Changes in rat myocardium associated with modulation of ischemic tolerance by diazoxide

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Abstract. Pretreatment with diazoxide, mitochondrial K_{ATP} channel opener, was found to protect the rat heart against ischemia/reperfusion injury. Our aim was also to characterize the effects of diazoxide on the alterations of regulatory myocardial proteins, on mitochondrial ultrastructure, integrity and induction of apoptotic responses. Isolated rat hearts were Langendorff perfused and subjected to index ischemia (II) induced by 25 min global ischemia and 35 min reperfusion. In diazoxide-treated hearts, diazoxide (50 μmol/l) was applied 15 min before II. The levels and activation of specific proteins were determined using specific antibodies, activities of matrix metalloproteinases by zymography using gelatin as a substrate. The ultrastructure of mitochondria was investigated by electron microscopy of ultrathin sections of mitochondrial fractions embedded in Epon812. In rat hearts pretreated with diazoxide we found better recovery of contractile function after II. Electron microscopy studies revealed that application of diazoxide was connected with better preservation of mitochondrial integrity at basal conditions and after II in comparison to control hearts. Ischemia induced activation of caspase-3 as well as decrease of mitochondria-associated Bcl-2 levels but diazoxide treatment did not significantly influence these changes. On the other hand, diazoxide pretreatment reduced the cytosolic levels of pro-apoptotic Bax protein. Western blot analysis revealed that application of diazoxide increased activation of both ERK-1 and ERK-2 as compared with control hearts. ERK-2 activities were also higher in diazoxide-treated hearts after II when compared to control hearts. Moreover, application of diazoxide inhibited the activities of tissue matrix metalloproteinases (MMP-2). The results suggest that the cardioprotection mediated by diazoxide in rats is associated with preservation of mitochondrial integrity and function. The effect of diazoxide on ERK pathway points to the involvement of this signaling cascade in diazoxide-mediated adaptive responses of myocardium to ischemia.

Key words: Heart — Diazoxide — Cell signaling — Regulatory proteins

Introduction

One approach of myocardial protection against ischemic damage is based on the idea to use the own protective mechanisms of heart, activated by a mild stress including a short-term ischemia (ischemic preconditioning, IP), hypoxia (hypoxic preconditioning), free oxygen radicals, heat stress, etc. (Murry et al. 1986; Kloner and Jennigs 2001; Kolář and Ošťádal 2004). This form of cardioprotection may be induced also pharmacologically (pharmacological preconditioning) (Kukreja et al. 2005). The main goal is to increase ischemic tolerance, expressed during long-term ischemia as slowdown of necrotic processes in cardiac cells, improved restoration of its function following ischemic load and a decrease in lethal arrhythmias (Murry et al. 1986; Ma et al. 1999). Ischemic and/or pharmacological preconditioning before prolonged ischemic insult induces a state of protection against ischemia/reperfusion injury but the precise mechanisms of this cardioprotection are not clear as yet. It is assumed that protective mechanisms include activation of the cell signaling system consisting of membrane receptors, postreceptor signal routes and

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effector system including ion-transport mechanisms (Cohen et al. 2000).

One of the crucial final processes in cell signalization cascades, resulting in cardioprotective effect, is connected with activation of ATP-dependent potassium (K_{ATP}) channels and an important role in heart adaptation to acute ischemic and also chronic oxidative stress is assumed for K_{ATP} channel activation in cardiac mitochondria (Fryer et al. 2001; Garlid et al. 2003). Diazoxide, opener of mitochondrial K_{ATP} channels, is cardioprotective drug which pharmacologically mimics IP. The underlying mechanisms of cardioprotection by diazoxide are still unclear but a dependence from activation of some protein kinases (PKC, MAPKs) (Cohen et al. 2000; Tsukamoto et al. 2005) and increased NO production in the heart (Oldenburg et al. 2004) has been suggested. It was found that signal routes mediated by some mitogen-activated protein kinases (MAPKs) play important but different role in the modulation of the course of ischemic damage by IP (Ma et al. 1999; Strohm et al. 2000; Strnisková et al. 2002). MAPKs represent family of serine/threonine protein kinases that regulate a wide range of cellular processes. Three major classes of MAPKs have been identified: extracellular signal-regulated kinases (ERKs), c-Jun-N-terminal kinases (JNKs) and p38-MAPK (Robinson and Cobb 1997). Intracellular signaling mechanisms mediated by reactive oxygen species, MAPKs, as well as several growth and transcription factors involve also regulation of expression and activities of matrix metalloproteinases (MMPs) (Siwik and Colucci 2004; Zhang et al. 2004). MMPs are essential for extracellular matrix turnover and their activation facilitates angiogenesis and development of cardiac fibrosis through breakdown of the extracellular matrix (Burbridge et al. 2002; Siwik and Colucci 2004). Moreover, suggested was also role of MMPs, especially MMP-2, in infarct size-limiting effect of IP and pharmacological inhibition of MMP-2 produced cardioprotection (Giricz et al. 2006).

In the present study, the efect of diazoxide on heart function (contractility) during ischemia/reperfusion and on the activities of MAPKs as well as MMPs was examined. Also the effects of diazoxide treatment on integrity of mitochondria and levels and/or activation of proteins involved in regulation of apoptosis were determined.

Materials and Methods

Experimental model

In the study, adult male Wistar rats (250–280 g body weight) were used. All animals had free access to water and a standard laboratory diet. The study was performed in accordance with Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Perfusion technique

The animals were sacrificed by cervical dislocation, hearts were rapidly excised, placed in ice-cold perfusion buffer, cannulated via the aorta and placed into the Langendorff system for perfusion at a constant pressure of 70 mm Hg and at 37°C. Perfusion solution was a modified Krebs–Henseleit buffer gassed with 95% O_2 and 5% CO_2 (pH 7.4) containing (in mmol/l): NaCl 118.0, KCl 3.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.18, $CaCl_2$ 2.5, glucose 7.0. Solution was filtered through a 5 μm porosity filter (Millipore) to remove contaminants. An epicardial electrogram was registered by means of two stainless steel electrodes attached to the the aortic cannula and the apex of the heart and continously recorded (Mingograph Elema-Siemens, Solna, Sweeden). Heart rate was calculated from the epicardial electrogram. After recording the baseline values, the hearts were electrically stimulated at 300 beats/min throughout the remainder of the protocol. Left ventricular pressure was measured by means of a latex water-filled balloon inserted into the left ventricle *via* the left atrium (adjusted to obtain end-diastolic pressure of 5–7 mm Hg) and connected to a pressure transducer (P23 Db model, Gould Statham Instruments, USA). Left ventricular developed pressure (LVDP, systolic minus diastolic pressure), maximal rates of pressure development and fall, $+(dP/dt)_{max}$ and $-(dP/dt)_{max}$, as the indexes of contraction and relaxation were monitored during stabilization and preischemia period for the evaluation of the effect of diazoxide on the haemodynamic parameters and were continuously recorded until the end of reperfusion. Recovery of contractile function after ischemia/reperfusion was expressed as percentage of preischemic baseline values.

Experimental protocols

The Langendorff-perfused hearts were allowed to stabilize (20 min) before further interventions. The hearts were then exposed to 25 min global ischemia induced by stop of aortal inflow and 35 min reperfusion (test ischemia). The protocol of IP included two cycles of ischemia/reperfusion (5 min each), prior to test ischemia. In drug studies, diazoxide dissolved in DMSO (50 μmol/l) was applied 15 min before test ischemia.

Isolation of mitochondria and preparation of protein fractions

The heart (left ventricle) was put into cold isolation medium A containing (in mmol/l): 20 Tris-HCl, 180 KCl, 4.0 EDTA (pH 7.4). Tissue was homogenized with pestle homogenizer

(1 g of heart/10 ml of solution) and to improve the homogenization of tissue was added pre-purified sand into the homogenization mixture. After homogenization, to remove sand, the homogenates were filtered through gauze and centrifuged at 1900 × *g* for 5 min at 4°C. After this first centrifugation, the pellets were discarded and supernatants were centrifuged again at 6900 × *g* for 10 min. To obtain mitochondria, pellets from the second centrifugation were resuspended in buffer A and centrifuged again at 10,000 × *g* for 10 min. Sediments after this centrifugation contained intact mitochondria and were resuspended in 500 μl of 50 mmol/l Tris-HCl containing 180 mmol/l KCl, pH 7.4. The part of fresh suspension of mitochondria was used immedially for preparation of samples for electron microscopic examinations, and part for preparation of samples of mitochondrial fraction for electrophoretical and immunochemical examinations.

The postmitochondrial supernatants after the last centrifugation were centrifuged again for additional 30 min at 13,600 × *g* and the resulting supernatants were termed and used as soluble fractions. The pellets from this centrifugation were resuspended in buffer A containing 0.2% Triton X-100 and represented the particulate fractions. The protein concentrations were estimated by the method of Bradford (1976).

Electron microscopic examination

For electron microscopy examinations, the isolated suspensions of mitochondria were used. They were centrifuged (6900 × *g*, 10 min) and pellets were fixed by addition of 2.5% glutaraldehyde in 0.1 mol/l sodium cacodylate (pH 7.2) for 2 h. After postfixation in sodium cacodylate-buffered 1% osmium tetroxide, the samples were dehydrated in series of ethanol and cleared with propylenoxide. After infiltration and embedding into Epon812, ultrathin sections were cut with a ultramicrotome, stained with uranyl acetate and lead phosphate and examined in the transmision electron microscope Tesla BS 500.

Electrophoresis and immunochemical Western blot analysis

Samples of soluble or particulate fractions containing equivalent amounts of ventricular proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Total contents or activities of some regulatory proteins were determined by Western blot analysis using specific antibodies. For Western blot assays, proteins after electrophoretic separation were transferred to nitrocellulose membrane. The protein loading and quality of transfer was controlled by Ponceau S staining of nitrocellulose membranes after transfer. Specific anti-p38-MAPK, anti-ERKs, anti-JNKs (all from Santa Cruz Biotechnology), anti-phospho-p38-MAPK, anti-phospho-ERKs, anti-phospho-JNKs, anti-caspase-3, anti-cleaved caspase-3, anti-Bax, anti-Bcl-2 (all from Cell Signaling) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, from Chemicon International) antibodies were used for primary immunodetection. Peroxidase-labelled anti-rabbit or anti-mouse imunoglobulins (Amersham Biosciences) were used as the secondary antibodies. Bound antibodies were detected by the enhanced chemiluminiscence method.

Measurement of MMPs activites by gelatin zymography

The gelatinolytic activities of MMPs were determined by the modified method of Schonbeck et al. (1997). Laemmli buffer without 2-mercaptoethanol in proportion 1 : 1 was added to protein samples and non-heated samples were subjected to electrophoresis on 10% SDS-polyacrylamide gels co-polymerized with gelatin (2 mg/ml). After electrophoresis, gels were washed twice for 20 min with 50 mmol/l Tris-HCl (pH 7.4), containing 2.5% Triton X-100, at 25°C. After washing, the gels were incubated overnight at 37°C in substrate buffer containing 50 mmol/l Tris-HCl, 10 mmol/l $CaCl_2$ and 1.25% Triton X-100, pH 7.4. After this incubation, the gels were stained with 1% Coomassie Brilliant Blue G-250, resolved in mixture of methanol : acetic acid : water (4 : 1 : 5) for 2 h at room temperature and then destained with 40% methanol and 10% acetic acid. Gelatinolytic activities of MMPs (MMP-2) were detected as transparent bands against dark blue background.

Statistical evaluation

Quantification of protein levels was done using Phosphorimager Thyphoon (Amersham Biosciences). Data were expressed as means ± S.E.M. Statistical significance of differences between the groups was analysed by the unpaired Student's *t*-test. Differences were considered as significant at $p < 0.05$.

Results

Effect of diazoxide on postischemic recovery of contractile function in the rat heart

The changes in LVDP during postischemic reperfusion in the control hearts and diazoxide-treated hearts are shown in Fig. 1A. Treatment with 50 μmol/l diazoxide significantly improved recovery of LVDP during whole reperfusion in comparison with its values in the control hearts. Fig. 1B shows maximal recovery of contraction after ischemia/reperfusion in control hearts, after diazoxide treatment and after ischemia/reperfusion procedure. Diazoxide treatment significantly improved recovery of

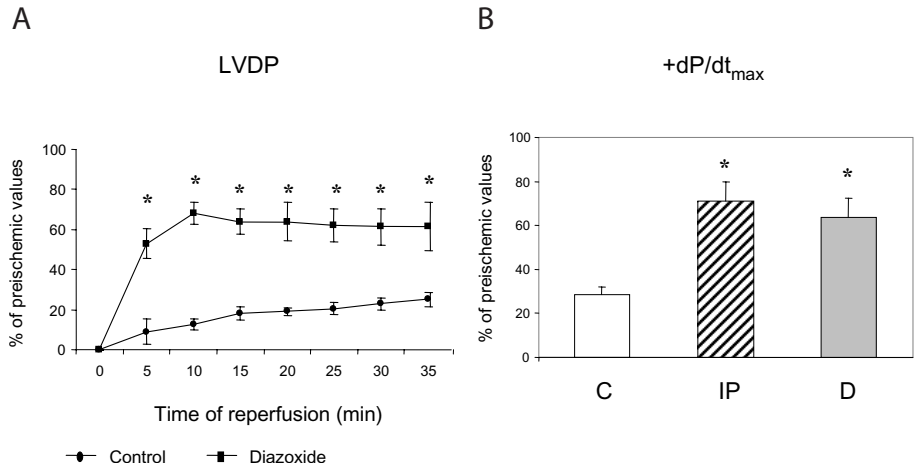

Figure 1. A. Effect of diazoxide on postischemic changes of left ventricular developed pressure (LVDP) in the rat hearts. **B.** Effect of diazoxide and ischemic preconditioning (IP, 4–6 animals *per* group) on the recovery of maximal rate of contraction (+d*P*/dt$_{max}$) after myocardial ischemia and reperfusion. Data are means ± SEM expressed in % of baseline values. C, control rat hearts, D, diazoxide-treated rat hearts, * $p < 0.05$ *vs.* control.

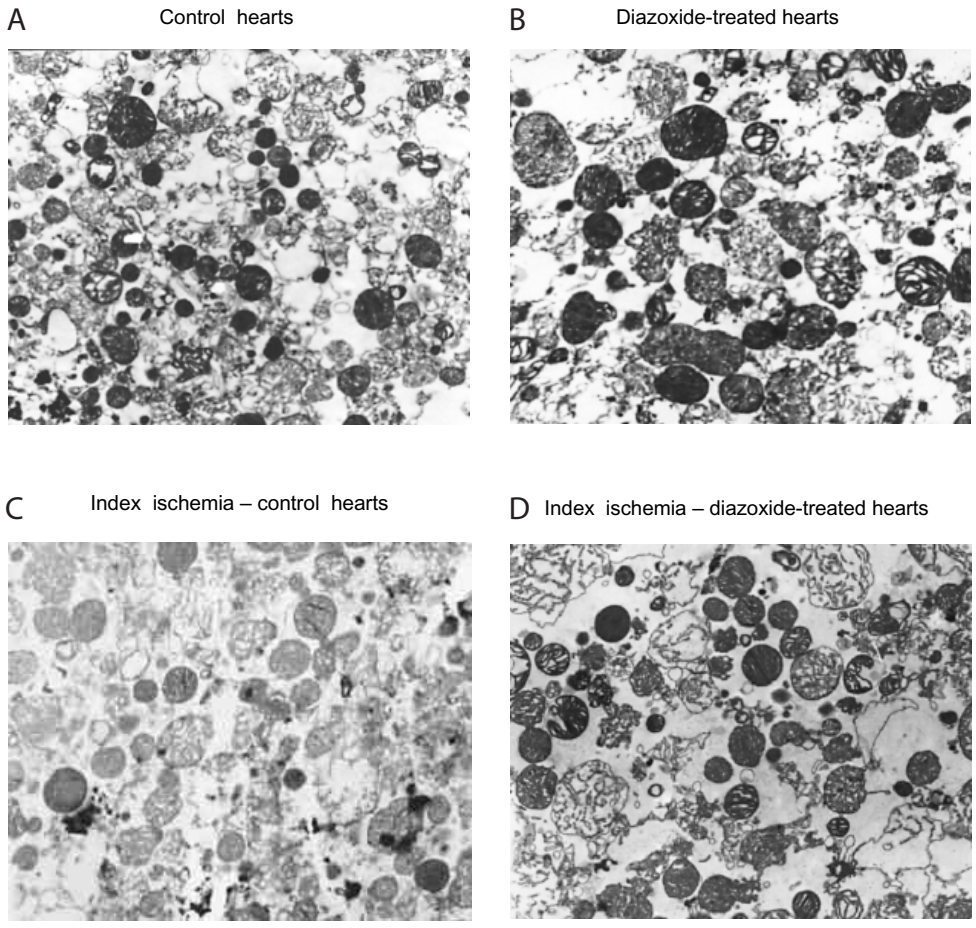

Figure 2. Electronograms of ultrastructure of mitochondria in control (A, C) and diazoxide-treated (B, D) rat hearts at basal conditions (A, B) and after ischemia (C, D). Note higher number of well preserved electron-dense mitochondria in diazoxide-treated ischemic rat hearts. Magnification ×6000.

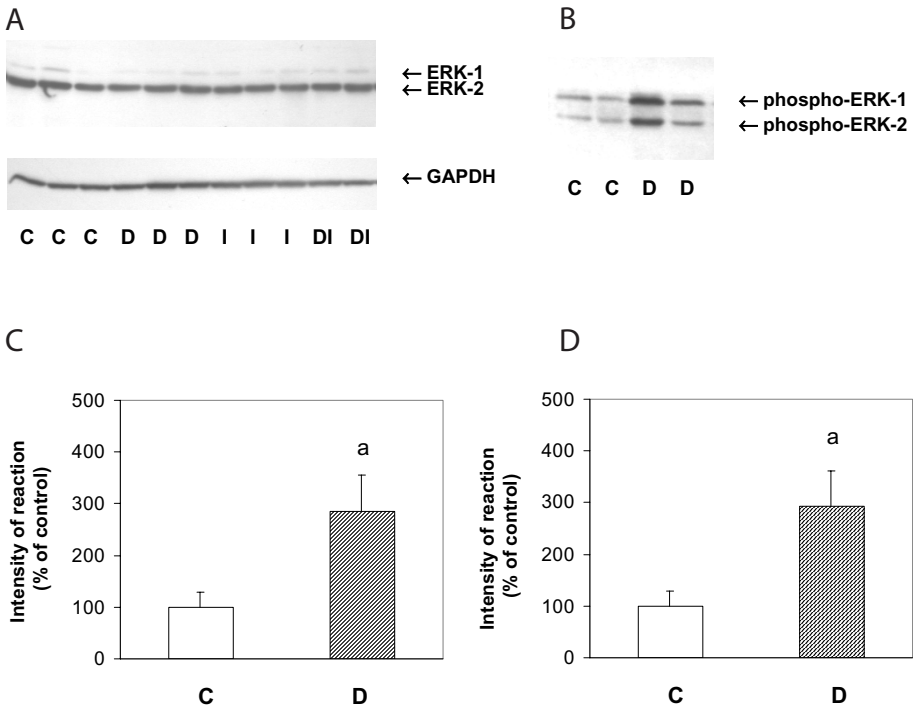

Figure 3. Effect of diazoxide treatment on protein levels and activation of extracellular signal-regulated protein kinases (ERKs). **A.** Upper blot record shows protein levels of ERKs in protein fractions isolated from the left ventricular tissue of control (C) and diazoxide-treated (D) rat hearts at the basal conditions (before ischemia) and after test ischemia (I). The ERKs levels were determined using specific antibody. Blot in lower part shows the protein levels of GAPDH. **B.** The changes in specific phosphorylation of ERKs in soluble fractions isolated from left ventricular tissue of control (C) and diazoxide-treated (D) rat hearts. The activation of ERKs was determined using phospho-specific antibody (Thr202/Tyr204). The arrows on the right show the position of these enzymes. **C.** Quantification of phospho-ERK-1 content in the left ventricular tissue after diazoxide treatment. Data are expressed as a percentage of values for control tissue. Each bar represents mean ± S.E.M. of 5 tissue samples *per* group. **D.** Quantification of phospho-ERK-2 content in the left ventricular tissue after diazoxide treatment. Data are expressed as a percentage of values for control tissue. Each bar represents mean ± S.E.M. of 5 tissue samples *per* group. [a] $p < 0.05$ *vs.* control group; DI, test ischemia after diazoxide treatment.

$+dP/dt_{max}$ after ischemia and reperfusion in comparison with control hearts. The effect of diazoxide treatment on recovery of contractile function after ischemia/reperfusion was similar to IP (Fig. 1B).

Influence of diazoxide on ultrastructure of mitochondria

Electron microscopic examination revealed higher number of larger electron dense mitochondria exhibiting normal appearance in isolated fraction from diazoxide-treated rat hearts (Fig. 2B) when compared to controls (Fig. 2A). Ischemia resulted in mitochondrial injury characterized by swollen electron lucent mitochondria with ruptured inner (cristae) and/or outer membrane of majority mitochondria (Fig. 2C). On the contrary, numerous mitochondria in the fraction isolated from diazoxide-treated rat hearts submitted to ischemia exhibited preserved ultrastructure and integrity of cristae (Fig. 2D).

Influence of diazoxide treatment on ERKs

Analysis with the antibody specific for ERKs revealed no differences in the levels of ERK-1 and ERK-2 between the control and diazoxide-treated hearts (Fig. 3A). Also ischemia did not influence the protein level of these kinases. On the other hand, using antibody that reacts specifically with dual phosphorylated ERKs (Thr202/Tyr204) we found that application of diazoxide increased activation of ERKs as compared with control (untreated) hearts (Fig. 3B). The phosphorylation of ERK-1 and activation of ERK-2 were increased about 2.9 times (Fig. 3C,D). Increased activation (phosphorylation) of ERK-2 was found also at the end of index ischemia and diazoxide treatment further increased this activation (Fig. 4A,C). For ERK-1, the phosphorylation was also partially increased but without siginicant changes (Fig. 4A,B).

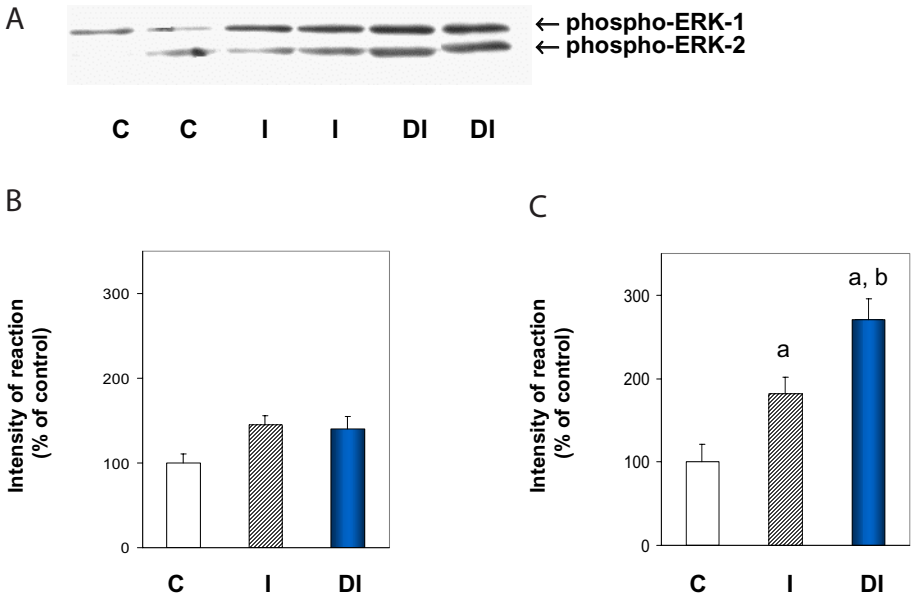

Figure 4. Effect of ischemia and diazoxide treatment on activation of extracellular signal-regulated protein kinases (ERKs). **A.** The changes in specific phosphorylation of ERKs in soluble fractions isolated from left ventricular tissue of control (C), after ischemia (I) and from diazoxide-treated rat hearts after ischemia (DI). The arrows on the right show the position of phosphorylated ERKs. **B.** Quantification of phospho-ERK-1 content in the left ventricular tissue after ischemia and diazoxide treatment. Data are expressed as a percentage of values for control tissue. Each bar represents mean ± S.E.M. of 5 tissue samples *per* group. **C.** Quantification of phospho-ERK-2 content in the left ventricular tissue after ischemia and diazoxide treatment. Data are expressed as a percentage of values for control tissue. Each bar represents mean ± S.E.M. of 5 tissue samples *per* group. [a] $p < 0.05$ *vs.* control; [b] $p < 0.05$ *vs.* ischemia.

Influence of diazoxide treatment on p38-MAPK and JNKs pathways

Analysis with antibody specific for p38-MAPK showed that there were not significantly influenced the levels of cytosolic p38-MAPK in hearts pretreated with diazoxide (Fig. 5A). We also did not observe changes in phosphorylation of p38-MAPK on Thr180/Tyr182. Specific phosphorylation at these sites reflects the activation of p38-MAPK (Fig. 5B). For cascade of JNKs, we did not observe changes in content of JNKs after diazoxide treatment (Fig. 5C). Analysis with phospho-specific antibody also did not show activation of these kinases during ischemia or after diazoxide treatment.

Influence of diazoxide treatment on levels and activation of proteins involved in regulation of apoptosis

Caspase-3 is a key executioner of apoptosis. We did not find significant differences in the levels of its inactive zymogen between control and diazoxide-treated hearts. Also ischemia did not influence the levels of inactive caspase-3. Activation of caspase-3 requires proteolytic processing of its inactive zymogen into activated p17 and p12 fragments. Levels of large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage adjacent to Asp175 were determined using specific antibody. By comparison of control and diazoxide-treated hearts we did not found changes in content of the cleaved caspase-3 at basal conditions (Fig. 6A). Ischemia induced increased activation of caspase-3 but diazoxide treatment did not significantly influence these ischemia-induced changes.

Bcl-2 has been demonstrated to exert a survival function in response to a wide range of apoptotic stimuli through inhibition of mitochondrial cytochrome c release. In soluble fractions, no significant differences were observed in levels of Bcl-2 (Fig. 6B) but during ischemia, the levels of mitochondria-associated Bcl-2 protein were considerably decreased (Fig. 6C). However, diazoxide treatment did not significantly influence these ischemia-induced changes. On the other hand, pretreatment with diazoxide reduced the cytosolic levels of pro-apoptotic Bax protein (Fig. 7A,C). By the study of mitochondria-associated Bax protein, the comparison of control and diazoxide-treated heart samples did not show any significant changes (Fig. 7B,D).

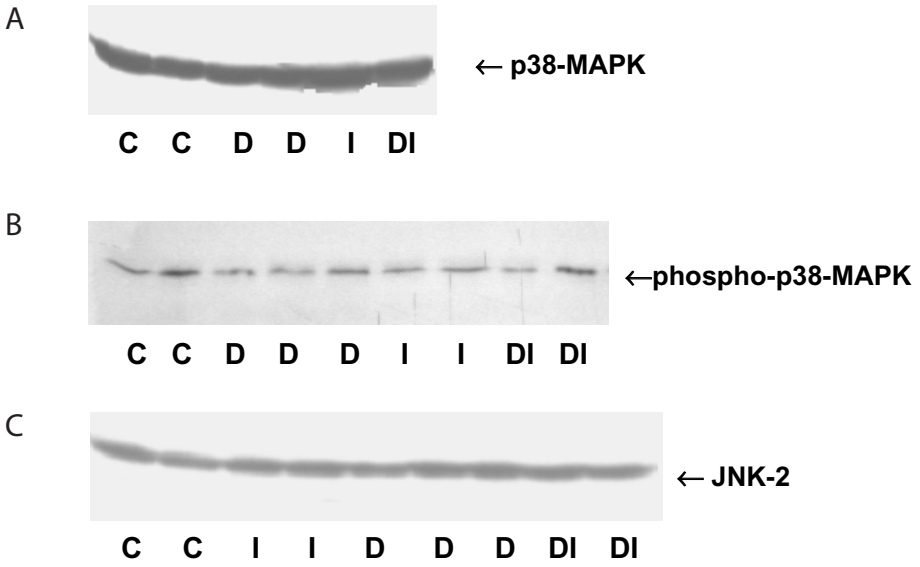

Figure 5. Effect of ischemia and diazoxide treatment on protein levels and activation of p38-MAPK and c-Jun-N-terminal protein kinases (JNKs). **A.** The record shows protein levels of p38-MAPK in protein fractions isolated from the left ventricular tissue of control (C) and diazoxide-treated (D) rat hearts at the basal conditions (before ischemia) and after test ischemia (I). The p38-MAPK levels were determined using specific antibody and the arrow on the right shows the position of this enzyme. **B.** The changes in specific phosphorylation of p38-MAPK in soluble fractions isolated from left ventricular tissue of control (C) and diazoxide-treated (D) rat hearts. The activation of p38-MAPK was determined using phospho-specific antibody (Thr180/Tyr182). **C.** Protein levels of JNK-2 in protein fractions isolated from the left ventricular tissue of control (C) and diazoxide-treated (D) rat hearts at the basal conditions (before ischemia) and after test ischemia (I). The JNKs levels were determined using specific antibody and the arrow on the right shows the position of this enzyme. DI, test ischemia after diazoxide treatment.

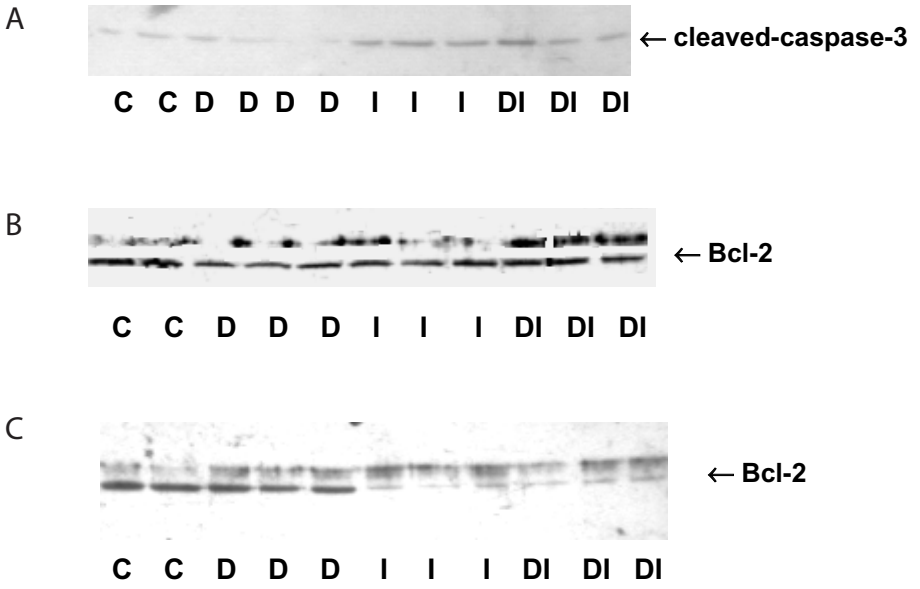

Figure 6. Influence of ischemia and diazoxide treatment on levels and activation of proteins involved in regulation of apoptosis. **A.** The record shows the effect of ischemia and diazoxide on activation of caspase-3. Levels of large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage adjacent to Asp175 was determined using specific antibody. The arrow on the right shows the position of this fragment. Effect of ischemia and diazoxide on protein levels of (**B**) soluble (cytosolic) and (**C**) mitochondria-associated Bcl-2 protein. C, control rat hearts; D, diazoxide-treated rat hearts; I, test ischemia; DI, test ischemia after diazoxide treatment.

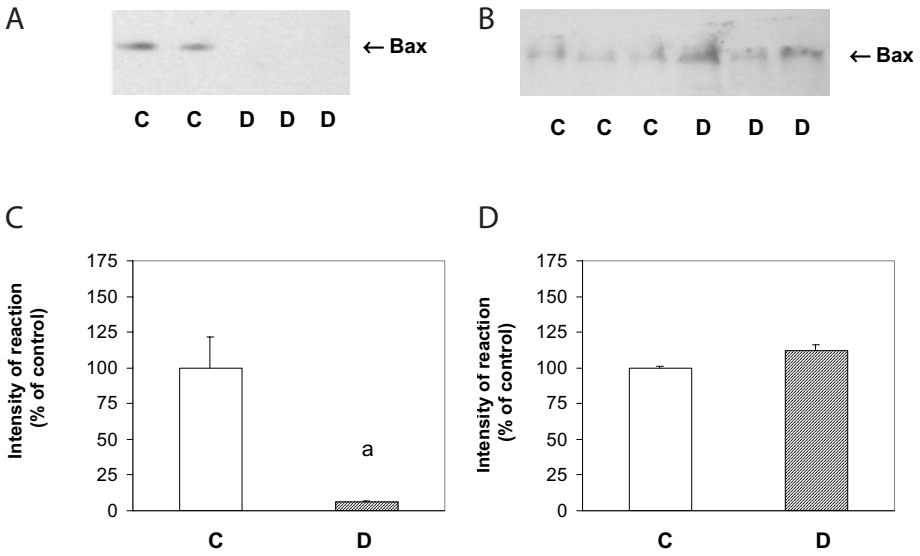

Figure 7. Influence of diazoxide treatment on protein levels of (**A**) soluble (cytosolic) and (**B**) mitochondria-associated pro-apoptotic Bax protein. **C**. Quantification of Bax content in soluble fractions. Data are expressed as a percentage of values for control tissue. Each bar represents mean ± S.E.M. of 4 tissue samples *per* group. **D**. Quantification of mitochondria-associated Bax protein. Data are expressed as a percentage of values for control tissue. Each bar represents mean ± S.E.M. of 4 samples *per* group. [a] $p < 0.05$ *vs.* control group.

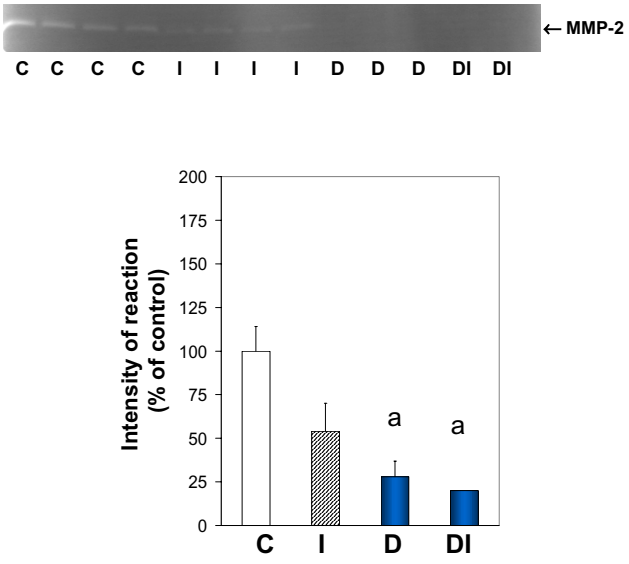

Figure 8. Effect of ischemia and diazoxide treatment on activities of matrix metalloproteinases (MMPs). Upper graph: the activities of MMPs were analyzed by zymography in 10% polyacrylamide gels containing 2% gelatine as a substrate. The arrow on the right shows the gelatinolytic activity in the range of 63 kDa (MMP-2). Lower graph: quantitative analysis of MMP-2 activities. Data are expressed as a percentage of values for control tissue. Each bar represents mean ± S.E.M. of 5 samples *per* group. [a] $p < 0.05$ *vs.* control group. C, control rat hearts; D, diazoxide-treated rat hearts; I, test ischemia; DI, test ischemia after diazoxide treatment.

Influence of diazoxide on MMPs

Enzymatic gelatinolytic activity in the range of approximatelly 63 kDa that corresponds to molecular weight of MMP-2 was significantly decreased in left ventricles of diazoxide-treated hearts as compared to control untreated hearts (Fig. 8). The decreased activity after diazoxide treatment was observed at basal conditions (before ischemia) as well as after ischemia.

Discussion

In the present study, we showed that the mitochondrial K_{ATP} channels opener, diazoxide, recovered the contractile function after ischemia/reperfusion similarly to IP and cardioprotective effects induced by diazoxide were connected with activation of anti-apoptotic cascade of ERKs. The results show that ERKs activation after application of diazoxide may be important in preventing cell injury in the ischemic myocardium. We did not observe the effects of diazoxide on JNKs or p38-MAPK pathways and so the effect of diazoxide is with regard to MAPKs cascades selective to ERKs and not shared by p38-MAPK or JNKs cascades. The present study also added evidence that application of diazoxide inhibited the activities of MMP-2.

The application of diazoxide was connected with better preservation of mitochondrial integrity at basal conditions

and after ischemia in comparison to control rat hearts. This finding is in agreement with observation that opening of these channels decreases mitochondrial calcium overload and this is connected with preservation of mitochondrial integrity in adult rabbit ventricular cardiomyocytes (Murata et al. 2001). It was also suggested that activation of mitochondrial K_{ATP} channels preserves mitochondrial function and the overproduction of cytotoxic reactive oxygen species (ROS) at the time of reperfusion (Ozcan et al. 2002). The cardioprotective effects elicited by diazoxide have been in several studies attributed to ROS formation (Forbes et al. 2001; Yue et al. 2002). Mitochondrial K_{ATP} channels can be a signal transduction element and may play a role in the production of ROS, which act as a signaling molecule. It was reported that increased production of oxidative stress by opening of mitochondrial K_{ATP} channels activates p38-MAPK and triggers the infarct size-limiting effect of IP (Yue et al. 2002). In non-cardiac THP-1 cells also ROS-dependent activation of ERKs by diazoxide has been demonstrated (Samavati et al. 2002). There exists possibility that opening of mitochondrial K_{ATP} channels directly influences the observed activation of ERKs also in our experimental system, and that the ERK-mediated phosphorylation of some specific target proteins contributes to diazoxide-induced cardioprotection. We have shown previously that ERKs signaling pathway is involved in cardioprotection of IP (Strohm et al. 2000) and increased activities of ERKs has been observed in diabetic myocardium at the acute stage of the disease, when these hearts became more resistant to necrotic changes in myocardium elicited by ischemia (Strnisková et al. 2003). Also other studies have assigned a role for the ERKs signaling system in cell survival processes during ischemia/reperfusion and ERKs were found to play a role by decreasing both the extent of necrotic damage (infarct size) and the level of apoptotic damage of cardiomyocytes (Strohm et al. 2000, 2002; Yue et al. 2000). Apoptosis plays a key role in ischemia/reperfusion injury, mitochondria are the main structures influenced during the ischemia-induced myocardial damage and apoptotic signals are integrated at the mitochondrial level. It has been demonstrated that also diazoxide may play a regulatory role in apoptotic cell death (Takashi et al. 1999). The release of cytochrome-c from mitochondria to the cytosol leads to the induction of apoptotic processes by activation of some intracellular proteases, particularly caspases (Blatt and Glick 2001). An important role in apoptosis is represented by Bcl-2 family of proteins. These proteins either inhibit apoptosis (Bcl-2) or promote the apoptotic processes (Bax, Bad). Their activities could be influenced also by phosphorylation mediated by protein kinase cascades such as ERKs and PI3K/Akt (Datta et al. 1997; Scheid et al. 1999). We found that ischemia/reperfusion stimulated the caspase-mediated apoptosis. This was connected with activation of caspase-3 as well as decrease in content of membrane-associated anti-apoptotic Bcl-2 protein. However, in our study (experimental model) we did not observe clear relationship between cardioprotective effects of diazoxide, activation of ERKs and modulation of ischemia-induced apoptosis. Application of diazoxide before ischemia did not influence ischemia-induced activation of caspase-3 and changes in Bcl-2 protein. On the other hand, treatment with diazoxide significantly reduced the cytosolic levels of pro-apoptotic Bax protein. However, we did not found corresponding changes (increase) in levels of mitochondria-associated protein. Further studies are necessary to clarify these changes and helpful could be the investigation of formation of complexes of Bcl-2/Bax proteins.

The actions of diazoxide were connected with upregulation of ERKs cascade as well as inhibition of matrix metalloproteinases (MMP-2). The enzyme system of MMPs may play an important role in realization of diazoxide action. Lalu et al. (2002) demonstrated that preconditioning inhibits ischemia-induced activation and release of MMP-2. The results suggested that preconditionig may exert part of its cardioprotective effects through reduction of MMP-2 release. In another study it was shown that administration of MMPs inhibitors attenuated the peroxynitrite-induced injury of myocardium (Wang et al. 2002). Morevover, a link between ERKs pathway and regulation of MMP-2 activities was demonstrated in the coronary endothelial cells (Donnini et al. 2004) as the induction of ERK-1 and ERK-2 phosphorylation resulted in MMP-2 upregulation and activation.

In summary, we have demonstrated that diazoxide pretreatment improved recovery of contractile function and preserved mitochondrial integrity after ischemia/reperfusion. The actions of diazoxide were connected with upregulation of ERKs cascade, inhibition of MMPs (MMP-2) and modulation of apoptotic Bax protein.

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