Remote Ischemic Preconditioning of the Heart: Protective Responses in Functional and Biophysical Properties of Cardiac Mitochondria

M. FERKO[1], I. KANCIROVÁ[1], M. JAŠOVÁ[1], S. ČARNICKÁ[1], M. MURÁRIKOVÁ[1], I. WACZULÍKOVÁ[2], Z. SUMBALOVÁ[3], J. KUCHARSKÁ[3], O. ULIČNÁ[3], T. RAVINGEROVÁ[1], A. ZIEGELHÖFFER[1]

[1]Institute for Heart Research, Centre of Excellence SAS NOREG, Slovak Academy of Sciences, Bratislava, [2]Division of Biomedical Physics, Department of Nuclear Physics and Biophysics, Faculty of Mathematics, Physics and Informatics, Comenius University, Bratislava, [3]Pharmacobiochemical Laboratory, Third Department of Internal Medicine, Faculty of Medicine, Comenius University, Bratislava, Slovakia

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Summary

Remote ischemic preconditioning (RIP)-induced protection of myocardial energetics was well documented on the level of tissue, but data concerning the involvement of mitochondria were missing. We aimed at the identification of changes in membrane properties and respiratory functions induced in rat heart mitochondria by RIP. Experiments were performed on 46 male Wistar rats divided into control and RIP-treated groups of 21 animals each. Blood flow in the occluded area was recorded by MRI angiography in four animals. RIP protocol comprised of three successive 5-min occlusions each followed by 5-min reperfusions of descending branches of the right hind limb femoral artery. The efficacy of RIP was evaluated as the extent of RIP-induced protection against damage to the functions of mitochondria isolated by differential centrifugation after 30-min global ischemia followed by 40-min reperfusion of the hearts in Langendorff mode. Assessments: mitochondrial membrane fluidity with a fluorescent probe DPH, CoQ_9 and CoQ_{10} with HPLC, mitochondrial respiration with the Oxygraph-2k (Oroboros). Results revealed that RIP was affecting the mitochondria. The immediate protection conferred by RIP involves beneficial and prognostically significant effects: a total elimination of ischemia/reperfusion-induced depression of mitochondrial membrane fluidity and a trend for better preservation of mitochondrial state 3 respiration.

Key words

Rat heart • Remote preconditioning • Ischemia • Reperfusion injury • Heart mitochondria • Oxygen consumption • Respiratory chain • Mitochondrial membrane fluidity

Corresponding author

M. Ferko, Institute for Heart Research, Slovak Academy of Sciences, Dúbravská cesta 9, 840 05 Bratislava, Slovakia. E-mail: usrdmife@savba.sk

Introduction

Remote ischemic preconditioning (RIP) represents a non-invasive model of cardioprotection conferred by one or more subsequent short episodes of ischemia applied in a distant tissue, such as in the limb (Przyklenk *et al.* 1993). A very recent review of the field (Čarnická *et al.* 2013) emphasized that already much effort was devoted not only to identification of afferent signals emitted from the distant ischemized area but also to probable association between these signals and the relevant beneficial responses observed in the heart after RIP. Recently it was also stressed that RIP exerts considerable protective effect on myocardial energetics (Abdul-Ghani *et al.* 2013). Therefore it is surprising that still very little attempts were made to specify the role of mitochondria in the sequence of reactions induced in the heart by RIP.

The aim of the present study was to identify the

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Fax +420 241 062 164, e-mail: physres@biomed.cas.cz, www.biomed.cas.cz/physiolres

changes in membrane properties, respiratory chain function and oxidative phosphorylation induced in the heart mitochondria by RIP realized *via* occlusion of descending branches of the right hind limb femoral artery. Another goal was attempting to reveal whether the receptor(s) catching the RIP-emitted signals to mitochondria would be situated on the mitochondrial membrane or elsewhere in the redox system.

Materials and Methods

Treatment of animals

All experiments were performed in accordance with the *Guide for Care and Use of Laboratory Animals* **(**ILAR 1996**)** and the rules issued by the State Veterinary and Alimentary Administration of the Slovak Republic, based on § 37 (6), legislation No. 488/2002 of the Slovak Parliament. The use and treatment of animals was also approved by the Animal Review Committee at the Institute for Heart Research, SAS where the experiments were carried out.

Experiments were performed on 46 male Wistar rats (280±20 g body weight) divided into two groups: a healthy control group and a group with RIP, 21 animals each. Separate investigation by MRI angiography was performed in four animals. All animals were kept under a standard 12 h light 12 h dark regimen at 22±2 °C, they were fed with a standard pellet diet *ad libitum* and had unrestricted access to drinking water.

Anesthesia

Prior to starting the experimental animals were anesthetized with thiopental (50-60 $mg.kg^{-1}$) administered intraperitoneally together with heparin (500 IU).

Remote ischemic preconditioning

RIP was induced by a brief occlusion applied on the right hind limb. Cessation of the blood flow in descending branches of the femoral artery was verified by magnetic resonance image (MRI) angiography in two animals from the control and two from the ischemized group. The protocol for RIP consisted of three cycles of five min limb ischemia followed by five min of reperfusion. Subsequently, hearts were rapidly excised and perfused in the Langendorff mode. Heart mitochondria were isolated and investigated after 15 min stabilization perfusion, 30 min global ischemia and 40 min of post-ischemic reperfusion (8 hearts in each situation in both experimental groups).

MRI angiography

MRI experiments were performed in 4.7 T horizontal scanner (Agilent, Yarton, UK) equipped with 400 mT/m gradient insert and DDR console. Quadrature birdcage volume coil with i.d. of 63 mm was used for excitation and signal detection. Angio3D sequence (Tr/TE/NEX=10/3ms/4) with FA of 20° 3D was used to obtain image of the vessels with pixel resolution of 0.3 mm and thk = 0.23 mm (192x192x128). The overall acquisition time was 16 min. The anesthetized animal was investigated in supine position. The ambient temperature was maintained at 33 °C by warm air during the whole measurement (SA Instruments). 3D visualization of hind leg blood vessels was performed by freely available software image 1.46r (http://imagrj.nih.gov) by maximum intensity projection method. For more details see Valkovič *et al.* (2012).

Isolation of mitochondria

Excised hearts were moistened with a small volume of ice-cold isolation solution containing in $mmol.l^{-1}$: 180 KCl, 4 EDTA, 1 % bovine serum albumin, at pH 7.4, and subsequently cut into small pieces with scissors. Minced tissue was then transferred to a beaker with 20 ml of isolation solution containing in addition 2.5 $mg.g^{-1}$ heart wet weight of protease (Sigma P-6141). After 20 min of protease treatment with mild stirring, the whole suspension was transferred to a teflon-glass homogenizer and homogenized gently for 2-3 min. After centrifugation at 1000g for 10 min, the protease containing supernatant was discarded together with the mitochondria that were in direct contact with the protease. The pellet was re-suspended in the same volume of the protease-free isolation solution, again homogenized and spun down as previously. This supernatant containing now predominantly mitochondria that were not in direct contact with protease was centrifuged at 6200g for 10 min. The pellet containing mitochondria was then again re-suspended in an albumin-free isolation solution containing in $mmol.l^{-1}$ 180 KCl and 4 EDTA. It was spun down at 6200g for 10 min and subsequently used for estimation of protein concentration according to Lowry *et al.* (1951) as well as for further biophysical and biochemical investigations.

Estimation of mitochondrial purity

Purity of the mitochondrial preparation was

tested by estimation of the markers – ATPase activities, one characteristic for sarcolemma (the Na^+/K^+-ATPase) and the another for sarcoplasmic reticulum ATPase (the Mg^{2+}/Ca^{2+}-ATPase) in the absence and presence of their specific inhibitors using the technique described in details by Ferko *et al.* (2006) and Máleková *et al.* (2007).

Estimation of membrane fluidity

Membrane fluidity of the hydrophobic region and the order parameters of isolated membranes from heart mitochondria of healthy and RIP-rats were measured at steady state conditions (at 20-24 °C) by means of a luminescence spectrometer LS 45 (Perkin Elmer, USA) using 1,6-diphenyl-1,3,5-hexatriene (DPH; 1 mmol) as a fluorescent probe (Incerpi *et al.* 1988). The steady state fluorescence anisotropy of DPH characterizes the mobility of phospholipid acyl chains in the lipid bilayer of mitochondrial membranes and it is inversely proportional to membrane fluidity (Waczulíková *et al.* 2010). Briefly, isolated mitochondria were diluted to a level at which no significant light scattering had been recorded (10 mg/ml). Each sample of mitochondrial suspension was mixed with a DPH solution (at a final concentration of 0.67 μmol.l^{-1}) and was equilibrated at 30±0.2 °C in the dark for 20 min. Then the sample was measured in a micro-cell and both the vertical and horizontal components of the intensity of the emitted light were collected. The steady-state anisotropy was determined as the mean of at least ten consecutive recordings.

Investigation of oxygen consumption, respiratory chain function and oxidative phosphorylation

Parameters of oxidative phosphorylation (OXP) in isolated heart mitochondria were determined using Oxygraph-2k (Oroboros Instruments, Austria). The measurements were performed at 37 °C in respiration medium MiRO6 (Fasching *et al*. 2014), in a 2 ml chamber. Datlab software (OROBOROS INSTRUMENTS) was used for data acquisition and analysis. State 2 and state 3 respiration with 2 combinations of substrates were evaluated. The combination of substrates glutamate (10 mmol.l^{-1}) plus malate (0.2 mmol.l^{-1}) was selected for determination of CI-linked respiration, the combination of malate (0.2 mmol.l^{-1}) plus octanoyl carnitine (0.2 mmol.l^{-1}) was used for evaluation of fatty acids oxidation.

State 2 respiration was evaluated after addition of a substrate combination to mitochondria in the Oxygraph chamber. State 3 respiration was induced by subsequent addition of saturating concentration of ADP (2 mmol.l^{-1}). Oxygen consumption was normalized to citrate synthase activity that was determined spectrophotometrically according Eigentler *et al.* (2012).

Estimation of CoQ_9 and CoQ_{10} in mitochondria

Content of oxidized isoforms of coenzyme Q (CoQ_{9ox} and CoQ_{10ox}) in the isolated mitochondria was estimated by means of high pressure liquid chromatography (HPLC, Beckmann Gold, USA) using a 250 x 4 mm i.d., 7 μm sephadex column (Sepharon SGX C18, Tessek, Czech Republic). Mobile phase consisted of the mixture methanol : acetonitril : ethanol 6:2:2 and it was applied at a flow ratio of 1 ml.min^{-1}; sample volume of 20 μl. Concentration of coenzyme isoforms was detected spectrophotometrically at λ=275 nm. All measurements were performed at room temperature. For more details see Kucharská *et al.* (1996).

Chemicals

All chemicals were purchase from Sigma-Aldrich or Lachema (Czech Republic) unless indicated otherwise.

Statistical analysis

Data were checked for normality with the Shapiro-Wilk W-test. Normally distributed variables are presented as mean ± SEM (standard error of the mean). Effect of treatment (stabilized, ischemic, reperfused) within the given group was tested with one-way analysis of variance, followed either with the Dunnett test for multiple comparisons with the control or Tukey-Kramer test for all pairwise comparisons. In the case of non-normality and/or unequal variances in between groups being compared, a non-parametric alternative (the Kruskal-Wallis test and the post hoc pairwise comparisons with the Connover-Inman test) was performed. Due to detected interaction in the analysis (see Fig. 5 and results) the treatment effects were analyzed within each group separately. The vertical group effects (i.e. within the same level of treatment) were analyzed with the unpaired t-test, if not otherwise stated. All statistical analyses were performed using StatsDirect version 2.7.8 (StatsDirect, UK).

Results

As a *conditio sine qua non* for a successful RIP

of the heart induced with limb occlusion is considered a complete cessation of blood flow in the occluded area. MRI angiographic investigation revealed a complete fulfilment of the latter demand. Figure 1 represents a typical recording from our MRI angiographic investigations. It documents the presence of blood flow in right hind limb of the rat prior to the occlusion (panel A) and its absence after onset of the ligature (panel B).

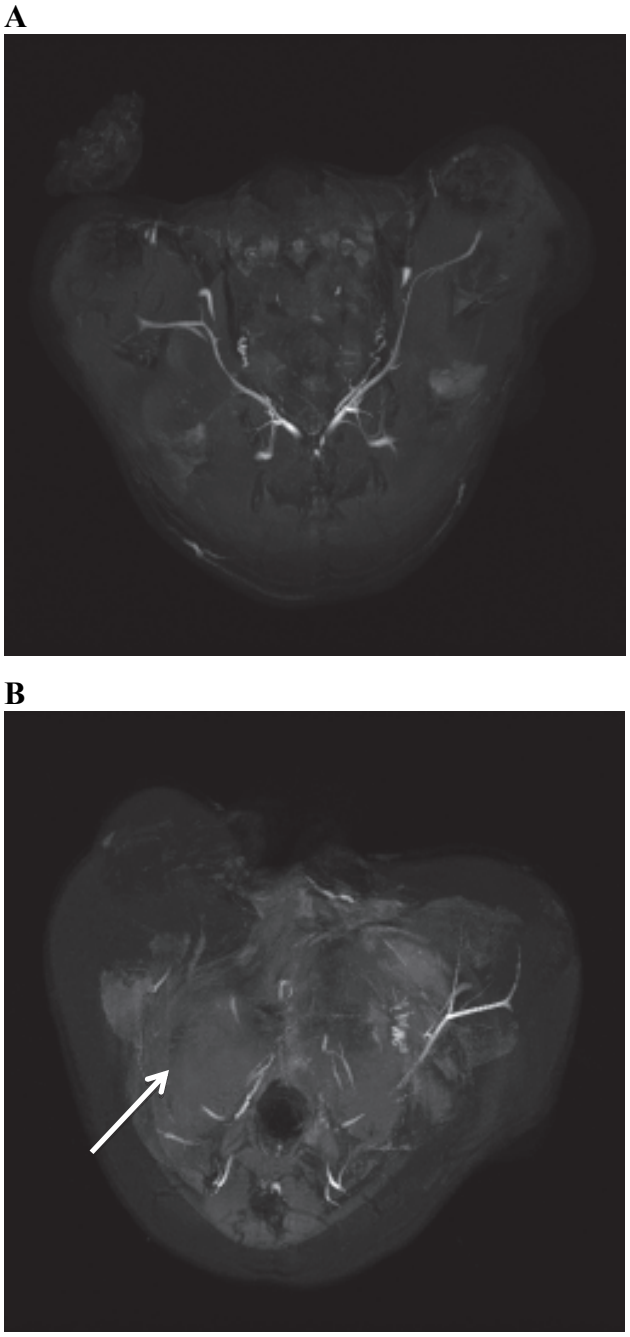

Fig. 1. Magnetic resonance images of blood flow in the occluded area of the right hind limb in rat. **Panel A** – prior to the occlusion, **panel B** – after the occlusion. Z projection of transversal slices of rat hind legs with the dorsal part of the body in a supine position. The arrow indicates stopped blood flow in descending branches of the femoral artery.

The degree of contamination of mitochondrial preparation by membranes of the sarcolemma and sarcoplasmic reticulum, estimated by presence of their marker enzymes amounted to 0.84 % and 1.59 %, respectively (data not shown).

State 2 oxygen consumption by isolated mitochondria from control- and RIP- treated hearts exposed to ischemia/reperfusion are demonstrated in Figure 2, with glutamate + malate as substrates (panel A) and with malate + octanoyl carnitine (to estimate the fatty acid oxidation) on the panel B. In state 2 respiration no significant changes were observed.

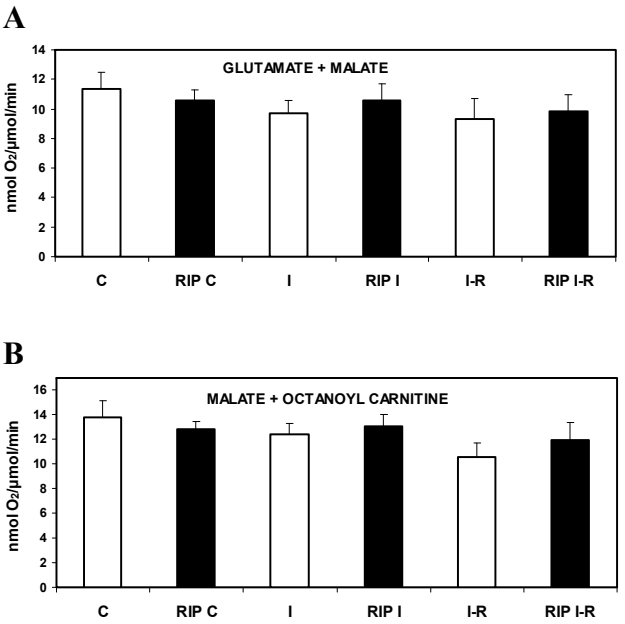

Fig. 2. State 2 oxygen consumption by isolated mitochondria from control- and RIP-treated hearts exposed to ischemia/reperfusion. Results are expressed in nmol of oxygen per µmol per min (oxygen consumption normalized to the citrate synthase activity) and presented as means ± SEM; n=7. Substrates: glutamate + malate (**panel A**), malate + octanoyl carnitine (**panel B**). Symbols: C – healthy control hearts, RIP C – remote preconditioned control hearts, I – healthy control hearts after 30 min global ischemia, RIP I – remote ischemic preconditioned hearts after 30 min global ischemia, I-R – healthy control hearts after 40 min postischemic reperfusion, RIP I-R – remote ischemic preconditioned hearts after 40 min postischemic reperfusion. Significances: none statistically significant differences between the healthy control- and RIP-treated hearts were found. For more information see the Materials and Methods.

Figure 3 presents state 3 oxygen consumption, coupled with ATP synthesis, in isolated mitochondria from control- and RIP-treated hearts exposed to ischemia/reperfusion. Data obtained with glutamate + malate are depicted on the panel A while those registered

after using malate + octanoyl carnitine are depicted on the panel B. This investigation brought about two interesting findings: i.) that mitochondria from preconditioned hearts were well preserving their capability to state 3 oxygen consumption during the ischemic phase of the ischemia/reperfusion loading test; ii.) that mitochondria oxidizing fatty acids exhibit slightly (by 13 %) higher resistance against the massive depression in mitochondrial oxygen consumption induced by postischemic reperfusion ($p<0.05$).

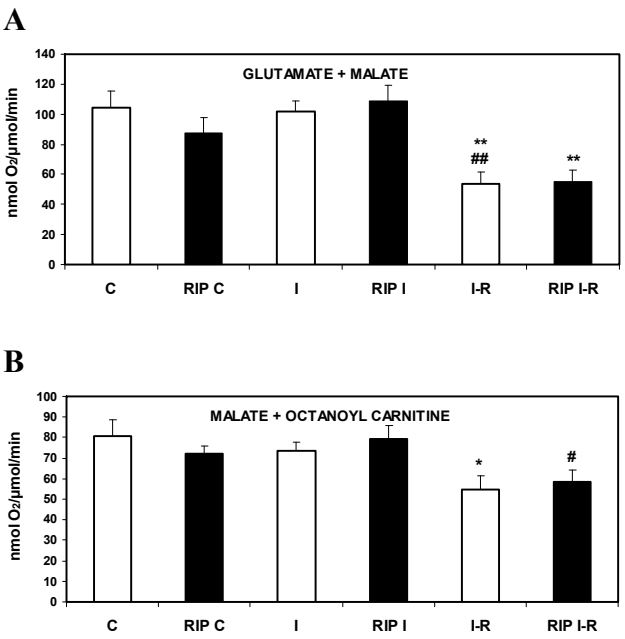

Fig. 3. State 3 oxygen consumption by isolated mitochondria from healthy control- and RIP-treated hearts exposed to ischemia/reperfusion. Results are expressed in nmol of oxygen per µmol per min (oxygen consumption normalized to citrate synthase activity) and presented as means ± SEM; n=7. Substrates: glutamate + malate (**panel A**), malate + octanoyl carnitine (**panel B**). Symbols: identical as in Fig. 2. Significances for panel A: ** $p<0.01$ C vs. I-R and RIP I vs. RIP IR, ## $p<0.01$ I vs. I-R; for panel B: * $p<0.05$ C vs. I-R and # $p<0.05$ RIP I vs. RIP I-R. For more details see the Materials and Methods.

It proved reasonable to assume that RIP might also provide some protection to the heart mitochondria against the attack of free radicals associated with both, application of the ischemia/reperfusion loading test and with the RIP itself. Our results depicted in Figure 4 (panel A) showed a marked trend for a gradual increase in the oxidation of the CoQ_{9ox} isoform of coenzyme Q present in the mitochondria from healthy control hearts during the ischemia/reperfusion test. Mitochondria from RIP treated hearts seemed to exhibit relatively high level of CoQ_{9ox}. However, it appears that the preconditioning prevents the ischemia/reperfusion-associated increase in CoQ_{9ox} oxidation. Investigation of CoQ_{10ox} formation in mitochondria from control and RIP-treated hearts (Fig. 4, panel B) yielded less marked results.

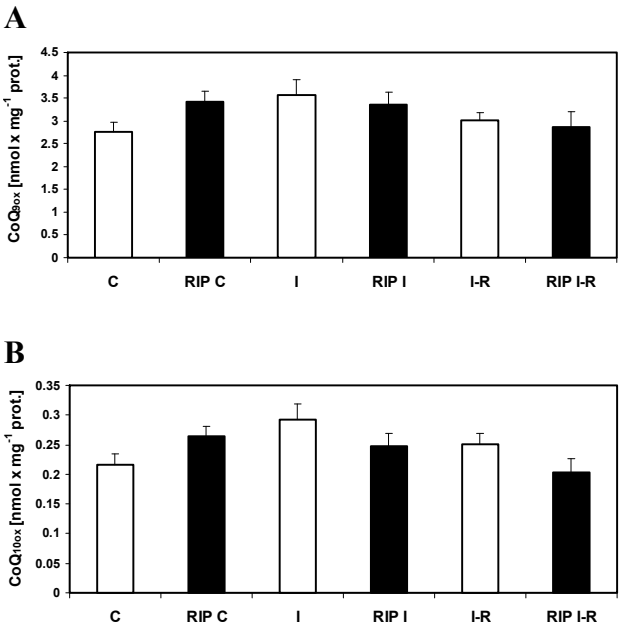

Fig. 4. Changes in oxidized form of coenzyme Q_9 (Q_{9ox}) (**panel A**) and Q_{10} (Q_{10ox}) (**panel B**) in isolated mitochondria from healthy control- and RIP-treated hearts exposed to ischemia/reperfusion. Results are means ± SEM; n=7, and are expressed in $nmol.mg^{-1}$ prot. Symbols: identical as in Fig. 2. Significances: none statistically significant differences between the healthy control and RIP treated hearts were found. For more details see the Materials and Methods.

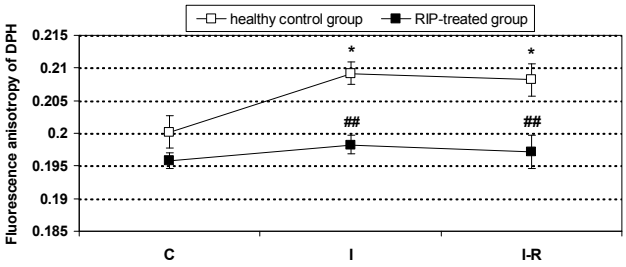

Fig. 5. RIP-induced changes in membrane fluidity of isolated heart mitochondria exposed to ischemia/reperfusion. Results are means ± SEM; n=7 and are expressed in units of optical anisotropy. Symbols: C – controls prior to induction of ischemia, I – results immediately after 30 min of ischemia, IR – results after 40 min of postischemic reperfusion, □ – healthy control group, ■ – RIP-treated group. Significances: C vs. RIP prior the onset of ischemia – borderline significance p-value (unpaired t test), combined standard error=0.002736; control hearts between themselves (using the global significance test): * $p<0.05$ C vs. I of healthy control group and C vs. IR of healthy control group; ## $p<0.01$ RIP I vs. I of healthy control group and RIP IR vs. IR of healthy control group.

The idea that RIP supposedly influences the properties and function of cardiac mitochondria was based on the notion that the signals released from the place of remote occlusion might reach the mitochondrial membrane. Hence, the function of respiratory chain localized in the mitochondrial membrane might be modulated by its properties. For this reason we focused our interest to find out whether RIP-induced modulation of mitochondrial functions would also involve changes in the mitochondrial membrane fluidity. Results achieved by means of the ischemia/reperfusion control test (Fig. 5) indicated significantly increased anisotropy i.e., decreased fluidity of the mitochondrial membranes from non-preconditioned hearts while RIP-treated hearts preserved the membrane fluidity of their mitochondria.

Discussion

Despite recent dramatic progress in the prevention and treatment, ischemic heart disease (IHD) still remains the leading cause of mortality in the developed countries. In more than 90 % of cases, IHD is a consequence of reduced coronary blood flow secondary to obstructive atherosclerotic vascular disease. The high mortality and morbidity rates substantiate the search for further innovative modes of treatment continued. Ischemic preconditioning (IP) of the heart, first described by Murry *et al.* (1986) constitutes such a promising modality for treatment of IHD. In the course of time IP has been developed in an effective procedure that increases the tolerance of the myocardium to ischemia.

In the view of this fact the IP has become an effective procedure in cardioprotective therapy. The effect of IP has been proved in animal experiments by reduced myocardial infarct size, suppressed arrhythmias, decreased ventricular tachycardia and etc. (Ravingerová *et al.* 2000). However, because of problems resulting from its invasive character IP is not devoid of complications (Čarnická *et al.* 2013). Despite the several studies pointing at cardioprotective effects of IP on animal experiments, proving in human is still needed. The effective clinical use of IP requires detailed understanding and monitoring of cellular and molecular mechanisms at the level of upregulation of cardioprotective genes (Guo *et al.* 1998, Bolli 2007). Nevertheless, it still represents a promising way of protective treatment even from a clinical perspective (Chinybayeva *et al.* 2013).

Przyklenk *et al.* (1993) introduced an alternative strategy to afford a similar increase in the ischemic tolerance of the myocardium in a non-invasive way – RIP of the heart. This technique is based on ischemic impulses induced by the occlusion of vessels in a distant locus, for instance on the limb or leg, without surgical intervention. Thus IP provides a biphasic cardioprotection: an immediate one spanning 3 h and reappearing again after 24 h in a second window lasting for 3 days. Since its introduction RIP drove much attention between experimenters and clinicians. There were described various techniques for induction of RIP activating several signaling pathways which might be involved in its mechanism of action and extensively investigated its protective effects on levels of the heart function and metabolism (Przyklenk and Whittaker 2011, Čarnická *et al.* 2013). Especially the protection of cardiac energetics is widely discussed by researchers, but only incomplete direct data are available about the preconditioning-induced changes in myocardial energy household. Even the classic studies of Murry *et al.* (1990) performed in hearts with IP as well as of Takaoka *et al.* (1999) and Abdul-Ghani *et al.* (2013) in RIP-treated hearts were dealing with myocardial energetics predominantly on the cellular (tissue) level.

Participation of mitochondria in the protection of the myocardium against hypoxia and ischemia was first demonstrated in rat hearts partially adapted to streptozotocin-induced experimental diabetes, a disease associated with pseudohypoxia. Pseudohypoxia is defined as the aberrant activation of hypoxia response pathways under normal oxygen conditions (Williamson *et al.* 1993, Ferko *et al.* 2006, Ziegelhöffer *et al.* 2002, 2009, 2012, Muráriková *et al.* 2013, Takiyama and Haneda 2014).

Cao *et al.* (2011) reported that the mitochondrial Ca^{2+} activated K^+ channel contributed to cardioprotection by the limb RIP in rat. Basing on these findings we have assumed that the cardioprotective mechanisms triggered by RIP might also involve increased ischemia resistance on the level of respiratory functions and membrane properties of mitochondria. Hence, from the aspect of cardiac mitochondria, our data about changes in the properties and function of heart mitochondria represent a pilot study on the involvement of these organelles into RIP-induced cardioprotection.

Simultaneously, in order to present investigation of biochemical parameters which would be focused on mitochondria and energetic metabolism, we also measured postischemic contractile restoration of hemodynamic parameters, arrhythmias and size of

infarction in the hearts of both groups, control animals and those subjected to RIP. RIP group in comparison with unaffected control group exerted better restoration of the contractile function as suggested by better restoration of the left ventricular developed pressure (left ventricular systolic pressure minus end-diastolic developed pressure). The decrease in restoration of end-diastolic developed pressure indicated reduction in postischemic contracture. In accordance with these preliminary results, we can conclude that hearts of RIP group exhibited better restoration of coronary flow, which indicates better endothelial function. Focusing on the infarct size, RIP applied to the right hind limb reduced the size of infarction up to three times in comparison with the control group. These results are in accordance with results obtained by other authors (Kristiansen *et al.* 2005, Lim *et al.* 2010, Costa *et al.* 2013).

Up to now, no optimal technique for induction of RIP of the heart has been proposed. In our study we employed the protocol of preconditioning with three 5 min cycles of occlusion, each followed by 5 min reperfusion of descending branches of the femoral artery on the right hind limb of the rat.

The protocol using 3 cycles of 5 min limb ischemia and reperfusion was evaluated by researchers as the most suitable based on evaluation of biochemical parameters (significantly decreased plasma creatine kinase-MB activity, significantly increased ATP/ADP ratio, content of ATP, myocardial content of total adenine nucleotides, etc.) and also on the arrhythmia diagnosis (significant delay in arrhythmia onset, decrease number of different types of ventricular arrhythmias) compared to I/R group (Ahmed *et al.* 2012). The 3-cycle model of RIP described in the Materials and Methods part seems to be effective also for clinical trials (Hoole *et al.* 2009, Xie *et al*. 2011, Ahmed *et al.* 2013, Slagsvold *et al*. 2014).

Efficacy of the occlusion was verified by means of MRI angiography which revealed a complete cessation of blood flow in the occluded area (Fig. 1).

We focused our interest to the investigation of the most sensitive functional parameters, namely those describing oxygen consumption by mitochondria. The investigation of the basal, state 2 respiration, as well as the activated, state 3 mitochondrial respiration coupled with ATP generation revealed only a non-significant trend for better preservation of oxygen consumption ability of the mitochondria from RIP-treated hearts in comparison with the controls. This trend was manifested by the higher state 3 oxygen consumption during the ischemic phase and by 13 % higher resistance against the depression in oxygen consumption during the reperfusion phase of the ischemia/reperfusion loading test and it was more apparent in experiments using malate + octanoyl carnitine as substrates. The less expressed effect of RIP observed in our experimental conditions may be explained by the fact discovered very recently by Prasad *et al.* (2013), who revealed that RIP applied immediately before percutaneous coronary intervention would not impact myocardial necrosis, inflammatory response and circulating endothelial progenitor cell counts. Analogously if similar events occured also in our experiments then a significant protective effect of RIP on the functional parameters of cardiac mitochondria could develop in the second window of this type of preconditioning only. The later assumption is strongly supported with the finding that in none of preconditioned controls the ischemia/reperfusion loading test induced a significant decrease in mitochondrial membrane fluidity while in RIP-treated hearts that decrease remained completely eliminated. It was shown that, *via* changes in the fluidity of the lipid bilayer, the heart mitochondria may exert a strong regulatory influence on the mobility of the membrane-bound systems and, in this way, to control their function (Ziegelhöffer *et al.* 2012). Therefore, *via* maintaining of the membrane fluidity at an optimum, RIP may eliminate at least a part of the ischemia/reperfusion injury and in this way may exert protective effect on the heart mitochondria.

Conclusion

Our study presents, for the first time, an evidence that the endogenous protective mechanisms triggered in the myocardium by RIP also concern cardiac mitochondria. We assume that the observed immediate protective effect manifested by a trend for better preservation of mitochondrial oxygen consumption coupled with ATP generation (S3) is more developed in the second window of RIP-induced cardioprotection. A significant and prognostically beneficial protective effect of RIP on the heart mitochondria is reflected in a total elimination of ischemia/reperfusion-induced massive depression of mitochondrial membrane fluidity.

Conflict of Interest

There is no conflict of interest.

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