Participation of Heart Mitochondria in Myocardial Protection Against Ischemia/Reperfusion Injury: Benefit Effects of Short-Term Adaptation Processes

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Summary
Acute streptozotocin diabetes mellitus (DM) as well as remote ischemic preconditioning (RPC) has shown a favorable effect on the postischemic-reperfusion function of the myocardium. Cardioprotective mechanisms offered by these experimental models involve the mitochondria with the changes in functional properties of membrane as the end-effector. The aim was to find out whether separate effects of RPC and DM would stimulate the mechanisms of cardioprotection to a maximal level or whether RPC and DM conditions would cooperate in stimulation of cardioprotection. Experiments were performed on male Wistar rats divided into groups: control, DM, RPC and DM treated by RPC (RPC+DM). RPC protocol of 3 cycles of 5-min hind limb ischemia followed by 5-min reperfusion was used. Ischemic-reperfusion injury was induced by 30-min ischemia followed by 40-min reperfusion of the hearts in Langendorff mode. Mitochondria were isolated by differential centrifugation, infarct size assessed by staining with 1 % 2,3,5-triphenyltetrazolium chloride, mitochondrial membrane fluidity with a fluorescent probe DPH, CoQ9 and CoQ10 with HPLC. Results revealed that RPC as well as DM decreased the infarct size and preserved mitochondrial function by increasing the mitochondrial membrane fluidity. Both used models separately offered a sufficient protection against ischemic-reperfusion injury without an additive effect of their combination.

Key words
Remote ischemic preconditioning • Acute streptozotocin diabetes mellitus • Heart mitochondria • Membrane fluidity • Infarct size

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Introduction
Both acute streptozotocin-induced diabetes mellitus (DM) and remote ischemic preconditioning (RPC) represent a suitable model for investigation of adaptive mechanisms leading to the increase of ischemic tolerance and to the reduction of extent of ischemic-reperfusion injury in the myocardium (Ferdinandy et al. 2014, Przyklenk et al. 1993, Ravingerová et al. 2000, Tosaki et al. 1996). The rationale for studying the experimental models is based on the established knowledge of the cardioprotection method induced by ischemic preconditioning. RPC, which is induced by short ischemic and reperfusion periods on a distant tissue such as a limb, prevents myocardial ischemic-reperfusion injury by the activation of humoral (Lim et al. 2010), neural (Mastitskaya et al. 2012) and/or systemic pathways.
involving crosstalk with immune cells (Przyklenk and Whittaker 2011). Up-regulation of survival signal pathway converges on the mitochondria as an RPC end-effector (Veighhey and MacAllister 2012). Preconditioning (PC)-like protection observed in the experimental model of streptozotocin-induced DM was manifested in the acute phase of the disease, when the alterations caused by complications are not fully developed (Ravingerová et al. 2000). Increased resistance to ischemia of the diabetic myocardium is attributed to the alterations in the function of subcellular structure and metabolism of cardiac cells including resistance to Ca$$^{2+}$$ overload (Tani and Neely 1988, Tribulová et al. 1996), formation of mitochondrial permeability transition pores (mPTP) (Bakker et al. 1993, Ziegelhöffer-Mihalovičová et al. 1997) and remodellation of sarcolemmal (Ziegelhöffer-Mihalovičová et al. 2003) and mitochondrial membranes (Ferko et al. 2008) associated with the change in their fluidity. Apparently, the membrane fluidity seems to be critical to the function and adaptation of cardiomyocytes thus forming a part of the process of the endogenous protective mechanisms. The concept of fluidity as applied to membranes usually refers to a molecular mobility and ordering of phospholipid acyl chains inside the lipid bilayer. Fluidity essentially depends on the protein and lipid composition, especially on the cholesterol-to-phospholipid content, as well as on the extent of free radicals-induced lipid peroxidation (Waczulíková et al. 2010). In diabetic rat hearts, the sarcolemmal membrane was proved to be less fluid owing to nonenzymatic glycosylation of membrane biomolecules which, on the other hand, protects the myocardium against Ca$$^{2+}$$ overload (Ziegelhöffer-Mihalovičová et al. 2003). Unlike the sarcolemma, mitochondrial membrane, exhibits an increased fluidity thus improving ATP transport from the mitochondrial matrix into the cytosol of the cardiomyocytes. Such a positive fluidising effect observed in DM (Ferko et al. 2008), in the mitochondrial membrane could also be observed in the protective mechanism induced by RPC (Ferko et al. 2014). The described cardioprotective effect of RPC and PC-like protection have usually been accompanied by a reduced infarct size (Heusch 2015, Ravingerová et al. 2010). The presented paper is aimed to verify whether RPC and DM separately would offer a maximal possible cardioprotective effector and whether they would cooperate in stimulation of cardioprotection. For this purpose the simultaneous action of streptozotocin-induced DM and RPC has been investigated with the attention focused on the functional properties of mitochondrial membrane, i.e. membrane fluidity and formation of oxidized form of coenzyme Q (CoQ). At the same time, the postischemic-reperfusion functional properties of myocardium were investigated by measuring the myocardial infarct size.

**Methods**

**Animals**

Male Wistar rats (9-11 weeks old, 220±20 g b. wt.) were used for this study. They were randomly divided into four groups of seven animals each to form a control group (C), a diabetic group (DM), a remote ischemic preconditioning group (RPC) and a diabetic group treated by remote ischemic preconditioning (DM+RPC). All animals were kept under the standard light regimen (D:L, 12:12) at 22±2 °C in a cage (max 5 animals per cage) with access to water and to a standard pellet food ad libitum. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Animal Health and Animal Welfare Division of the State Veterinary and Food Administration of the Slovak Republic.

**Induction of diabetes mellitus**

Acute diabetes mellitus was induced by a single dose of streptozotocin (65 mg.kg$$^{-1}$$, i.p.) dissolved in 0.1 mol.l$$^{-1}$$ citrate buffer, pH 4.0. Diabetic state of rats was monitored daily during eight days by measuring of glycosuria using Gluko PHAN strips (Erba-Lachema, Brno, Czech Republic) and by estimation of glucose after excision of heart (MultiCare, Biochemical system international, Florence, Italy) and glycohemoglobin (BIO-LA-TEST, GHB 100, Pliva-Lachema, Brno, Czech Republic) in the blood, as well as cholesterol and triacylglycerols (MultiCare, Biochemical system international, Florence, Italy) in the serum. Serum insulin was determined by the commercial RIA kit (Linco Research USA).

**Induction of remote ischemic preconditioning**

Animals were anesthetized by intraperitoneal injection of thiopental (50-60 mg.kg$$^{-1}$$) administered together with heparin (500 IU). RPC was evoked by inflation (to 200 mm Hg) of pressure-cuff placed around the right hind limb followed by cuff deflation. This intervention led to cessation and renewal of the blood flow in descending branches of the femoral artery. RPC
protocol of 3 cycles of 5 min hind limb ischemia followed by 5 min of reperfusion was used.

**Perfusion of the isolated heart**

After anesthetization of the animals (as described in the previous paragraph) and opening the chest cavity, hearts were rapidly excised, placed in ice-cold perfusion buffer and Langendorff perfused with oxygenated (95 % O₂ and 5 % CO₂) Krebs-Henseleit buffer containing (in mM): NaCl 118, KCl 3.2, MgSO₄ 1.2, NaHCO₃ 25, NaH₂PO₄ 1.18, CaCl₂ 2.5, glucose 11.1, pH 7.4 at 37 °C. Perfusion was maintained at a constant pressure of 70 mm Hg and perfusion protocol consisted of 25 min stabilization perfusion, 30 min global ischemia and 40 min of post-ischemic reperfusion. Isolated hearts of all groups were taken at the end of each stage of perfusion protocol for further investigation. For the investigation of mitochondrial processes utilized in the present study, the model of global ischemia is more appropriate for technical reasons. While in the model of regional ischemia, left anterior descending (LAD) occlusion, ischemic (risk) area represents approximately 40 % of the size of the left ventricle, in the model of global ischemia, the whole heart is ischemic.

**Isolation of mitochondria**

Isolated hearts were cut into small pieces by scissors and damped with 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 tissues of hearts were suffused with 20 ml of ice-cold IS with addition of protease 2.5 mg.g⁻¹ of tissue (Sigma P-6141) and mild stirred for 20 min. Subsequently tissues were homogenized gently in teflon/glass homogenizer for 2-3 min. After 10 min centrifugation at 1000g the obtained supernatant together with the part of mitochondria, which were in direct contact with protease, was poured out. Pellet containing mitochondria was homogenized again in the same volume of ice-cold IS without protease and spinned down as previously. Obtained supernatant containing predominantly mitochondria was centrifugated at 6200g for 10 min and pellet was re-suspended again in an albumin free isolation solution (IS, containing in mmol.l⁻¹: 180 KCl, 4 EDTA). The final mitochondrial fraction 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. The whole isolation procedure was performed at 4 °C.

**Membrane fluidity**

For evaluation of the membrane fluidity of isolated heart mitochondria the parameter of steady-state fluorescence anisotropy of a lipophilic fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH, Sigma-Aldrich) (Incerpi et al. 1988) was used. A degree of steady state DPH fluorescence anisotropy characterizes the mobility of phospholipid acyl chain in the lipid bilayer of a membrane and is inversely proportional to membrane fluidity (Shinitzky 1984). For DPH measurements, mitochondria were resuspended in an isotonic buffer containing KCl 180 mmol.l⁻¹, EDTA 4 mmol.l⁻¹ (adjusted to pH 7.4) at a level at which no significant light scattering had been recorded (10 mg.ml⁻¹). Diluted mitochondrial suspension was subsequently labeled with a 0.25 µmol.l⁻¹ solution of DPH and stored at 30±0.2 °C in the dark for 20 min to allow a complete incorporation of the probe molecules into the membranes. Steady-state fluorescence anisotropy was measured with a luminescence spectrometer LS 45 (Perkin Elmer, USA) with the excitation wavelength set at 360 nm and with the wavelength of 425 nm for detection of emission. Fluorescence anisotropy of DPH as an indicator inversely proportional to the membrane fluidity was determined as the mean of at least ten consecutive recordings.

**Determination of coenzyme Q (CoQ₉-ox and CoQ₁₀-ox)**

Content of oxidized isoforms of coenzyme Q (CoQ₉-ox and CoQ₁₀-ox) 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 SGXC18, Tessek, Czech Republic). The mobile phase consisted of the mixture methanol : acetonitrile : ethanol 6:2:2 and was applied at a flow ratio of 1 ml.min⁻¹; sample volume of 20 µl. Concentration of coenzyme isoforms was detected spectrophotometrically at the wavelength of 275 nm. All measurements were performed at room temperature. For more details see Kucharská et al. (1996).

**Infarct size determination**

The infarct size (IS) area was delineated by staining with 1 % 2,3,5-triphenyltetrazolium chloride dissolved in 0.1 M phosphate buffer (pH 7.4). The hearts were cut perpendicularly to the long axis of the ventricle into the 1 mm thick slices and stored overnight in 10 %
neutral formaldehyde solution. IS was determined by a computerized planimetric method as described earlier (Ravingerová et al. 2007) and was expressed as a percentage of the area at risk (AR) size.

Statistical analysis

Data were checked for normality with the Shapiro-Wilk W-test. Precision of the estimated mean was evaluated by standard error. 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 with the Tukey-Kramer test for all pairwise comparisons. In the case of non-normality and/or unequal variances among groups being compared, a non-parametric alternative (the Kruskal-Wallis test and the post hoc pairwise comparisons with the Conover-Inman test) were performed. The effects 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).

Table 1. Metabolic parameters of rats with experimental streptozotocin-induced diabetes mellitus.

<table>
<thead>
<tr>
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<th>Control group</th>
<th>Diabetic group</th>
</tr>
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<tbody>
<tr>
<td>Glucose (mmol.L⁻¹)</td>
<td>5.19 ± 0.14</td>
<td>17.94 ± 0.86*</td>
</tr>
<tr>
<td>Triacylglycerols (g.L⁻¹)</td>
<td>1.27 ± 0.11</td>
<td>4.54 ± 0.37*</td>
</tr>
<tr>
<td>Cholesterol (g.L⁻¹)</td>
<td>1.73 ± 0.10</td>
<td>2.65 ± 0.12*</td>
</tr>
<tr>
<td>Glycohemoglobin (%Hb)</td>
<td>4.09 ± 0.10</td>
<td>7.62 ± 0.96*</td>
</tr>
<tr>
<td>Insulin (ng.ml⁻¹)</td>
<td>1.08 ± 0.18</td>
<td>0.48 ± 0.07*</td>
</tr>
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</table>

Results are given as means ± SEM; n=12, * p<0.01 diabetic vs. control group.

Results

In order to investigate a potential simultaneous impact of streptozotocin-induced DM and RPC on the postischemic-reperfusion functional properties of myocardium and its subcellular organelles mitochondria the myocardial infarct size area and mitochondrial membrane fluidity was measured and the content of coenzyme Q in oxidized form was determined. The main purpose of this study was to assess whether the experimental model of acute streptozotocin-induced DM and RPC separately would offer cardioprotection in a higher level or whether their simultaneous action would result in the additive effect in cardioprotection.

Diabetic state of experimental animals was confirmed by a significant (p<0.01) increase in blood glucose, triacylglycerols, cholesterol and content of glycohemoglobin as well as in a decrease in blood levels of insulin (Table 1).

The results shown in Figure 1 represent the infarct size normalized to the size of the left ventricle from control, diabetic, RPC and RPC+DM group. In the diabetic rats, the IS/LV was smaller (46.19 %), but did not differ significantly from RPC rats (35.26 %). The protective combined effect of DM and RPC was also observed (decrease in IS/LV by 35.8 %), but was less explicit compared to RPC and diabetic group affected separately.
Table 2. Changes in the functional properties of rat heart mitochondria induced by DM-, RPC-, RPC+DM-caused remodeling.

<table>
<thead>
<tr>
<th></th>
<th>Stabilization</th>
<th>Ischemia</th>
<th>Reperfusion</th>
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<tr>
<td><em>Fluidity of mitochondrial membrane [arb. unit]</em></td>
<td></td>
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</tr>
<tr>
<td>C</td>
<td>4.88 ± 0.07</td>
<td>4.71 ± 0.06</td>
<td>4.63 ± 0.05</td>
</tr>
<tr>
<td>DM</td>
<td>5.08 ± 0.06</td>
<td>4.72 ± 0.04</td>
<td>4.71 ± 0.04</td>
</tr>
<tr>
<td>RPC</td>
<td>4.99 ± 0.04</td>
<td>4.90 ± 0.05*</td>
<td>4.87 ± 0.07**</td>
</tr>
<tr>
<td>RPC+DM</td>
<td>4.88 ± 0.05</td>
<td>4.76 ± 0.05</td>
<td>4.78 ± 0.04</td>
</tr>
</tbody>
</table>

| *Content of oxidized form of coenzyme Q9 in mitochondria [nmol/mg of protein]* |       |              |             |
| C                | 2.60 ± 0.09   | 3.72 ± 0.33** | 3.02 ± 0.17 |
| DM               | 3.48 ± 0.49**  | 4.08 ± 0.26   | 3.66 ± 0.244 |
| RPC              | 3.42 ± 0.23+  | 3.34 ± 0.29   | 3.04 ± 0.22 |
| RPC+DM           | 3.31 ± 0.14++ | 4.25 ± 0.48   | 3.45 ± 0.27 |

| *Content of oxidized form of coenzyme Q10 in mitochondria [nmol/mg of protein]* |       |              |             |
| C                | 0.207 ± 0.01  | 0.278 ± 0.03* | 0.258 ± 0.01 |
| DM               | 0.259 ± 0.03  | 0.291 ± 0.02  | 0.291 ± 0.02 |
| RPC              | 0.255 ± 0.01  | 0.247 ± 0.02  | 0.213 ± 0.02 |
| RPC+DM           | 0.248 ± 0.01  | 0.324 ± 0.04  | 0.263 ± 0.26 |

Results are given as means ± SEM; n=8. DM – diabetic group, RPC – group with remote ischemic preconditioning, C – control group, DM+RPC – group of streptozotocin-induced d. mellitus affected by remote ischemic preconditioning. * p<0.05 RPC after 30 min ischemia vs. C after 30 min ischemia; ** p<0.01 DM after 40 min reperfusion vs. C after 40 min reperfusion, *** p<0.01 RPC after 40 min reperfusion vs. C after 40 min reperfusion. * p<0.05 RPC after stabilization perfusion and C after 30 min ischemia vs. C after stabilization perfusion, ** p<0.01 DM after stabilization perfusion, RPC+DM after stabilization perfusion and C after 30 min ischemia vs. C after stabilization perfusion

Membrane fluidity was decreased due to ischemic-reperfusion injury in all groups. DM, RPC and RPC+DM prevented the decline observed in the control group. Isolated mitochondrial membranes from diabetic and RPC rat hearts after reperfusion phase exhibited a 5.18 % (p<0.01) and 1.73 % increase in the membrane lipid fluidity in comparison with control group (Table 2). RPC+DM caused a 3.24 % increase in the fluidity of mitochondria isolated from hearts after 40 min reperfusion (Fig. 2). However, the observed effect did not reach the level of RPC.

We observed a 33.85 %, 31.54 % and 27.31 % increase in the concentration of CoQ9-ox caused by DM, RPC and RPC+DM, respectively, in comparison with
control group after the stabilization perfusion (Table 2, Fig. 3). The content of CoQ$_{9\text{-ox}}$ was significantly increased (by 43.70 %) in the control group, while it was not changed in the group of DM, RPC, RPC+DM after the ischemic phase. The ischemic-reperfusion injury had no significant effect on CoQ$_{9\text{-ox}}$ concentration in the heart mitochondria isolated from control, RPC and RPC+DM group. Unlike these groups, DM group exhibited a significant increase in CoQ$_{9\text{-ox}}$ concentration under the ischemic-reperfusion condition (Table 2). The similar results were observed in the measurement of CoQ$_{10\text{-ox}}$ concentration in the mitochondria isolated from heart (Fig. 4).

**Discussion**

As it has already been mentioned, impulses such as acute streptozotocin-induced DM and RPC trigger mechanisms of endogenous protection that help reduce myocardial injury caused by ischemia (Birnbaum et al. 1997, Kharbanda et al. 2002, Oxman et al. 1997, Ravingerová et al. 2010, Shi and Vinten-Johansen 2012). These cardioprotective mechanisms have not been fully elucidated, even if they are known to be associated with the changes at the level of mitochondrial membrane (Ferko et al. 2008, Kristiansen et al. 2005, Turrell et al. 2014). Further, there is no unambiguous information about sufficiency of cardioprotection offered by streptozotocin-induced DM and by RPC adaptation processes. Therefore, we aimed to find out the limits of the separate action of DM and RPC and to verify if their effect can be stimulated by each other.

For this purpose we examined the alterations in the fluidity of mitochondrial membranes using a fluorescent probe as a marker of membrane fluidity and in the free radicals production evaluated by the content of oxidized forms CoQ$_{9\text{-ox}}$ and CoQ$_{10\text{-ox}}$ in RPC and DM conditions. We were also interested in whether the observed changes would correlate with physiological state of myocardium evaluated by the determination of the infarct size.

After ischemic-reperfusion phase, we observed a significant anti-infarct effect of RPC on the investigated physiological parameters. Similarly, the results from diabetic hearts showed a significant reduction in the infarct size in comparison with the control group. The combination of DM and RPC suppressed the anti-infarct effect, although the size of infarct area was lower than that in the control group not subjected to the RPC. The important finding is that both RPC and DM induced an increased ischemic tolerance to the ischemic-reperfusion injury. Moreover, the acute phase of type I DM was accompanied by an increased tolerance to ischemia. Nevertheless, RPC in diabetic hearts had no notable additive protective effect. We assume that the protective cascade responsible for the RPC was already activated during the acute phase of diabetes and therefore the additional activation RPC could not be so explicit.

In the previous study, it was documented that mitochondria isolated from acute diabetic hearts exhibit increased membrane fluidity (Ferko et al. 2008). The observed membrane fluidization, which can be interpreted as enhanced flexibility of acyl-chains in the hydrocarbon core of lipid bilayer, can be viewed as the process of endogenous protective mechanisms (Ziegelhöffer et al. 2012, Ferko et al. 2008). This finding was associated with an increase in the mitochondrial ATP-synthase activity, in oxidized forms of the coenzyme Q and with a decrease in the basal and ADP-stimulated mitochondrial respiration, in the respiratory control index, in the rate of phosphorylation (Ferko et al. 2006) and in the mitochondrial transmembrane potential (Waczuliková et al. 2007). It has been suggested that these alterations participate in the remodeling of mitochondrial membrane, which leads to preservation of myocardial postischemic function. The results of the present study reporting the increased mitochondrial fluidity from acute diabetic hearts exposed to ischemic-reperfusion injury are in accordance with the previous suggestion. The improved functional parameters during post-ischemic reperfusion were also confirmed by a study of Muráriková et al. (2013), where a significant increase in the activity of ATP synthase was observed. RPC, as well as DM conditions completely eliminated the significant decrease in mitochondrial membrane fluidity caused by ischemia and postischemic reperfusion. Moreover, the average change in the membrane fluidity caused by RPC was higher in its absolute value than the change caused by DM. We assume that the permanent persistence of myocardium in the state of pseudohypoxia might be associated with the minor fluidising effect of acute streptozotocin-induced DM (Ziegelhöffer et al. 2002, 2009, Williamson et al. 1993). Moreover, DM with RPC did not affect additively, nevertheless their combination evoked a significant increase in mitochondrial membrane fluidity in comparison with the control group.

Our data from all groups exhibited an increase in
the mitochondrial content of CoQ$_{9}$-ox and CoQ$_{10}$-ox after the stabilization phase, which persisted after the reperfusion phase except for DM group where we found a significantly increased content of the coenzyme. The DM-caused increase in free radical production was moderated by RPC, which was evidenced by a decrease in CoQ$_{9}$-ox and CoQ$_{10}$-ox contents in the heart mitochondria after ischemic-reperfusion injury. However, the increased free radical production was not accompanied with a rigidization of the mitochondrial membrane. It suggests that formed free radicals acted as signal molecules leaving the mitochondria through the chloride channel and triggering the expression of hypoxic genes (Kalakech et al. 2013, Ziegelhöffer et al. 2009).

The results show that the mitochondrial membrane fluidization as a part of the process of endogenous protection is limited and its maximal level was observed in separate action of RPC and DM.

**Conclusion**

In general, the concept of fluidity is interpreted as permissiveness of the architecture to continuous, dynamic restructuring of the clusters according to the needs of the cell and as evoked by the alterations in the environment conditions (Vereb et al. 2003). Our findings confirm the positive participation of biophysical membrane remodeling of heart mitochondria in the process of endogenous protection resulting in a partial adaptation to ischemic conditions. The fluidizing effect of the mitochondrial membrane observed in the acute diabetic and RPC cardiac mitochondria appears to be a significant component of cardioprotection. Membrane fluidization facilitates transport of ATP from mitochondria to cytoplasm in the cardiomyocytes. Proven compensatory mechanisms are reflected in the sustained higher capacity of the mitochondria to produce ATP for the increased energy demand during posts ischemic reperfusion.

Processes of a short-term adaptation such as acute streptozotocin DM and RPC induced a beneficial signal sufficient enough to trigger the process of protection myocardium against ischemic-reperfusion injury. No additive effect was observed in the group subjected to the combination of DM and RPC, which points to the possibility of triggering the same chain of events by both conditions.

**Conflict of Interest**

There is no conflict of interest.

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