Stimulation of Mitochondrial ATP Synthase Activity – a New Diazoxide-Mediated Mechanism of Cardioprotection

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Summary

Pharmacological preconditioning by diazoxide and a model of experimental streptozotocin-induced acute diabetes mellitus (STZ-DM) provided similar levels of cardioprotection assessed as limiting myocardial infarct size. The aim was to explore the possibility of existence of another *in vitro* mechanism, which could be contributory to cardioprotection mediated by diazoxide treatment. Mitochondrial membrane fluidity and ATP synthase activity in isolated heart mitochondria were determined under the influence of two factors, STZ-DM condition and treatment with diazoxide. Both factors independently increased the ATP synthase activity (p<0.05), as no interaction effect was observed upon the combination of STZ-DM with diazoxide. On the other hand, the mitochondrial membrane fluidity was significantly increased by STZ-DM only; no significant main effect for diazoxide was found. Based on the results from measurements of enzyme kinetics, we assume a direct interaction of diazoxide with the molecule of ATP synthase stimulated its activity by noncompetitive activation. Our present work revealed, for the first time, that cardioprotection induced by diazoxide may not be caused exclusively by mitochondrial K_{ATP} opening, but presumably also by a direct interaction of diazoxide with ATP synthase, although the mechanisms for achieving this activation cannot be fully delineated.

Key words

Diazoxide • Mitochondrial ATP synthase • Mitochondrial membrane fluidity • Adaptation • Experimental diabetes mellitus

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Introduction

Several phenomena have been identified that are effective in providing a powerful cardioprotection, such as ischemic preconditioning (IPC) (Murry *et al.* 1986), ischemic postconditioning (Zhao *et al.* 2003), remote ischemic conditioning (Przyklenk *et al.* 1993) and pharmacological preconditioning (Piot *et al.* 2008, Galano *et al.* 2011).

Myocardial adaptability to pathological conditions as well as a key role of mitochondria in the process of cardioprotection have been frequently reported. Despite some gaps and uncertainties in the findings by the studies focusing on adaptation mechanisms at the molecular level, the involvement of signaling pathways mediated by opening of mitochondrial permeability transition pores (mPTP) (Kristian *et al.* 2001), protein kinase C (PKC) activation (Ytrehus and Downey 1994), free oxygen species, or by the mitochondrial ATP-dependent K^+ channels (K_{ATP}) opening (Liu *et al.* 1998) is widely accepted. Activation of these essential mechanisms by cardioprotective phenomena leads to a decreased susceptibility of the heart to ischemia/reperfusion injury (I/R) manifesting in better preservation of contractile function and in infarct

size reduction (Ravingerová *et al.* 2003, Ferko *et al.* 2015).

Preconditioning-like effect induced by experimental model of acute streptozotocin-induced diabetes mellitus (STZ-DM) is characterized by better coping of the heart with the increased pathological load (Ziegelhöffer *et al.* 1996). STZ-DM hearts are characterized by the lack of oxygen consumption due to damage of electron transport chain resulting in a situation similar to ischemia or hypoxia. With regard to the adaptation of the heart to pathological conditions induced by STZ-DM, there were observed many different changes in structural and functional properties of both, cardiomyocytes and heart mitochondria (Pierce and Dhalla 1981, Ferko *et al.* 2006, 2008, Malfitano *et al.* 2015). Some of these changes, like an increase in ATP synthase activity and in mitochondrial membrane fluidity, or the protection against Ca^{2+} overload, were considered to be an adaptation mechanisms of heart mitochondria exposed to an acute stage of STZ-DM (Ferko *et al.* 2006, 2008, Ziegelhöffer *et al.* 1996).

Pharmacological preconditioning is one of effective tools for induction of heart adaptation by a chemical agent that influences cardioprotective pathway. Several pharmacological substances for preventing myocardial reperfusion injury were already tested. Inhibiting the formation and opening of mPTP by cyclosporin-A (Piot *et al.* 2008), free radicals scavenging by melatonin (Galano *et al.* 2011) or reducing the production of inflammatory mediators (Ebrahimi *et al.* 2014) have been found to participate in the infarct size reduction and better recovery after I/R injury.

Since the effect of mitochondrial K_{ATP} channels openers was assumed to be cardioprotective, one of them, diazoxide, has often been used as a pharmacological tool inducing preconditioning (Garlid *et al.* 1997, Sanada *et al.* 2001). Consequences of the induced opening of the mitochondrial K_{ATP} channels results in depolarization of intramitochondrial membrane by K^+ entry which simultaneously brings about reduction in mitochondrial calcium overload (Holmuhamedov *et al.* 1998).

Diazoxide has also been recognized as an inhibitor of succinate dehydrogenase (Lenzen and Panten 1983), therefore, the defect in the electron transport chain, succinate accumulation likewise the decrease in intracelular pH can be attributed to the effect of diazoxide. These metabolic changes contribute to the prevention of mPTP opening and simulate conditions equivalent to those of IPC (Garlid *et al.* 1997).

The question remains whether there is another mechanism – similar to STZ-DM – by which diazoxide could contribute to the endogenous cardioprotection. Investigation of diazoxide effect in terms of changes in structural and functional characteristics of mitochondrial membrane can provide new information about the possible mechanism of diazoxide action in healthy and diabetic myocardium. The aim was therefore to explore the possibility of existence of another *in vitro* mechanism, which could be contributory to cardioprotection mediated by diazoxide treatment and assessed in terms of an increase in the mitochondrial membrane fluidity and ATP synthase activity.

Material and Methods

All experiments were performed in accordance with the European Guidelines on Laboratory Animal Care. The use and treatment of animals were also approved by the Ethics Committee of the Institute for Heart Research, SAS where the experiments were carried out.

Male Wistar rats of age 9-11 weeks (250±20 g) were kept under conditions of standard light regimen (D:L, 12:12) at 22±2 °C in cages (max 5 animals per cage) with access to water and standard pellet food *ad libitum*.

Experimental diabetes mellitus was induced by a single dose of streptozotocin (STZ) (65 mg.kg^{-1}, i.p.) dissolved in 0.1 mol.l^{-1} citrate buffer, pH 4.0. Progress of disease was monitored during 8 days by measuring of glykosuria using Gluko PHAN strips (Erba-Lachema, Brno, Czech Republic).

Eight days after STZ injection, experimental animals were anesthetized with thiopental (50-60 mg.kg^{-1}, i.p.) together with heparin (500 IU, i.p.).

Metabolic state of animals was determined by estimation of glucose, cholesterol, triacylglycerols and glycohemoglobin (MultiCare, Biochemical system internation, Florence, Italy) in the blood as well as insulin in the serum (RIA kit, Linco Researech USA).

Excised heart was cut into small pieces by scissors in the presence of small volume of ice-cold isolation solution (IS, containing in mmol.l^{-1}: 180 KCl, 4 EDTA, 1 % bovine serum albumin, pH 7.4). Minced heart tissue was suffused with 20 ml of ice-cold IS with addition of protease 2.5 mg.g^{-1} of tissue (Sigma P-6141) and subsequently, during 2-3 min, homogenized in teflon/glass homogenizer on the ice. Tissue homogenate

was processed by differential centrifugation. After 10 min centrifugation at 1000 g, the obtained supernatant together with the part of mitochondria which were in direct contact with protease were poured out and the pellet containing mitochondria was homogenized in 20 ml of ice-cold IS without protease and centrifuged again at 1000 g for 10 min. Resulting supernatant was centrifuged at 6200 g for 10 min and pellet containing mitochondria was resuspended in an albumin free IS (containing in mmol.l^{-1}: 180 KCl, 4 EDTA). Obtained homogenate was spunned down at 6200 g for 10 min and subsequently used for the estimation of protein concentration according to Lowry, as well as for further biophysical and biochemical investigations (Lowry *et al.* 1951). The procedure of isolation of heart mitochondria was performed at 4 °C (Ziegelhöffer *et al.* 2009).

Mitochondrial membrane fluorescence anisotropy was determined by using a lipophilic fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH). The degree of DPH fluorescence anisotropy characterizes the mobility of phospholipid acyl chains in the membrane. The measured fluorescence anisotropy of DPH thus reflects the membrane microviscosity which is a reciprocal of the membrane fluidity. Isolated mitochondria were resuspended in an isotonic buffer containing 180 KCl (mmol.l^{-1}), 4 EDTA (mmol.l^{-1}), pH 7.4 and in the presence and absence of 7 diazoxide (μmol.l^{-1}). Diluted mitochondrial fraction was labeled with a 0.25 μmol.l^{-1} of DPH solution. Samples were incubated at 30±0.2 °C in the dark for 20 min to allow a complete incorporation of the probe into the membrane. Fluorescence anisotropy was measured with luminiscence spectrometer LS 45 (Perkin Elmer, USA) at the excitation wavelength of 360 nm and the emission wavelength of 425 nm according to Shinitzky (1984).

The activity of mitochondrial ATP synthase was determined in the presence of Mg^{2+} cations in the reverse direction of reaction as Mg^{2+}-ATPase, i.e. as a splitting of ATP molecules. Enzyme activity was determined by adding of 50 μg mitochondrial fraction (1 μg.μl^{-1}) into the 950 μl of incubation medium containing: 200 μl imidazole buffer (250 mmol.l^{-1}), 100 μl MgCl$_2$ (40 mmol.l^{-1}), 100 μl ATP-Tris (40 mmol.l^{-1}), in the presence and absence of 100 μl dinitrophenol (DNP) (0.1 mmol.l^{-1}) and 200 μl diazoxide (0.5-7 μmol.l^{-1}) and destilled water added to a total volume of 950 μl. Addition of DNP disrupts all mitochondrial membranes, thus allowing influx of Mg^{2+} cations into the

mitochondria. In this way it is possible to measure the total activity of mitochondrial Mg^{2+}-ATPase. The reaction started by ATP addition after 10 min of pre-incubation of mitochondrial fraction at 37 °C. Termination of reaction was performed by addition of 1 ml ice-cold 12 % trichloroacetic acid, after 20-min incubation of mitochondrial fraction with ATP (Ferko *et al.* 2006). Finally, the mitochondrial Mg^{2+}-ATPase activity was determined spectrophotometrically at 700 nm as the amount of inorganic phosphate (P$_i$) liberated by ATP splitting per unit of time (μmol.P$_i$.g^{-1}.h^{-1}) according to Taussky and Shorr (1953).

Determining the type of diazoxide-enzyme interaction is possible by measuring of the dependence of mitochondrial Mg^{2+}-ATPase activity on the increasing concentration of ATP, based on the changes in kinetic parameters: the maximal reaction rate (V$_{max}$) and Michaelis-Menten constant (K$_m$). Enzyme kinetics of mitochondrial ATP synthase activity was measured in the presence of diazoxide (at a concentration of 7 μmol.l^{-1}) and ATP (in the range of concentrations up to 40 mmol.l^{-1}) according to Ziegelhöffer *et al.* (1996).

Data are presented as a mean ± standard error of mean (SEM). Differences between groups were evaluated by the method of analysis of variance (ANOVA). For non-normally distributed data we used a nonparametric analysis of variance, Kruskal-Wallis test for an unpaired design or Friedman test for paired comparisons. Fitting enzyme kinetics data was made using nonlinear regression with a model of Michaelis-Menten enzyme kinetics equation. The results were compared with those obtained after standard data transformation yielding linearised dose-response curves. Method of linear regression together with testing of influential points was then used for testing the significance of relations. Statistical analyses were performed using StatsDirect 2.8.0 (3.0.161) software (Stats Direct Ltd., UK) and GraphPad Prism 6.01 (GraphPad Software, Inc., USA). Two-sided P-values are reported.

Results

Diabetic state of experimental animals was confirmed by a significant (p<0.01) increase in the levels of blood glucose, triacylglycerols, cholesterol and the content of glycohemoglobin as well as by decreased levels of blood insulin on the eighth day after the STZ injection (Table 1).

Table 1. Comparison of the metabolic parameters of control healthy and acute streptozotocin-induced diabetic rats.

	Control group	Diabetic group
Glucose (mmol/l)	5.20 ± 0.14	17.90 ± 0.84*
Triacylglycerols (g/l)	1.22 ± 0.11	4.56 ± 0.34*
Cholesterol (g/l)	1.75 ± 0.10	2.69 ± 0.14*
Glycohemoglobin (% Hb)	4.12 ± 0.10	7.68 ± 0.92*
Insulin (ng/ml)	1.16 ± 0.18	0.48 ± 0.06*

Results are given as mean ± SEM; n=14, * p<0.01 vs. control healthy group.

Mitochondrial membrane fluidity was measured under a combination of two factors: STZ-DM condition and treatment with diazoxide (DZX). When the effects of factors were considered separately, only the acute stage of STZ-DM yielded statistically significant increase in the membrane fluidity. The second factor, diazoxide, did not have a significant impact on this biophysical parameter. Thus, the observed significant increase in membrane fluidity upon the combination of the two factors can be attributed to the main effect of STZ-DM. Diazoxide did not independently increase membrane fluidity (Fig. 1).
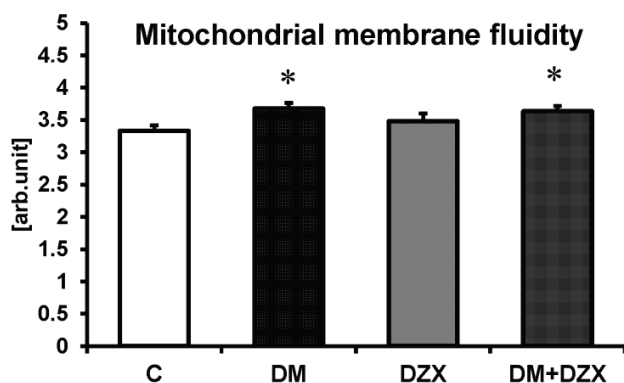
Fig. 1. Modulation of mitochondrial membrane fluidity in control healthy hearts and hearts exposed to acute streptozotocin-induced diabetes mellitus and diazoxide. Experimental groups: C, isolated mitochondria from control healthy group; DM, isolated mitochondria from group with acute stage of streptozotocin-induced diabetes mellitus; DZX, isolated mitochondria from control healthy hearts exposed to 7 µmol.l^{-1} diazoxide (*in vitro*); DM+DZX, isolated mitochondria from hearts with acute streptozotocin-induced diabetes mellitus exposed to 7 µmol.l^{-1} diazoxide (*in vitro*). Results are given as mean ± SEM; n=6 for each group, * p<0.05 vs. control healthy group.
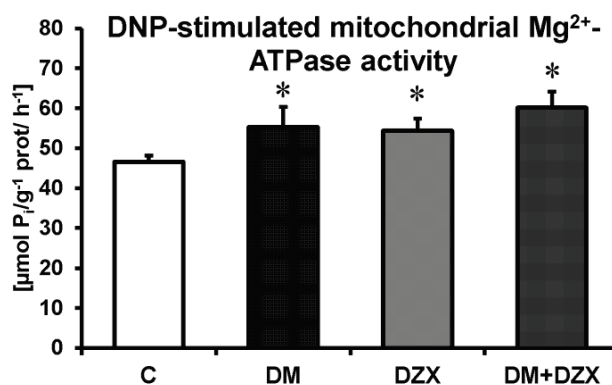
Fig. 2. Activity of DNP-stimulated Mg^{2+}-ATPase in mitochondria isolated from control healthy and acute streptozotocin-induced diabetic rat hearts exposed to diazoxide. Experimental groups: C, isolated mitochondria from control group; DM, isolated mitochondria from group with streptozotocin-induced diabetes mellitus; DZX, isolated mitochondria from control healthy hearts exposed to 7 µmol.l^{-1} diazoxide (*in vitro*); DM+DZX, isolated mitochondria from hearts with acute streptozotocin-induced diabetes mellitus exposed to 7 µmol.l^{-1} diazoxide (*in vitro*). Results are given as mean ± SEM; n=7 for each group. * p<0.05 vs. control healthy group.

Separate effect of acute STZ-DM was manifested by a significant (p<0.05) increase in the activity of mitochondrial Mg^{2+}-ATPase by 18.93 %. Stimulatory effect of diazoxide on the mitochondrial Mg^{2+}-ATPase activity was proved within the whole monitored range of diazoxide concentrations (0-7 µmol.l^{-1}) with the statistical significance (p<0.05) up to 5 µmol.l^{-1} diazoxide including (data not shown). Exposure of mitochondria isolated from healthy and acute STZ-DM hearts to 7 µmol.l^{-1} diazoxide (under *in vitro* conditions) induced a significant increase (p<0.05) in Mg^{2+}-ATPase activity by 16.74 % and 29.09 % in healthy and in acute STZ-DM group, respectively (Fig. 2).

In order to determine the type of diazoxide-enzyme interaction, the dependence of ATP synthase activity on the increasing ATP concentration (so called dose-response curves) in the presence of constant diazoxide concentration (7 µmol.l^{-1}) were measured in both experimental groups (Fig. 3A,B). Statistically significant increase (p<0.05) in V_{max} in control as well as in diabetic group and no statistically significant change of the K_m value were observed in the control as well as in the SZT-DM group (Table 2). No significant interaction between the two factors, STZ-DM and diazoxide, was observed, which denotes that the effects of these factors on the V_{max} can be considered separately.
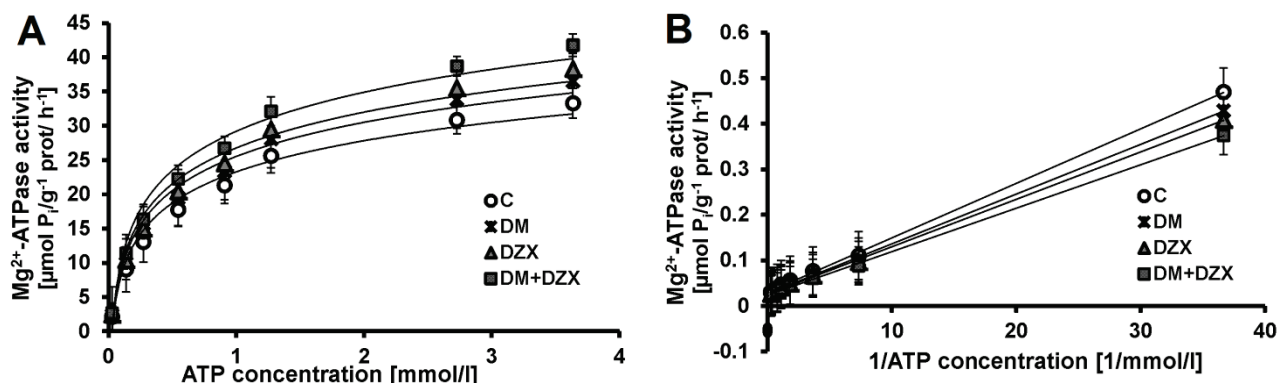
Fig. 3. (A) Activation of mitochondrial Mg^{2+}-ATPase by increasing concentration of ATP in the presence and absence of 7 $\mu mol.l^{-1}$ diazoxide in control healthy hearts and hearts exposed to acute streptozotocin-induced diabetes mellitus. **(B)** Lineweaver-Burk plot of mitochondrial Mg^{2+}-ATPase activation. Experimental groups: C, isolated mitochondria from control healthy group; DM, isolated mitochondria from group with streptozotocin-induced diabetes mellitus; DZX, isolated mitochondria from control healthy hearts exposed to 7 $\mu mol.l^{-1}$ diazoxide (*in vitro*); DM+DZX, isolated mitochondria from hearts with acute streptozotocin-induced diabetes mellitus exposed to 7 $\mu mol.l^{-1}$ diazoxide (*in vitro*). Results are given as mean ± SEM; n=7 for each group.

Table 2. Kinetics parameters of mitochondrial Mg^{2+}-ATPase activation by increasing concentration of ATP in the presence and absence of 7 $\mu mol.l^{-1}$ diazoxide in the hearts of control healthy and streptozotocin-induced diabetes mellitus.

	V_{max}	K_m
C	33.674 ± 0.861	0.148 ± 0.004
DIA	38.752 ± 1.080 *	0.147 ± 0.008
DZX	36.903 ± 1.134 *	0.148 ± 0.005
DM+DZX	42.160 ± 0.982 *	0.148 ± 0.007

Results are given as mean ± SEM. Experimental groups: C, isolated mitochondria from control healthy hearts; DM, isolated mitochondria from hearts of animals with acute streptozotocin-induced diabetes mellitus; DZX, isolated mitochondria from control healthy hearts exposed to 7 $\mu mol.l^{-1}$ diazoxide (*in vitro*); DM+DZX, isolated mitochondria from hearts of animals with acute streptozotocin-induced diabetes mellitus exposed to 7 $\mu mol.l^{-1}$ diazoxide (*in vitro*). n=7 for each group, * p<0.05 vs. control healthy group.

Discussion

Our attention in this study was focused on the possibilities of pharmacological influencing of already recognized cardioprotective mechanisms induced by acute stage of STZ-DM (Tani and Neely 1988, Ziegelhöffer *et al.* 1996, Ferko *et al.* 2006, Malfitano *et al.* 2015). Diazoxide is considered to be an appropriate agent for modulation of the endogenous protective mechanisms. Whereas diazoxide has proven its protective effect in the physiological parameters, specifically an antiarrhythmic protection in terms of the ventricular premature beats and the total duration of ventricular tachyarrhythmia) (Matejíková *et al.* 2009), we also focused on the mechanism of diazoxide action.

Acute stage of streptozotocin-induced diabetic animal models has become a very useful platform for understanding cardioprotective mechanisms (Malfitano *et al.* 2015, Liu *et al.* 1993). Despite using a wide range of STZ doses (Bhadada and Goyal 2015, Mukundwa *et al.* 2016) the application of 65 $mg.kg^{-1}$ STZ has been shown to be sufficient to cause DM as confirmed by altered metabolic parameters on the eighth day after STZ aplication (Table 1). Moreover, we have already reported that induction of acute stage of STZ-DM by doses of 65 $mg.kg^{-1}$ STZ results in a damage of mitochondrial electron transport chain without disconnection of oxidative phosphorylation (Ferko *et al.* 2006). The decrease in oxygen utilization due to defect in electron transport chain is partially compensated by an increase in mitochondrial membrane fluidity (Ferko *et al.* 2008, Waczulíková *et al.* 2007). The degree of membrane fluidity is a very important parameter influencing functional properties of proteins incorporated in the membranes, such as that of activity of mitochondrial ATP synthase (Waczulíková *et al.* 2002).

In accordance with our recent data, a significant increase in the mitochondrial membrane fluidity was observed in the hearts exposed to STZ-DM (Fig. 1) (Ferko *et al.* 2008, Waczulíková *et al.* 2007). An elevated formation of mPTP could be one of the possible explanations of the observed mitochondrial membrane fluidization. Enhanced formation and a short-term mPTP opening observed during the acute STZ-DM resulted in mitochondrial membrane fluidization and facilitation of ATP transport without lethal damage (Ziegelhöffer-Mihalovičová *et al.* 1997).

The increase in membrane fluidity of mitochondrial membranes isolated from the hearts exposed to STZ-DM could presumably result in reducing of local friction, thus facilitating ATP synthase rotation, which was manifested by enhanced ATP synthase activity, measured as DNP-stimulated Mg^{2+}-ATPase activity (Fig. 2). The activity of mitochondrial Mg^{2+}-ATPase is measured in the presence of DNP that makes mitochondrial membranes permeable to Mg^{2+} ions which enter the mitochondria thus allowing determination of the total enzyme activity. Similarly, the results presented in studies by Ferko *et al.* (2006, 2008) point to the ability of myocardium to adapt on the acute pathological stimulus, STZ-DM, by using the compensative mechanisms originated in mitochondria (increase in mitochondrial membrane fluidity and Mg^{2+}-ATPase activity). The effect of diazoxide did not manifest in an alteration of mitochondrial membrane lipid fluidity. Moreover, combination of STZ-DM with diazoxide did not result in an independent effect of diazoxide. The observed increase can be attributed solely to the effect of STZ-DM (Fig. 1). Based on these results, we can assume that diazoxide did not incorporate into the lipid bilayer, neither affected its composition.

The effect of diazoxide as a cardioprotective agent imitating IPC was observed in the increase of mitochondrial DNP-stimulated Mg^{2+}-ATPase activity (Fig. 2) within the whole diazoxide concentration range 0 to 7 $\mu mol.l^{-1}$ (data not shown) as was confirmed in studies by Dzeja *et al.* (2003) and Portenhauser *et al.* (1971). Interestingly, diazoxide was able to stimulate the increase in enzyme activity independently of STZ-DM condition (Fig. 2).

Based on the results, it can be deemed reasonable to anticipate the involvement of different mechanisms for each of the phenomena leading to cardioprotection. In the model of STZ-DM we observed the supportive effect of mitochondrial membrane fluidization on the increase in mitochondrial DNP-stimulated Mg^{2+}-ATPase activity. On the other hand, the increase in the enzyme activity after diazoxide treatment was not associated by changes in mitochondrial membrane fluidity, which implied existence of a different mechanism by which diazoxide stimulated the Mg^{2+}-ATPase.

Focusing on the elucidation the mechanism of diazoxide action, the kinetics measurements were performed. Dependence of Mg^{2+}-ATPase activity on the increasing concentration of ATP in the presence or absence of diazoxide revealed a significant elevation in V_{max} values in all experimental groups compared to the control group (Fig. 3A,B). No statistically significant interaction between the effects of STZ-DM and DZX was proved, and so the effect of DZX can be assumed identical irrespective of the level of the second factor (diabetic and healthy condition). Thus, we can conclude that the factors STZ-DM and DZX are related to V_{max} completely independently. Unchanged K_m values of all groups exposed to diazoxide action pointed to the fact that diazoxide did not cause any alterations in afinity of the ATP-binding site of the enzyme to the substrate (Table 2). The observed changes in kinetic parameters are characteristic for a noncompetitive type of enzyme activation.

In the kinetic parameters determination, the priority was given to the measurement of actual Mg^{2+}-ATPase activity, i.e. the enzyme activity of that part of mitochondria with membrane permeabilisation. Addition of DNP into the incubating medium was omited in order to avoid a possible nonspecific interaction with diazoxide. Since diazoxide is known to have a wide range of the effects depending on the used concentration (Murata *et al.* 200, Kowaltowski *et al.* 2001), we selected one of the lowest concentrations that were proved to induce the evident protective effect in determination of DNP-stimulated Mg^{2+}-ATPase activity, i.e. 7 $\mu mol.l^{-1}$ diazoxide (Fig. 2).

We assume that acute STZ-DM seems to be a strong stimulus able to induce cardioprotective mechanisms such as modulation of mitochondrial membrane fluidity and mitochondrial ATP synthase activity in comparison with the effect of diazoxide. A combination of both experimental factors, STZ-DM+DZX, bring about a moderate increase in the parameters mentioned above with a considerable contribution of STZ-DM. Based on our experimental results we propose a new view of cardioprotection induced by diazoxide that does not act through the mitochondrial K_{ATP} opening only, but presumably also through a direct interaction of diazoxide with the molecule of mitochondrial ATP synthase. That explanation is in accordance with the opinion by Contessi *et al.* (2004) who consider the stabilisation of Mg-ADP binding in catalytic site of the β subunit of mitochondrial ATP synthase as a prerequisite for the general diazoxide action.

In conclusion, the experimental findings of our study provide evidence for different mechanisms

of cardioprotection induced by pharmacological preconditioning with diazoxide and those induced by acute diabetes mellitus in terms of mitochondrial membrane properties. While acute STZ-DM stimulates ATP synthase activity indirectly, presumably *via* changes in the local lipid environment, the effect of diazoxide seems to be enabled by a direct interaction of diazoxide with the molecule of ATP synthase. These results point to a possibility of existence of a new, previously not recognized, mechanism of diazoxide's action on the induction of myocardial protection, which is independent of its effect on K_{ATP} channels.

Conflict of Interest
There is no conflict of interest.

References

BALYKIN MV, SAGIDOVA SA, ZHARKOV AV: Changes blood gas and of free radical oxidation of lipids in the myocardium during adaptation to physical stress. *Ross Fiziol Zh Im I M Sechenova* **101**: 1007-1012, 2015.

BHADADA SV, GOYAL RK: Effect of aqueous extract of Tephrosia purpurea on cardiovascular complications and cataract associated with streptozotocin-induced diabetes in rats. *Indian J Pharm Sci* **77**: 522-529, 2015.

CONTESSI S, METELLI G, MAVELLI I, LIPPE G: Diazoxide affects the IF1 inhibitor protein binding to F1 sector of beef heart F0F1ATPsynthase. *Biochem Pharmacol* **67**: 1843-1851, 2004.

CRESTANELLO JA, DOLIBA NM, BABSKY AM, DOLIBA NM, NIIBORI K, OSBAKKEN MD, WHITMAN GJ: Mitochondrial function during ischemic preconditioning. *Surgery* **131**: 172-178, 2002.

DZEJA PP, BAST P, OZCAN C, VALVERDE A, HOLMUHAMEDOV EL, VAN WYLEN DG: Targeting nucleotide-requiring enzymes: implications for diazoxide-induced cardioprotection. *Am J Physiol Heart Circ Physiol* **284**: H1048-H1056, 2003.

EBRAHIMI H, BADALZADEH R, MOHAMMADI M, YOUSEFI B: Diosgenin attenuates inflammatory response induced by myocardial reperfusion injury: role of mitochondrial ATP-sensitive potassium channels. *J Physiol Biochem* **70**: 425-432, 2014.

FERKO M, GVOZDJAKOVÁ A, KUCHARSKÁ J, MUJKOŠOVÁ J, WACZULÍKOVÁ I, STYK J, RAVINGEROVÁ T, ZIEGELHÖFFER-MIHALOVIČOVÁ B, ZIEGELHÖFFER A: Functional remodeling of heart mitochondria in acute diabetes: interrelationships between damage, endogenous protection and adaptation. *Gen Physiol Biophys* **25**: 397-413, 2006.

FERKO M, HABODÁSZOVÁ D, WACZULÍKOVÁ I, MUJKOŠOVÁ J, KUCHARSKÁ J, ŠIKUROVÁ L, ZIEGELHÖFFER B, STYK J, ZIEGELHÖFFER A: Endogenous protective mechanisms in remodeling of rat heart mitochondrial membranes in the acute phase of streptozotocin-induced diabetes. *Physiol Res* **57**: 67-73, 2008.

FERKO M, KANCIROVÁ I, JAŠOVÁ M, WACZULÍKOVÁ I, ČARNICKÁ S, KUCHARSKÁ J, ULIČNÁ O, VANČOVÁ O, MURÁRIKOVÁ M, RAVINGEROVÁ T, ZIEGELHÖFFER A: Participation of heart mitochondria in myocardial protection against ischemia/reperfusion injury: benefit effects of short-term adaptation processes. *Physiol Res* **64** (Suppl 5): S617-S625, 2015.

GALANO A, TAN DX, REITER RJ: Melatonin as a natural ally against oxidative stress: a physicochemical examination. *J Pineal Res* **51**: 1-16, 2011.

GARLID KD, PAUCEK P, YAROV-YAROVOY V, MURRAY HN, DARBENZIO RB, D'ALONZO AJ: Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K+ channels. Possible mechanism of cardioprotection. *Circ Res* **81**: 1072-1082, 1997.

GOTZSCHE O: Myocardial catecholamine uptake and catecholamine sensitivity in experimental diabetes. In: *The Diabetic Heart*. NAGANO M, DHALLA NS (eds), Raven Press, New York, 1991, pp 199-208.

HOLMUHAMEDOV E, JOVANOVIC S, DZEJA P, JOVANOVIC A, TERZIC A: Mitochondrial ATP-sensitive K1 channels modulate cardiac mitochondrial function. *Am J Physiol* **275**: H1567-H1576, 1998.

KOBARA M, TATSUMI T, MATOBA S, YAMAHARA Y, NAKAGAWA C, OHTA B, MATSUMOTO T, INOUE D, ASAYAMA J, NAKAGAWA M: Effect of ischemic preconditioning on mitochondrial oxidative phosphorylation and high energy phosphates in rat hearts. *J Mol Cell Cardiol* **28**: 417-428, 1996.

KOWALTOWSKI AJ, SEETHARAMAN S, PAUCEK P, GARLID KD: Bioenergetic consequences of opening the ATP-sensitive K(+) channel of heart mitochondria. *Am J Physiol Heart Circ Physiol* **280**: H649-H657, 2001.

KRISTIAN T, BERNARDI P, SIESJÖ BK: Acidosis promotes the permeability transition in energized mitochondria: implications for reperfusion injury. *J Neurotrauma* **18**: 1059-1074, 2001.

KÜBLER W, HAASS M: Cardioprotection: definition, classification, and fundamental principles. *Heart* **75**: 330-333, 1996.

LENZEN S, PANTEN U: Characterization of succinate dehydrogenase and α-glycerophosphate dehydrogenase in pancreatic islets. *Biochem Med* **30**: 349-356, 1983.

LIU Y, THORNTON JD, COHEN MV, DOWNEY JM, SCHAFFER SW: Streptozotocin-induced non-insulin-dependent diabetes protects the heart from infarction. *Circulation* **88**: 1273-1278, 1993.

LIU Y, SATO T, O'ROURKE B, MARBAN E: Mitochondrial ATP-dependent potassium channels: novel effectors of cardioprotection? *Circulation* **97**: 2463-2469, 1998.

LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265-275, 1951.

MALFITANO C, De SOUZA JUNIOR AL, CARBONARO M, BOLSONI-LOPES A, FIGUEROA D, De SOUZA LE, SILVA KA, CONSOLIM-COLOMBO F, CURI R, IRIGOYEN MC: Glucose and fatty acid metabolism in infarcted heart from streptozotocin-induced diabetic rats after 2 weeks of tissue remodeling. *Cardiovasc Diabetol* **14**: 149, 2015.

MATEJÍKOVÁ J, RAVINGEROVÁ T, PANCZA D, ČARNICKÁ S, KOLÁŘ F: Mitochondrial KATP opening confers protection against lethal myocardial injury and ischaemia-induced arrhythmias in the rat heart via PI3K/Akt-dependent and -independent mechanisms. *Can J Physiol Pharmacol* **87**: 1055-1062, 2009.

MUKUNDWA A, MUKARATIRWA S, MASOLA B: Effects of oleanolic acid on the insulin signaling pathway in skeletal muscle of streptozotocin-induced diabetic male Sprague-Dawley rats. *J Diabetes* **8**: 98-108, 2016.

MURATA M, AKAO M, O'ROURKE B, MARBAN E: Mitochondrial ATP-sensitive potassium channels attenuate matrix Ca(2+) overload during simulated ischemia and reperfusion: possible mechanism of cardioprotection. *Circ Res* **89**: 891-898, 2001.

MURRY CE, JENNINGS RB, REIMER KA: Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* **74**: 1124-1136, 1986.

OZDEMIR O, AKALIN PP, BASPINAR N, HATIPOGLU F: Pathological changes in the acute phase of streptozotocin-induced diabetic rats. *Bull Vet Inst Pulawy* **53**: 783-790, 2009.

PIERCE GN, DHALLA NS: Cardiac myofibrillar ATPase activity in diabetic rats. *J Mol Cell Cardiol* **13**: 1063-1069, 1981.

PIOT C, CROISILLE P, STAAT P, THIBAULT H, RIOUFOL G, MEWTON N, ELBELGHITI R, CUNG TT, BONNEFOY E, ANGOULVANT D, MACIA C, RACZKA F, SPORTOUCH C, GAHIDE G, FINET G, ANDRÉ-FOUËT X, REVEL D, KIRKORIAN G, MONASSIER JP, DERUMEAUX G, OVIZE M: Effect of cyclosporine on reperfusion injury in acute myocardial infarction. *N Engl J Med* **359**: 473-481, 2008.

PORTENHAUSER R, SCHAFER G, TROLP R: Inhibition of mitochondrial metabolism by the diabetogenic thiadiazine diazoxide. II. Interaction with energy conservation and ion transport. *Biochem Pharmacol* **20**: 2623-2632, 1971.

PRZYKLENK K, BAUER B, OVIZE M, KLONER RA, WHITTAKER P: Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. *Circulation* **87**: 893-899, 1993.

RAVINGEROVA T, STETKA R, VOLKOVOVA K, PANCZA D, DZURBA A, ZIEGELHÖFFER A: Acute diabetes modulates response to ischemia in isolated rat heart. *Mol Cell Biochem* **210**: 143-151, 2000.

RAVINGEROVÁ T, NECKÁŘ J, KOLÁŘ F: Ischemic tolerance of rat hearts in acute and chronic phases of experimental diabetes. *Mol Cell Biochem* **249**: 59-65, 2003.

RERUP CC: Drugs producing diabetes through damage of the insulin secreting cells. *Pharmacol Rev* **22**: 485-518, 1970.

SANADA S, KITAKAZE M, ASANUMA H, HARADA K, OGITA H, NODE K: Role of mitochondrial and sarcolemmal K(ATP) channels in ischemic preconditioning of the canine heart. *Am J Physiol Heart Circ Physiol* **280**: H256-H263, 2001.

SHINITZKY M: Membrane fluidity and cellular functions. In: *Physiology of Membrane Fluidity.* M SHINITZKY (ed), CRC Press, Boca Raton, 1984, pp 1-51.

TANI M, NEELY JR: Hearts from diabetic rats are more resistant to in vitro ischemia: possible role of altered Ca2+ metabolism. *Circ Res* **62**: 931-940, 1988.

TAUSSKY HH, SHORR E: A microcolorimetric method for determination of inorganic phosphorus. *J Biol Chem* **202**: 675-685, 1953.

VOGEL WM, APSTEIN CS: Effects of alloxan-induced diabetes in ischemia-reperfusion injury in rabbit hearts. *Circ Res* **62**: 975-982, 1988.

WACZULÍKOVÁ I, ZIEGELHÖFFER A, ORSZÁGHOVÁ Z, CÁRSKY J: Fluidising effect of resorcylidene aminoguanidine on sarcolemmal membranes in streptozotocin-diabetic rats: blunted adaptation of diabetic myocardium to Ca2+ overload. *J Physiol Pharmacol* **53**: 727-739, 2002.

WACZULIKOVA I, HABODASZOVA D, CAGALINEC M, FERKO M, ULICNA O, MATEASIK A, SIKUROVA L, ZIEGELHÖFFER A: Mitochondrial membrane fluidity, potential, and calcium transients in the myocardium from acute diabetic rats. *Can J Physiol Pharmacol* **85**: 372-381, 2007.

YTREHUS K, LIU Y, DOWNEY JM: Preconditioning protects ischemic rabbit heart by protein kinase C activation. *Am J Physiol* **266**: H1145-H1152, 1994.

ZHAO ZQ, CORVERA JS, HALKOS ME, KERENDI F, WANG NP, GUYTON RA, VINTEN-JOHANSEN J: Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. *Am J Physiol Heart Circ Physiol* **285**: H579-H588, 2003.

ZIEGELHÖFFER A, RAVINGEROVÁ T, STYK J, TRIBULOVÁ N, VOLKOVOVÁ K, ŠEBOKOVÁ J, BRIERER A: Diabetic cardiomyopathy in rats: biochemical mechanisms of increased tolerance to calcium overload. *Diabetes Res Clin Pr* **31**: 93-103, 1996.

ZIEGELHÖFFER A, WACZULÍKOVÁ I, FERKO M, KINCELOVÁ D, ZIEGELHÖFFER B, RAVINGEROVÁ T, CAGALINEC M, SCHÖNBURG M, ZIEGELHOEFFER T, SIKUROVÁ L, ULICNÁ O, MUJKOSOVÁ J: Calcium signaling-mediated endogenous protection of cell energetics in the acutely diabetic myocardium. *Can J Physiol Pharmacol* **87**: 1083-1094, 2009.

ZIEGELHÖFFER-MIHALOVIČOVÁ B, OKRUHLICOVÁ L, TRIBULOVÁ N, RAVINGEROVÁ T, VOLKOVOVÁ K, SEBOKOVÁ J, ZIEGELHÖFFER A: Mitochondrial contact sites detected by creatine phosphokinase activity in the hearts of normal and diabetic rats: is mitochondrial contact sites formation a calcium-dependent process? *Gen Physiol Biophys* **16**: 329-338, 1997.