Abstract. – OBJECTIVE: Ischemic postconditioning (IPO) reduces lethal reperfusion injury under normal conditions, but its effectiveness is blocked by hypercholesterolemia (HC). This study aims to determine whether blocking the mitochondrial permeability transition pore (mPTP) with cyclosporine-A (CsA) can restore cardioprotection of IPO in hypercholesterolemic rat heart.

MATERIALS AND METHODS: Isolated rat hearts underwent 30 min global ischemia and 120 min reperfusion. Postconditioning protocol was induced by six cycles of 10s ischemia and 10s reperfusion at the onset of the reperfusion. CsA (0.5 µM or 5 µM) was administered 15 minutes before ischemia. Myocardial infarct size was estimated by triphenyltetrazolium chloride (TTC) staining. Cardiomyocyte apoptosis was assessed by TUNEL staining and creatine kinase-MB (CK-MB) was analyzed from coronary effluent.

RESULTS: In normocholesterolemia (NC) groups, infarct size, cardiomyocyte apoptosis rate and release of CK-MB were significantly reduced after IPO. These reductions were completely abolished by HC, as evidenced by a similar infarct size, cardiomyocyte apoptosis rate and release of CK-MB observed between IPO-HC group and control-NC group, but were restored by IPO combined with CsA treatment. However, CsA treatment alone could not restore cardioprotection in a state of HC. CONCLUSIONS: Ischemic postconditioning, blocked by hypercholesterolemia may due to the excessive opening of the mPTP. Inhibiting of the mPTP with CsA is able to reverse this loss of cardioprotection.

Key Words: Mitochondrial permeability transition pore, Cyclosporine-A, Ischemic postconditioning, Hypercholesterolemia.

Introduction

Short cycles of ischemia-reperfusion performed at the onset of reperfusion, which is termed ischemic postconditioning (IPO), has been found to reduce myocardial ischemia reperfusion injury. The cardioprotection of IPO has been proved in several animal models and even in the human heart. Although existing data in the literature are still somewhat contradicting, the most of studies show that hypercholesterolemia (HC) interferes with the cardioprotective effect of IPO. However, the mechanism of attenuation of the cardioprotective effect of IPO in the condition of hypercholesterolemia is not well understood.

The opening of the mitochondrial permeability transition pore (mPTP) is associated with the pathogenesis of necrosis and apoptosis. Many studies have shown that mPTP opens during myocardial reperfusion injury due to oxidative stress, Ca²⁺ overload, decreased ATP levels and increased matrix pH, which causes more necrosis and apoptosis of cardiac myocyte. Inhibiting the opening of mPTP by cyclosporine-A (CsA) produces cardioprotection against ischemia reperfusion. Recent study has shown that the attenuation of cardioprotective effect of ischemic preconditioning in hyperlipidaemic heart may be involved in inhibition of protective signaling pathways upstream of glycogen synthase kinase-3β (GSK-3β) and the opening of mPTP. Therefore, we hypothesized that the protective effect of IPO abolished by hypercholesterolemia is involved in excessive opening of mPTP and inhibiting the opening of mPTP with CsA could restore the cardioprotection of IPO in the presence of hypercholesterolemia.

Based on this hypothesis, in our present study, we tested whether inhibiting the opening of mPTP by administration of CsA shortly before the ischemia could restore the cardioprotection of IPO in the presence of hypercholesterolemia.
**Materials and Methods**

**Animals**

The rats were purchased from the Center of Experimental Animals, China Medical University. All animals used in this study were treated in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH). The study protocol was approved by the Institutional Ethics Committee.

**Induction of Experimental Hypercholesterolemia**

One hundred male Wistar rats weighing 180 ± 10 g were randomly assigned to two different dietary groups: animals in the control diet group (n=40) were fed with normal diet, whereas those in the high fat diet group (n=60) received diet enriched with 1.5% cholesterol, 5% egg yolk powder, 10% lard, 0.5% sodium cholate, 3% sugar and 80% normal feedstuff for 8 weeks, and this formula was modified based on that reported previously \(^{14}\). At the end of 8-week feeding period, blood samples were taken from the rats’ vena caudalis for determination of plasma levels of total cholesterol (TC), high density lipoprotein (HDL) and low density lipoprotein (LDL) using commercial kits [(Total Cholesterol Assay Kit - Fluoro Cholesterol, CellTech, Santa Monica, CA, USA), (Biosystems S.A., Cholesterol HDL direct, Barcelona, Spain) and (Biosystems S.A., Cholesterol LDL direct, Barcelona, Spain)] in order to judge the success of hypercholesterolemic models.

**Drugs and Chemicals**

CyclosporineA (CsA) was purchased from Sigma Aldrich, St Louis, MO, USA. Triphenyltetrazolium chloride (TTC) was purchased from Sigma, St Louis, MO, USA.

**Heart Preparation**

Rats were anesthetized with an intraperitoneal injection with 4 mg/kg 10% chloral hydrate and then heparinized by injecting 3 mg/ml heparin via the inferior vena cava to prevent intracoronary clot formation. After one minute, the rats were fully heparinized and the heart was dissected and placed in Krebs Henseleit (KH) solution containing (in mmol) 127NaCl, 17.7NaHCO\(_3\), 5.1KCl, 1.5CaCl\(_2\), 1.26MgCl\(_2\), 11D-glucose (pH7.4), at 4°C for trimming. The heart was mounted on a Langendorff-perfusion apparatus and retrogradely perfused through the aorta with recirculating KH solution saturated with 95% O\(_2\)-5% CO\(_2\) at 37°C. The heart was maintained in a thermostatic chamber at 37°C. Perfusion was maintained at a constant pressure of 75 mmHg. A fluid-filled latex balloon was inserted in the leftventricle (LV) via the left atrium for pressure measurement. The balloon was connected to a pressure transducer and inflated to an initial LV end-diastolic pressure between 8 and 10 mmHg.

**Experimental Protocol**

Rats were divided into 10 groups as shown in Figure 1. In all groups, the isolated rat hearts were perfused with K-H solution and allowed for 10 min of stabilization. Control-normocholesterolemia group (Control-NC) (n=10): global ischemia for 30 min, and reperfusion for 120 min. Ischemic postconditioning-normocholesterolemia group (IPO-NC) (n=10): ischemia for 30 min, followed by six cycles of reperfusion and ischemia, both with equal lengths of 10 s, and reperfusion for 120 min. Cyclosporine A-normocholesterolemia group (CsA-NC) (n=10): 0.5 µM cyclosporine-A restores postconditioning in hypercholesterolemic rat heart.
cyclosporine A was added to the perfusate for 15 min before ischemia. Then global ischemia for 30 min and reperfusion for 120 min. Ischemic postconditioning+cyclosporineA-normocholesterol-terolemia group (IPO+CsA-NC) (n=10): 0.5 µM cyclosporine A was added to the perfusate for 15 min before ischemia. After 30 min ischemia, followed by 6 cycles of reperfusion and ischemia, both with equal lengths of 10 s and reperfusion for 120 min. Control-hypercholesterolemia group (Control-HC) (n=10): rats underwent ischemia and reperfusion as described in Control-NC. Ischemic postconditioning-hypercholesterolemia group (IPO-HC) (n=10): rats received ischemic postconditioning as described in IPO-NC. Cyclosporine A-hypercholesterolemia group (CsA-HC) (n=10): rats received 0.5 µM CsA as described in CsA-NC. Ischemic postconditioning+cyclosporine A-hypercholesterolemia group (IPO+CsA-HC) (n=10): rats received ischemic postconditioning and 0.5 µM CsA as described in IPO+CsA-NC.

To investigate whether more cycles of IPO or a higher concentration of CsA alone could restore cardioprotection during HC, we added two more groups with IPO induced by 12 cycles of reperfusion and ischemia and 5 µM CsA.

Ischemic postconditioning2-hypercholesterolemia group (IPO2-HC) (n=10): rats received twelve cycles of ischemic postconditioning as described above. Cyclosporine A-hypercholesterolemia group (CsA2-HC) (n=10): rats received 5 µM CsA as described above.

**Measurement of Infarct Size**

Infarct size was determined as previously described15. After perfusion, the heart was removed from the perfusion apparatus and placed in the -20°C freezer for one hour. The frozen left ventricle was cut into six equal sections from the apex to the bottom along the direction of the atrioventricular groove. The sections were placed in 1% triphenyltetrazolium chloride (TTC) solution (TTC dissolved in pH7.8 Na2HP04/NaHPO4 buffer) for 15 min and then fixed in 10% formaldehyde for another 15 min. The red non-infarcted area and gray-white infarcted area were seen in the sections. The sections were scanned and the infarct area was calculated using imageJ analysis software.

**Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay**

At the end of two hours' reperfusion, the heart was removed as described earlier. Myocardial apoptosis was detected by in situ DeadEndTM Colorimetric Apoptosis Detection System (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Briefly, tissue sections were washed in phosphate buffered saline (PBS) and then fixed in 4% paraformaldehyde solution before incubation in 20 lg/ml proteinase K for 10 min. After washed in PBS for three times, tissue sections were incubated with terminal deoxynucleotidyl transferase enzyme in a humidified chamber at 37°C for 60 min for incorporation of biotinylated nucleotides at the 3′-OH DNA ends. The reaction was terminated by transferring the slides to 2 × sodium citrate saline solution. Endogenous peroxidase activity was quenched by incubation in 0.3% hydrogen peroxide. Finally, streptavidin horseradish peroxidase (HRP) was bound to th biotinylated nucleotides and peroxidase activity was demonstrated in each section by the application of a stable chromogen diaminobenzidine. In this technique, apoptotic nuclei are stained dark brown. The sections were counter stained with methyl green for total nuclei. Three sections from each myocardial sample were randomly selected and 10 microscopic fields (Olympus BX51 microscope, Tokyo, Japan) per section were evaluated by two independent blind observers. In each field, the nuclei were counted and the percentage of TUNEL-positive nuclei was calculated.

**Statistical Analysis**

The data were expressed as mean ± SD values. Statistical analysis was performed by using
Cyclosporine-A restores postconditioning in hypercholesterolemic rat heart

SPSS 17.0 statistical software. One-way ANOVA was applied in analyzing the difference between the groups. If the difference was statistically significant, Student-Newman-Keuls post hoc test (SNK) was applied in further pairwise comparisons. All \( p \) values < 0.05 were considered statistically significant.

Results

The Levels of Plasma Lipid

Table I shows the average values for total cholesterol, HDL, and LDL obtained from the plasma of animals fed with a normal diet and of those fed with a cholesterol-enriched diet. An increase in total cholesterol and LDL cholesterol values was observed in the animals fed with the cholesterol-enriched diet versus the normal diet group (\( p < 0.05 \)).

<table>
<thead>
<tr>
<th></th>
<th>Total cholesterol (mg/dl)</th>
<th>HDL-C (mg/dl)</th>
<th>LDL-C (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal diet fed group</td>
<td>51.2 ± 5.8</td>
<td>43.2 ± 4.5</td>
<td>17.5 ± 5.3</td>
</tr>
<tr>
<td>Cholesterol-enriched diet fed group</td>
<td>227.1 ± 7.6*</td>
<td>45.1 ± 5.3</td>
<td>49.5 ± 6.3*</td>
</tr>
</tbody>
</table>

*\( p < 0.05 \), vs. Normal diet fed group.

Hemodynamic Changes

Table II shows the values of HR, LVDP, +dp/dt and -dp/dt at baseline and during different times of reperfusion. No significant differences in HR, LVDP, +dp/dt and -dp/dt were observed among the experimental groups during baseline. In normocholesterolemia groups, IPO group, CsA group and IPO+CsA group significantly increased values of HR, LVDP, +dp/dt and -dp/dt at different times of reperfusion. However, in hypercholesterolemia groups, only IPO+CsA group significantly increased values of HR, LVDP, +dp/dt and -dp/dt at different times of reperfusion. But there were no significant differences in the values of HR, LVDP, +dp/dt and -dp/dt at baseline and during different times of reperfusion in other groups.

Release of CK-MB Measurement

Figure 2 shows the release of CK-MB of isolated heart after 30 minutes of global no-flow ischemia, and 120 minutes reperfusion. IPO significantly reduced release of CK-MB in normal rat hearts as compared with Control-NC group (85.4 ± 6.2 vs. 157.0 ± 10.1, \( p < 0.05 \)). CsA treatment could also decrease release of CK-MB in normal rat hearts as compared with Control-NC group (83.5 ± 13.5 vs. 157.0 ± 10.1, \( p < 0.05 \)). However, IPO and CsA treatment alone failed to significantly decrease the release of CK-MB in hypercholesterolemic rat hearts (167.2 ± 13.2, 164.5 ± 11.8 vs. 157.0 ± 10.1, \( p > 0.05 \)). In addition, more cycles of IPO (IPO2) and higher concentration of CsA (CsA2) were also blocked by hypercholesterolemia (169.4 ± 6.4, 168.0 ± 10.9 vs. 157.0 ± 10.1, \( p > 0.05 \)). But IPO combined with CsA treatment restored the effect of decreasing release of CK-MB in hypercholesterolemic rat hearts as compared with Control-NC group (87.7 ± 12.4 vs. 157.0 ± 10.1, \( p < 0.05 \)).

Infarct Size Measurement

Figure 3 shows the infarct size after 30 minutes of global no-flow ischemia, and 120 minutes reperfusion. In normocholesterolemia rats, infarct size was reduced after ischemic postconditioning as compared with Control-NC group (28.4 ± 7.6% vs. 38.4 ± 9.1%, \( p < 0.05 \)). CsA had a similar infarct reducing effect as IPO (27.0 ± 4.2% vs. 38.4 ± 9.1%, \( p < 0.05 \)), but a combination of IPO did not further reduce infarct size (25.8 ± 7.0% vs. 27.0 ± 4.2%, \( p > 0.05 \)). However, hypercholesterolemia abolished the infarct sparing effect of ischemic postconditioning (45.3% ± 7.0% vs. 38.4 ± 9.1%, \( p > 0.05 \)). Meanwhile, the cardioprotective effect of CsA alone was also blocked by hypercholesterolemia (43.8 ± 10.8% vs. 38.4 ± 9.1%, \( p > 0.05 \)). However, the combination of CsA and IPO provided an infarct sparing effect against hypercholesterolemic heart (28.4 ± 8.1% vs. 38.4 ± 9.1%, \( p < 0.05 \)).

TUNEL Staining for Apoptosis

TUNEL-positive cardiomyocyte nuclei are shown in Figure 4. In NC groups, IPO, CsA treatment, and IPO combined with CsA treatment all significantly reduced the number of TUNEL-positive cells with corresponding values of 8.5% ±
In HC groups, the anti-apoptosis effect of IPO was abolished by hypercholesterolemia (25.8 ± 4.3% vs. 24.8% ± 3.7%, p > 0.05). However, the combination of CsA and IPO provided an anti-apoptosis effect against hypercholesterolemic heart during ischemia reperfusion (12.4 ± 3.1% vs. 24.8% ± 3.7%, p < 0.05).

<table>
<thead>
<tr>
<th>Time Baseline</th>
<th>R-10</th>
<th>R-30</th>
<th>R-120</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heart rate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control-NC</td>
<td>226 ± 23</td>
<td>150 ± 15</td>
<td>164 ± 11</td>
</tr>
<tr>
<td>IPO-NC</td>
<td>217 ± 20</td>
<td>165 ± 11*</td>
<td>185 ± 13*</td>
</tr>
<tr>
<td>CsA-NC</td>
<td>225 ± 17</td>
<td>168 ± 12*</td>
<td>188 ± 10*</td>
</tr>
<tr>
<td>IPO+CsA-NC</td>
<td>220 ± 18</td>
<td>166 ± 9*</td>
<td>186 ± 12*</td>
</tr>
<tr>
<td>Control-HC</td>
<td>215 ± 16</td>
<td>145 ± 11</td>
<td>160 ± 12</td>
</tr>
<tr>
<td>IPO-HC</td>
<td>227 ± 21</td>
<td>142 ± 16</td>
<td>159 ± 9</td>
</tr>
<tr>
<td>CsA-HC</td>
<td>221 ± 22</td>
<td>139 ± 22</td>
<td>162 ± 22</td>
</tr>
<tr>
<td>IPO+CsA-HC</td>
<td>230 ± 18</td>
<td>176 ± 17*</td>
<td>189 ± 14*</td>
</tr>
<tr>
<td>IPO2-HC</td>
<td>212 ± 26</td>
<td>146 ± 14</td>
<td>168 ± 15</td>
</tr>
<tr>
<td>CsA2-HC</td>
<td>222 ± 21</td>
<td>140 ± 18</td>
<td>170 ± 18</td>
</tr>
<tr>
<td><strong>LVDP (mm Hg)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control-NC</td>
<td>86 ± 6.4</td>
<td>34 ± 5.4</td>
<td>38 ± 5.1</td>
</tr>
<tr>
<td>IPO-NC</td>
<td>84 ± 6.5</td>
<td>44 ± 4.3*</td>
<td>48 ± 4.5*</td>
</tr>
<tr>
<td>CsA-NC</td>
<td>83 ± 6.6</td>
<td>46 ± 4.9*</td>
<td>51 ± 6.0*</td>
</tr>
<tr>
<td>IPO+CsA-NC</td>
<td>88 ± 7.5</td>
<td>48 ± 6.4*</td>
<td>54 ± 5.7*</td>
</tr>
<tr>
<td>Control-HC</td>
<td>84 ± 6.9</td>
<td>34 ± 6.7</td>
<td>39 ± 4.2</td>
</tr>
<tr>
<td>IPO-HC</td>
<td>86 ± 4.4</td>
<td>34 ± 5.4</td>
<td>37 ± 5.1</td>
</tr>
<tr>
<td>CsA-HC</td>
<td>85 ± 7.3</td>
<td>32 ± 7.7</td>
<td>36 ± 6.5</td>
</tr>
<tr>
<td>IPO+CsA-HC</td>
<td>89 ± 5.6</td>
<td>45 ± 3.2**</td>
<td>50 ± 6.6**</td>
</tr>
<tr>
<td>IPO2-HC</td>
<td>87 ± 8.5</td>
<td>31 ± 9.4</td>
<td>35 ± 5.4</td>
</tr>
<tr>
<td>CsA2-HC</td>
<td>82 ± 6.5</td>
<td>31 ± 9.4</td>
<td>36 ± 6.4</td>
</tr>
</tbody>
</table>

**HR, +dp/dt, -dp/dt, and LVDP before and during reperfusion.**

HR = heart rate; +dp/dt = positive first order derivative of ventricular pressure; -dp/dt = negative first order derivative of ventricular pressure; LVDP = left ventricular developed pressure; NC = normocholesterolemia; HC = hypercholesterolemia; IPO = ischemic postconditioning for six cycles of reperfusion and ischemia; CsA = 0.5 µM cyclosporineA pretreatment; IPO2 = ischemic postconditioning for twelve cycles of reperfusion and ischemia; CsA2 = 5 µM cyclosporineA pretreatment. Data are presented as means ± SD and n = 10 for each. *p < 0.05 vs. Control-NC; **p < 0.05 vs. Control-HC.
Ischemic postconditioning is a powerful form of protection, but its effectiveness under the pathological conditions is in dispute. Some studies reported the cardioprotection of IPO is limited in the presence of diabetes mellitus, hyperlipidemia, uremia and so on\cite{16,17}. Among these disease conditions, hyperlipidemia, especially hypercholesterolemia, is regarded as an independent risk factor in the development of ischemic heart diseases including myocardial infarction. In this study, we found that the cardioprotection of IPO in rats heart is blocked by elevated level of blood hypercholesterolemia, which is induced by 8-week hypercholesterolemic diet fed. The result is consist with previous founding by other researchers\cite{5-7}. However, Donato et al\cite{18} showed that ischemic postconditioning reduces infarct size by activation of A1 receptors and K+ ATP channels in both normal and hypercholesterolemic rabbits. The discrepancies may be probably attributed to the animal species or duration of hyperlipidemia diet.

The mechanisms that hyperlipidemia/hypercholesterolemia abrogates the cardioprotective effects of ischemic postconditioning are not clear. Kupai et al\cite{7} found experimental hyperlipidemia induced by cholesterol-enriched diet impairs the cardioprotective effect of postconditioning by altering of nitrosative stress signal and increasing the production of several oxidants such as peroxynitrite and lipid peroxidation compounds. In addition, a long term (3-week) or short-term (3-day) statin administration could restore the infarct size-limiting effect of postconditioning in hypercholesterolemic rat heart potentially by increasing the expression and activity of endothelial nitric oxide synthase synthesis (eNOS)\cite{19,20}.

The mPTP opening might be regarded as a crucial step from reversible to irreversible cell death\cite{21}. IPO mediated its protective effects by inhibiting mPTP opening upon reperfusion and mPTP also is considered to be a critical end-effector of IPO\cite{22}. However, whether hypercholesterolemia block the cardioprotection of IPO involved in the opening of mPTP is still not known. In this work we demonstrated that CsA, a mPTP opening inhibitor, could restore the cardioprotection of IPO in hypercholesterolemic rat heart. Therefore, we speculated that ischemic postconditioning is blocked by hypercholesterolemia may due to the excessive the opening of mPTP and Inhibiting of the mPTP is able to reverse this loss of cardioprotection. We also found that the cardioprotective effect of CsA
alone was also blocked by hypercholesterolemia and only the combination of CsA and IPO provided an infarct sparing effect against myocardial ischemia reperfusion injury. Probable explanation is that the sole cardioprotective intervention with CsA or postconditioning is not strong enough to protect the hypercholesterolemic myocardium, but possibly the threshold for cardioprotection is lowered after combination of both protective pathways.

Compared with preconditioning which must be applied before an ischemic event, postconditioning has the advantage that it may be applied after sustained ischemia. It has much more extensive clinical applicability. However, mounting randomized controlled trials RCTs results shown that ST-elevation myocardial infarction (STEMI) patients perform IPO during primary percutaneous coronary intervention does not reduce myocardial damage, and it may even aggravate myocardial reperfusion injury23,24. The reason for such results may be that clinical patients tend to have many complications, such as diabetes, hyperlipidemia, renal dysfunction etc. which may influence the effectiveness of IPO. Several animal experiments have also proved that the effectiveness of IPO is influenced in some disease states such as hypertension, diabetes, heart failure, hypercholesterolemia and atherosclerosis, renal dysfunction, etc.16,25,26. Among these com-

Figure 4. Apoptosis after myocardial infarction. A, Control-NC. B, IPO-NC. C, CsA-NC. D, IPO+CsA-NC. E, Control-HC. F, IPO-HC. G, CsA-HC. H, IPO+CsA-HC. I, IPO2-HC. J, CsA-HC. Photomicrographs were taken at 200 × magnification. Apoptotic cardiomyocyte nuclei appear brown stained whereas terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-negative nuclei appear green. Histogram shows the percent of the TUNEL-positive cells (brown staining). NC = normcholesterolemia; HC = hypercholesterolemia; IPO = ischemic postconditioning for six cycles of reperfusion and ischemia; CsA = 0.2 µM cyclosporineA pretreatment; IPO2 = ischemic postconditioning for twelve cycles of reperfusion and ischemia; CsA2 = 5 µM cyclosporineA pretreatment. Data are presented as means ± SD and n = 8 for each. *p < 0.05 vs. Control-NC.
Conclusions

Hypercholesterolemia abolishes cardioprotection of ischemic postconditioning involved in the opening of mPTP and inhibition of mPTP with CsA may reverse this loss of cardioprotection.

Acknowledgements

These studies are supported by Liaoning Provincial Science and Technology Projects, China (2009225051-1).” Should correct to “These studies are supported by Liaoning Provincial Science and Technology Projects, China (2009225051-1) and (2013021011).

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References


Cyclosporine-A restores postconditioning in hypercholesterolemic rat heart


26) **Ren JY, Song JX, Lu MY, Chen H.** Cardioprotection by ischemic postconditioning is lost in isolated perfused heart from diabetic rats: involvement of transient receptor potential vanilloid 1, calcitonin gene-related peptide and substance P. Regul Pept 2011; 169: 49-57.