuged ($350 \times g$, 40 min). The myocyte-enriched fraction, which sedimented between the two layers of discontinuous Percoll gradient (1.059 g/ml and 1.082 g/ml), was saved and washed twice. The final cell pellets were resuspended in DMEM/medium 199 (4 : 1, v/v) supplemented with antibiotics, 5% FBS, and 10% HS, preplated for 30 min and then cultured for 2–3 days. 24 h before the experiment, the medium was changed to serum-free, high glucose (4.5 g/l) DMEM with vitamin B_{12}, transferrin, and insulin.

In vitro model of ischemia/reperfusion injury in H9c2 cardiac myoblasts

H9c2 cells were cultured up to about 70% confluence in a DMEM medium (as described above in cell culture paragraph). 24 h before the experiment, the medium was changed to serum-free DMEM. After 24 h culture, the medium was changed into DMEM without glucose and pyruvate, and the cells were incubated for 15 min with diazoxide and/or 5-HD (no drugs were added to the control cells). Then, the cells were placed in a Plexiglas airtight container which was gassed with 95% Ar/5% CO_2 to simulate ischemia (10 min gas flow, 10 min stop-flow, 12 min gas flow). There was less than 1 ppm of O_2 in the culture medium at the end of this protocol. The container was subsequently placed at 37℃ and the cells were incubated for 10 h. Then the container was opened, the culture medium was changed into serum-free DMEM and the cells were subjected to simulated reperfusion by incubating for additional 2 h in an atmosphere containing 5% CO_2/95% air at 37℃.

In vitro model of ischemia/reperfusion injury in neonatal cardiomyocytes

Neonatal cardiomyocytes were incubated for 15 min with diazoxide and/or 5-HD (no drugs were added to the control cells) in serum-free DMEM. Subsequently, cells were subjected to 2 h-simulated ischemia by incubation with ischemic buffer (in mmol/l: 118 NaCl, 24 $NaHCO_3$, 16 KCl, 1 KH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 0.5 EDTA-Na, 20 sodium lactate, pH 6.2) containing drugs, equilibrated with 95% Ar/5% CO_2 and supplemented with 750 μmol/l sodium dithionite. Control cells were incubated in control buffer (in mmol/l: 118 NaCl, 24 $NaHCO_3$, 4 KCl, 1 KH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 0.5 EDTA-Na, 20 pyruvate, 10 D-glucose, pH 7.4) Subsequently, cells were subjected to simulated reperfusion by reoxygenation for 24 h in serum-free DMEM in an atmosphere containing 5% CO_2/95% air at 37°C.

Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) enzyme activity was assayed using a commercial kit (Roche Molecular Biochemicals, Germany). The absorbance at 492 nm was measured on a spectrophotometer (UV-160A, Shimadzu, Japan). The reference wavelength was 690 nm. LDH enzyme release into the medium expressed an index of cell injury, defined as the percentage of total releasable LDH, yielded by lysis of H9c2 cells or cardiomyocytes in the presence of 0.1% Triton X-100.

Flow cytometric measurements of mitochondrial membrane potential ($\Delta\Psi_m$)

Changes in $\Delta\Psi_m$ were monitored by the uptake of the fluorescent dye JC-1. Cells were incubated with 2 μg/ml JC-1 for 10 minutes at 37°C in DMEM. Then, the cells were washed twice with ice-cold PBS, harvested by trypsinisation and analysed on a FACS Calibur instrument (Becton Dickinson, San Jose, CA, USA). The excitation wavelength was 488 nm, and the emission fluorescence for JC-1 was monitored at 530 nm (FL-1) and 617 nm (FL-2). The flow cytometry data were analysed using Cell Quest. Red fluorescence (FL-2) corresponds to the J-aggregate of JC-1 and represents mitochondria with high $\Delta\Psi_m$. Green fluorescence (FL-1) reflects the monomeric form of JC-1, appearing in the cytosol after mitochondrial membrane depolarisation. A minimum of 10,000 cells *per* sample were acquired and analysed with the Cell Quest software package.

Quantification of mRNA expression by RT-PCR

H9c2 cells were cultured until 70–80% confluent in culture medium. 24 h before the experiment, the medium was replaced with serum-free DMEM. Then, the cells were incubated with 100 μmol/l diazoxide or 100 μmol/l diazoxide plus 100 μmol/l 5-HD for 30 min and subjected to 3 h ischemia and 2 h reperfusion. Total RNA was isolated from those cells using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas). Semi-quantitative multiplex PCR was then performed using Cytoxpress, Quantitative PCR Detection Kit (BioSource International). Fragments of bax, bcl-2, caspase-3 and bcl-x_L genes were amplified. PCR products were resolved on 2% agarose

gel and visualized by ethidium bromide staining. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene fragment was used as an internal control.

Western blotting

Cells were washed twice with ice-cold PBS and then lysed in ice-cold lysis buffer (50 mmol/l Tris-HCl, pH 8.0; 137 mmol/l NaCl; 10% glycerol; 1% Nonidet P-40 (v/v); 1 mmol/l NaF; 10 μg/ml leupeptin; 2 mmol/l Na_3VO_4; 1 mmol/l phenylmethylsulfonyl fluoride) for 30 min at 4°C. The lysate was centrifuged at $14,000 \times g$ for 15 min at 4°C to remove cell debris. Proteins were boiled in Laemmli loading dye, and equal amounts of 30 μg of protein were resolved on 12% SDS-PAGE and electroblotted onto nitrocellulose membrane (Amersham Pharmacia Biotech, UK). Membranes were blocked for 2 hours at room temperature with 10% non-fat milk in TBS-T buffer (20 mmol/l Tris, 137 mmol/l NaCl, 0.1% Tween 20, pH 7.6) and incubated overnight with a specific primary antibody. After washing, the blots were incubated for 1 hour with a horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, UK) and viewed using an ECL detection system (Amersham Pharmacia Biotech, UK). Quantitative immunoreactivity was determined by densitometry using Molecular Imager FX (BIO-RAD).

Results

In the search for a cellular model to study the consequences of mitoK_{ATP} channel activation, the cardiac myoblast cell line H9c2 and neonatal rat ventricular cardiomyocytes were studied. Recently, we have shown that in the skeletal muscle cell line L6, potassium channel openers such as diazoxide and nicorandil caused stimulation of cellular oxygen consumption, implying mitochondrial effects of these drugs (Debska et al. 2001). Hence, in our studies we applied the cardiac myoblast cell line H9c2 to establish whether the potassium channel opener diazoxide is able to protect these cells against simulated ischemia/reperfusion injury. As shown in Figure 1A, incubation of H9c2 cells under the atmosphere of 95% argon and 5% CO_2 for 10 h followed by 2 hours of reoxygenation (for details see Materials and Methods) increased cell injury, measured as LDH release, from 14 ± 1% in untreated cells to 51 ± 4% after simulated ischemia/reperfusion. Application of 100 μmol/l diazoxide did not influence cell injury either in control (14 ± 1%) cells, or in cells that underwent the simulated ischemia/reperfusion protocol (48 ± 6%). The values obtained for cells treated additionally with 100 μmol/l 5-HD were also not significantly different from untreated controls, 12 ± 1% and 51 ± 2%, for control and simulated ischemia/reperfusion cells, respectively (Figure 1A). In Figure 1B simulated ischemia, simulated ischemia + 100 μmol/l diazoxide, simulated ischemia + 100 μmol/l diazoxide, 100 μmol/l 5-HD treatment are also shown not to influence mRNA expression level of apoptosis-related proteins in H9c2 cells. Cells were subjected to 3 h-simulated ischemia followed by 2 h-simulated reperfusion and the total RNA was isolated. Using multiplex RT-PCR, we studied the mRNA levels of

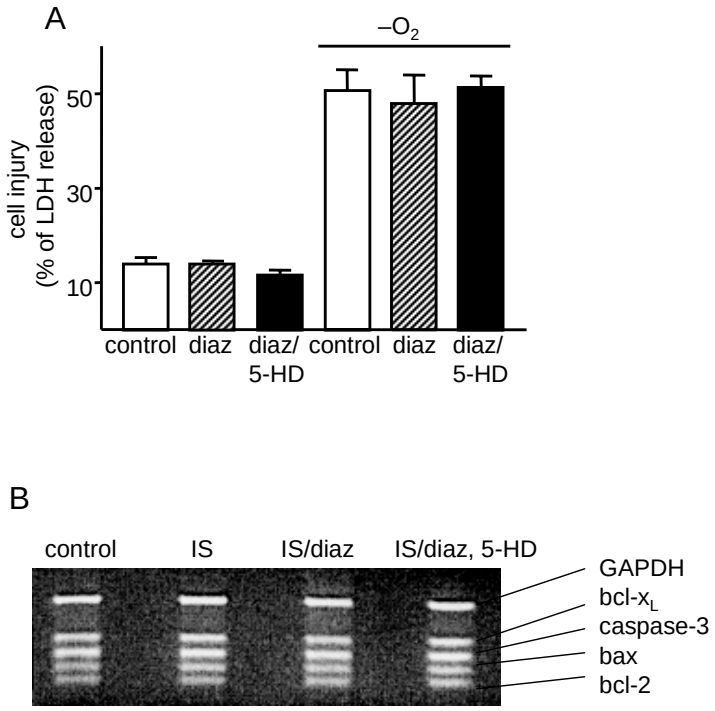


Figure 1. The effect of simulated ischemia/reperfusion on LDH release and mRNA level in H9c2 cells. **A.** Cell injury was estimated using LDH measurements. LDH activity was measured in the medium and cell lysates. Results are expressed as the percentage of total releasable LDH. Results are shown for: control, untreated cells; diaz, cells treated with 100 μmol/l diazoxide; diaz/5-HD, cells treated with 100 μmol/l diazoxide plus 100 μmol/l 5-HD; $-O_2$, indicates cell subjected to 10 h-simulated ischemia and 2 h-simulated reperfusion (as described in Materials and Methods). Experiments were performed in triplicate and the results are shown as mean ± S.E.M. **B.** mRNA expression of apoptosis-related proteins. Levels of bax, bcl-x_L, caspase-3 and bcl-2 mRNA were assessed as described in Methods. GAPDH gene fragment was used as an internal control. Fragments of the studied genes, amplified by RT-PCR, were resolved in 2% agarose gels and visualised by ethidium bromide staining. Control. untreated cells; IS, cells subjected to 3 h-simulated ischemia and 2 h-simulated reperfusion; IS/diaz, cells treated with 100 μmol/l diazoxide prior to simulated ischemia; IS/diaz, 5-HD, cells treated with 100 μmol/l diazoxide and 100 μmol/l 5-HD prior to simulated ischemia.

bax, bcl-2, bcl-x_L, and caspase-3. A fragment of constitutively expressed GAPDH was additionally amplified and used as an internal control (Figure 1B).

A similar ischemia/reperfusion protocol was applied to neonatal rat ventricular cardiomyocytes. Figure 2 shows the percentage of cell injury after 2 hours of simulated ischemia followed by 24 h of simulated reperfusion. These experiments were also performed in the presence of 100 μmol/l diazoxide and 100 μmol/l dia-

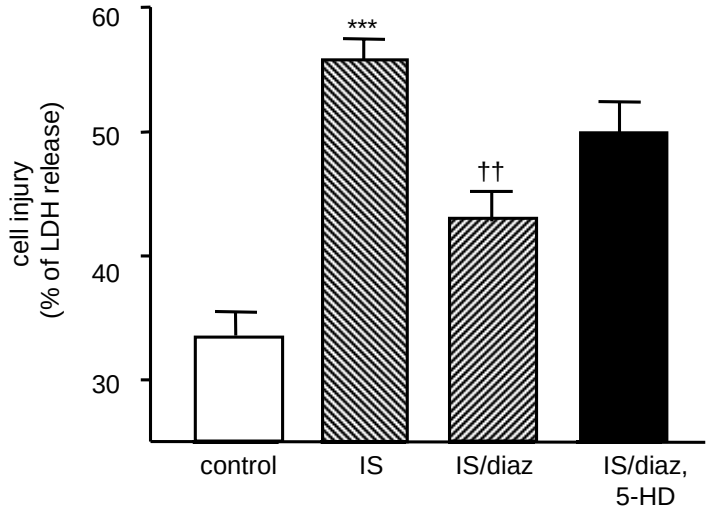
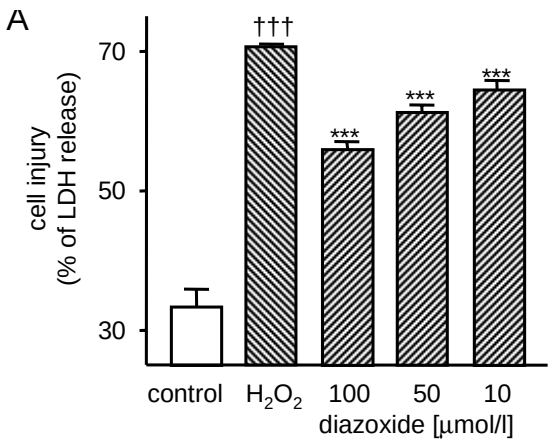


Figure 2. The effect of ischemia on rat neonatal cardiomyocytes. Cell injury was estimated using LDH measurements. LDH activity was measured in the medium and cell lysates. Results are expressed as a percentage of total releasable LDH. IS, the effect of 120 min simulated ischemia and of 24 h-simulated reperfusion on cell injury; IS/diaz, cells were incubated with 100 μmol/l diazoxide for 30 min prior to simulated ischemia; IS/diaz, 5-HD, cells incubated with 100 μmol/l diazoxide and 500 μmol/l 5-HD for 30 min prior to simulated ischemia; $^{***}p < 0.0001$ *vs.* control; $^{\dagger\dagger}p < 0.01$ *vs.* IS; control, untreated cells; diaz, cells treated with 100 μmol/l diazoxide, diaz/HD, cells treated with 100 μmol/l diazoxide and 500 μmol/l 5-HD. Experiments were performed in triplicate and the results are shown as mean ± S.E.M.

zoxide plus 500 μmol/l 5-HD. Cell injury was measured by estimating the level of LDH released from the cells. We show that simulated ischemia increased the percentage of damaged cells from $33 \pm 2\,\%$ in control to $56 \pm 2\,\%$ in cells incubated with the ischemic buffer. The level of injury of cells exposed to 100 μmol/l diazoxide from 15 min before inducing simulated ischemia until the end of ischemia decreased significantly to $43 \pm 2\,\%$. 500 μmol/l 5-HD applied together with diazoxide partly abolished diazoxide protection with cell injury at $50 \pm 3\,\%$ (Figure 2).

We have also studied injury of neonatal rat cardiomyocytes after exposure to oxidative stress induced by 16 h incubation with 100 μmol/l hydrogen peroxide, which is known to be a strong oxidant and an agent leading to hydroxyl radical production. In Figure 3A, we show that diazoxide protected cardiomyocytes against oxidative stress in a dose-dependent manner. Cell injury in control cells, as measured by LDH release, was equal to $33 \pm 3\,\%$. 100 μmol/l H_2O_2 increased this value to $70.7 \pm 0.3\,\%$. In the presence of 100 μmol/l diazoxide, LDH was released from $56 \pm 1\,\%$ cells. Incubation with 50 μmol/l and 10 μmol/l diazoxide gave the values of $62 \pm 1\,\%$ and $65 \pm 1\,\%$, respectively, which were still significantly different



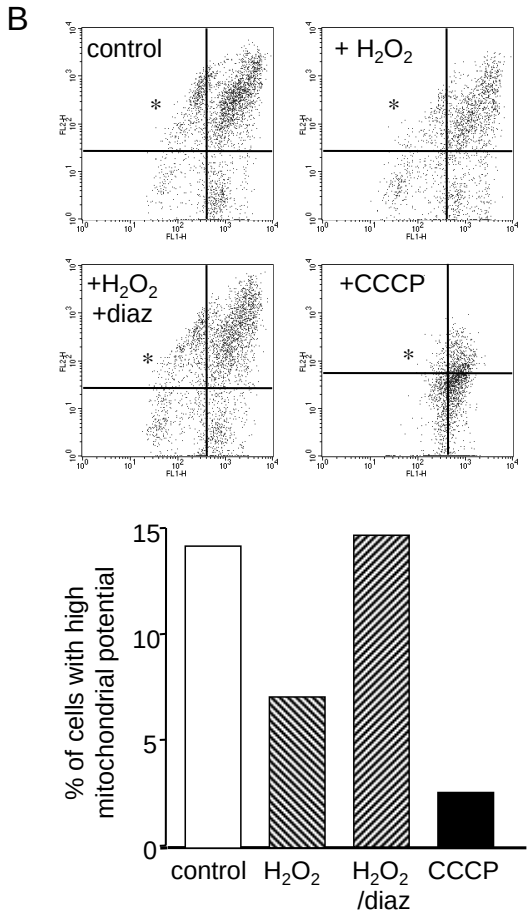


Figure 3. The effect of oxidative stress on neonatal rat cardiomyocytes. **A.** Cell injury measurements. Cardiac myocytes were incubated with 100 μmol/l H_2O_2 and diazoxide (as indicated) for 16 h. LDH activity was measured in the medium and cell lysates. Results are expressed as the percentage of total releasable LDH. $^{\dagger\dagger\dagger}p < 0.0001$ *vs.* control, $^{***}p < 0.0001$ *vs.* H_2O_2. Experiments were performed in triplicate and the results are shown as mean ± S.E.M. **B.** Measurements of mitochondrial potential. Mitochondrial potential was measured by flow cytometry. The cells were stained with JC-1 (2 μg/ml). High values of red fluorescence (upper left corner on panels, marked with $*$) indicate cells with high mitochondrial membrane potential. Control, control cardiomyocytes; $+H_2O_2$, cells incubated for 2 h with 200 μmol/l H_2O_2; $+H_2O_2$ + diaz, cells incubated for 2 h with 200 μmol/l H_2O_2 plus 100 μmol/l diazoxide; +CCCP, cells treated with the uncoupler, 1 mmol/l CCCP. The quantification of the results is shown on the lower panel. The result shown in the panel is representative of three independent experiments.

from the value obtained for cells treated with 100 μmol/l H_2O_2 alone. Figure 3B shows the results of mitochondrial membrane potential measurements in neonatal rat cardiomyocytes after exposure to oxidative stress. Cells were incubated with 200 μmol/l H_2O_2 for 2 h, loaded with the fluorescent mitochondrial potential indicator JC-1, and analysed using flow cytometry. We show that the percentage of cells with high mitochondrial potential (upper left corners of panels, marked with *) in control cells was 14.3%. In cells treated with H_2O_2, this value was reduced to 7.1%. 100 μmol/l diazoxide reversed the effect of oxidative stress and again 14.7% of cells had high mitochondrial membrane potential. As expected, only 2.6% of cells incubated for 10 min with the uncoupler, 1 μmol/l CCCP, had high mitochondrial membrane potential. The results shown in Figure 3 are representative of three independent experiments.

We have shown that diazoxide protected neonatal rat cardiomyocytes against injury caused by simulated ischemia and oxidative stress. Previous reports have shown that this protective action might be related to inhibition of apoptosis, and so we decided to study the influence of diazoxide on the expression of the anti-apoptotic protein bcl-2. Using Western blotting, we show (Figure 4A) that incubation of neonatal rat cardiomyocytes with 100 μmol/l diazoxide for 2, 3, 6, 8, 10, 16, 24 and 40 hours did not significantly change the level of bcl-2 protein present

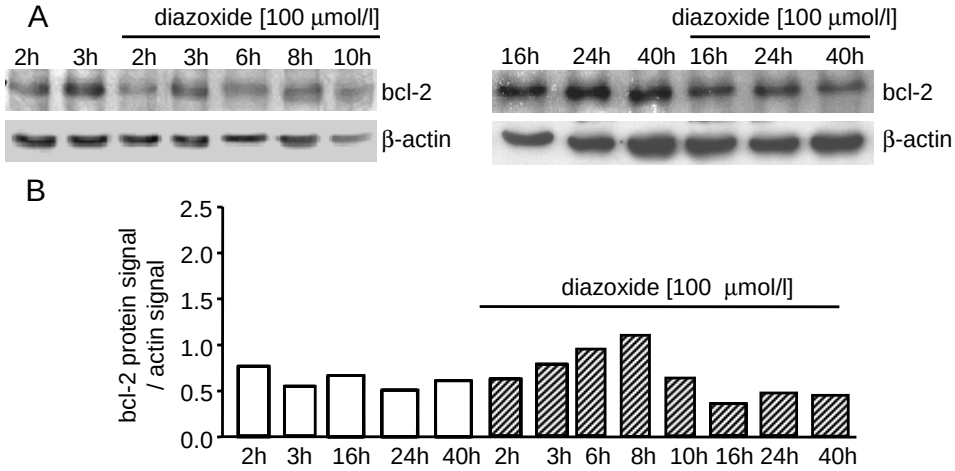


Figure 4. The effect of diazoxide on bcl-2 protein expression in neonatal rat cardiomyocytes. **A.** Cardiomyocytes were incubated with 100 μmol/l diazoxide for the times indicated. Cell lysates were collected and analysed by Western blot using antibodies against bcl-2 protein and β-actin (to ensure equal loading). The result shown in the panel is representative of three independent experiments. **B.** bcl-2 protein signal changes after cardiomyocyte treatment with 100 μmol/l diazoxide. Densitometric analysis of bcl-2 protein signal (normalised to β-actin signal) from Western blots shown in panel A. Untreated cells, white bars; diazoxide-treated cells, striped bars.

in the treated cells with respect to controls. Densitometric analysis of the result, normalized to the β-actin signal, is shown in Figure 4B.

Discussion

It is well established that activation of the mitoK_{ATP} channel in cardiac myocytes plays an important role in protection against cell injury (Liu et al. 1999; O'Rourke 2000). It is also believed that the mitoK_{ATP} channel is a key element in ischemic preconditioning (O'Rourke 2000). Since the discovery of the role of mitochondrial channel in cardioprotection (Garlid et al. 1997; Liu et al. 1998), the pharmacological properties of the mitoK_{ATP} channels have been clarified. Many questions, however, remain about the biochemical mechanisms leading from channel activation to cytoprotection. One of the possibilities is the involvement of the mitoK_{ATP} channel in regulating ischemia-induced cellular apoptosis. Recently, it has been reported that apoptosis may significantly contribute to irreversible myocyte injury during ischemia and reperfusion (Gottlieb et al. 1994; Fliss et al. 1996). The appearance of apoptotic cells in the peri-necrotic zone during reperfusion suggests that apoptosis may be important in the process of extending infraction over time, after the onset of reperfusion (Zhao et al. 2001).

In this study, we have used the cardiac myoblast cell line H9c2 and primary culture of rat ventricular cardiomyocytes. Recently, we have shown that activation of potassium transport upon application of KCOs in the rat skeletal muscle myoblast cell line L6 caused stimulation of cellular oxygen consumption, implying a mitochondrial target for KCOs (Debska et al. 2002). Moreover, the effect induced by diazoxide and nicorandil was blocked by the K_{ATP} channel blocker glibenclamide (Debska et al. 2002). Hence, we have explored the protective properties of diazoxide in the myoblast cell line H9c2 upon simulated ischemia/reperfusion. The myogenic cell line H9c2 was previously applied to study cell death mechanisms in response to oxidative stress (Neuss et al. 2001), hypoxia (Ekhterae et al. 1999), and overexpression of calreticulin (Kageyama et al. 2002). In our studies, no protective effect of diazoxide on cardiac myoblast H9c2 cells was observed. Moreover, diazoxide was not able to modulate the mRNA level of bax, bcl-x_L, caspase 3 and bcl-2 proteins. The above observations suggest that the mitoK_{ATP} channel is not present in the mitochondria of H9c2 cells. However, we have shown that the mitochondrial potassium channel opener diazoxide was able to protect neonatal rat ventricular cardiomyocytes against simulated ischemic/reperfusion injury. Moreover, it was able to protect neonatal cardiac cells against oxidative injury, similar as it was observed previously (Akao et al. 2001). At the same time, the protective action of diazoxide cannot be explained by its putative antioxidant properties, because its action is not mimicked by other antioxidants (Debska et al. 2001). Recently, similar results were obtained with another potassium channel opener – nicorandil (Akao et al. 2002). Protection against the simulated ischemic/reperfusion insult was suppressed by the potassium channel blocker 5-HD, confirming that activation of mitoK_{ATP} channel is a key event upon diazoxide application. Only partial effect of 5-HD is probably

due to low penetration of this drug through cell membranes, which diminishes its effective concentration in the cytosol.

Reperfusion of ischemic myocardium results in apoptotic cell death that can be blocked by adapting the heart to ischemic stress induced by cyclic episodes of brief ischemia and reperfusion, i.e., by ischemic preconditioning. It has been shown that ischemic preconditioning reduces (Maulik et al. 1999; Hattori et al. 2001). Studies using *in vivo* viral apoptosis in isolated rat heart by up-regulating bcl-2 protein and mRNA gene transfer of bcl-2 and transgenic mice overexpressing bcl-2 have also shown that this protein is efficient in blocking apoptosis and is able to preserve ventricular geometry and function after ischemia/reperfusion (Chen et al. 2001; Chatterjee et al. 2002). The use of antisense bcl-2 oligodeoxynucleotides reversed the protective effect of ischemic preconditioning by eliminating the antideath signal from bcl-2 (Hattori et al. 2001). Our experiments with neonatal cardiac myocytes, however, exclude that changes of bcl-2 protein expression are involved in diazoxide-induced cytoprotection.

In summary, the findings presented in this study provide functional data showing that diazoxide is able to protect neonatal cardiac myocytes against simulated ischemic/reperfusion and oxidative injury and suggest that activation of the mitochondrial ATP-regulated potassium channel plays an important role in protection of these cells against injury.

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