The effect of adrenergic receptor–adenyl cyclase system on myocardial ischemic preconditioning in rats

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Abstract In order to study the effects of every part of adrenergic receptor-adenyl cyclase system on ischemic preconditioning of myocardium in rats *in vivo*, SD rats were divided into three groups: IP group, I/R group and CON group. Rats were received surgical procedure and undergone left coronary artery occlusion and reperfusion. Hearts were extracted to analyze the infarct size by TTC staining, to measure serum myocardial enzymes, to study β-AR Bmax and Kd by radioligand binding assay of receptors (RAB), and to check the activity of AC and the content of cAMP by radioimmunoassay (RIA). The infarct area was found much smaller in IP group than I/R group (p <0.001); CK,CK MB and LDH were found significantly higher in I/R group (p <0.001). The Bmax of β AR in IP group were higher than in I/R group (p <0.001). No difference of Kd could be seen between IP and I/R group. In IP group, the activity of AC and the content of cAMP were higher than I/R group (p <0.05 and 0.001, respectively). It is concluded that ischemic preconditioning can protect the hearts from necrosis and reduce endo-enzyme leakage. The system of adrenergic receptor- adenyl cyclase system probably takes part in the protection of the IP.

Keywords Ischemic preconditioning, β-adrenergic receptor, cAMP, Rat

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1 INTRODUCTION

Repeated episodes of ischemia and reperfusion dramatically limit infarct size of myocardium caused by subsequent prolonged periods of ischemia. This phenomenon was called "ischemic preconditioning (IP)". The protection of IP was considered the strongest protection *in vivo*.

It is generally accepted that it is a receptor–mediate process with protein kinase C (PKC) activation as the common final pathway.[1] However, the role of PKC could not be corroborated by all workers.[2] As knowledge of preconditioning accumulated, it became apparent that several signal transduction pathways may participate in the protection of IP, including β-adrenergic receptor–adenyl cyclase pathway.[3] But they

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had only received little experimental attention and proof. In the present study, we use the ischemia/reperfusion model in rats *in vivo* to show the variety and effects of every part of β-adrenergic-adenyl cyclase system on IP.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Animals

Female SD rats (supplied by Animal Laboratory Center of China Medical University) weighing 270 to 320 g were used. Before anesthesia, rats were allowed free access to food and water.

2.1.2 Reagents

Triphenyltetrazolium chloride (TTC, from Shanghai Chemical Co.), ^{3}H-dihydroalprenolol (^{3}H-DHA, from China Institute of Atomic Energy, specific activity 1.7 TBq/mL), propranolol (from Sigma Co.), glass fiber filter (from Shanghai HongGuang Papermaking Co.), cAMP RIA kit (from Shanghai University of Traditional Chinese Medicine) were used in the work. Other reagents were from Shanghai Chemical Co. and all were of reagent grade.

2.1.3 Instruments

Animal respirator (from Zhejiang Medical University Instrument Co.), Nihon Konden electrocardiograph (ECG, from Shanghai Medical Electronic Instrument Co.), stereoscopic microscope, and Olympus DP10 camera were used in the work.

2.2 Methods

2.2.1 Experimental protocol

Rats were randomly divided into three groups. Ischemia/reperfusion insult was made by occlusion/reperfusion of the left major coronary artery. Experimental protocols are shown in Fig.1.

(1) Ischemic preconditioning group (IP group, n=12): The rats were subjected three cycles of five minutes of ischemia followed by five minutes of reperfusion and then subjected to 30 min of ischemia followed by 90 min of reperfusion.

(2) Ischemia/Reperfusion group (I/R group, n=12): After surgery, the rats were balanced for 35 min and then subjected to 30 min of ischemia followed by 90 min of reperfusion.

(3) Control group (CON group, n=6): After surgery, no procedures were followed. After 155 min, the experiment was finished.

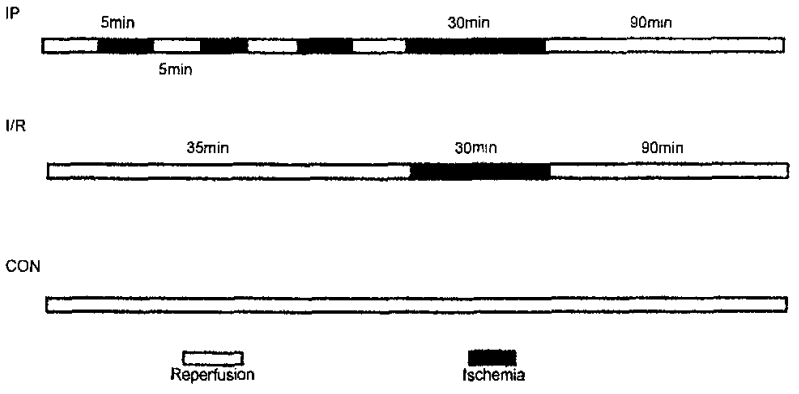

Fig.1 Experimental protocol

2.2.2 Animal model preparation

Rats were anesthetized with 3% sodium pentobarbital (60 mg/kg), intubated, and mechanically ventilated with a respirator with room air (ventilation was 70 to 80 breaths per minute and the tide volume was about 1–1.5 mL/100 g weight). ECG was monitored by bipolar limb leads. Left anterolateral thoracotomy was performed from the second to the fourth intercostals space, and the pericardium was opened. A 3–0 silk thread was then passed around the proximal of the left major coronary artery with a small curved needle, and its ends were threaded through a small polyethylene tube. Rats were allowed 5 min after surgical preparation to reach a steady state. Coronary occlusion was produced by pulling the snare and clamping it with a mosquito hemostat. Reperfusion was produced by releasing the clamp. Myocardial ischemia was confirmed by ST segment elevation of the ECG as well as observation of regional cyanosis over the myocardial surface.

2.2.3 Infarct size and myocardial enzymes determination

At the end of the experiment, 2 mL blood was drawn to measure the serum CK, CK-MB and LDH. Six rats were selected from IP and I/R groups to check infarct size. We excised the hearts and sliced the ventricles transversely into 2 mm-thick slices. Then the slices were incubated at 37°C for 10 min in a 1% TTC solution made up with 0.2 mol/L Tris buffer (pH 7.4). TTC stains viable myocardium brick red, whereas areas of necrosis appear white. By using the Metamorph system, infarct areas were defined and calculated. Other hearts of each group were excided quickly, frozen in liquid nitrogen and stored at -80°C until membrane and cytoplasm preparation.

2.2.4 Cytomembrane and cytoplasm preparation

The heart tissue was minced and homogenized in 0.25 mol/L cold sucrose buffer, with endo-homogenizer (6000 r/min, 20 s×3). This crude homogenate was centrifuged at 5000 g for 10 min in low temperature. The supernatant was then centrifuged at 40000 g for

20 min. The resulting supernatant was cytoplasm, which was used to check the activity of adenyl cyclase (AC) and cAMP level. The deposition was washed in cold Tris-HCl buffer. Then we got cytomembrane to check β-adrenergic receptor (β-AR). The protein content of cytoplasm and cytomembrane were determined by Lowry technique.

2.2.5 Radioligand binding assays (RBA) of β-AR

RBA was performed in Tris-HCl buffer (pH 7.4). In a final volume of 300 μL containing 100 μL cytomembrane, 0.5–4 nmol/L ^{3}H-DHA was added. The incubation was conducted at 37°C for 20 min. At the end of incubation, binding was stopped by throwing the sample into ice-bath, and immediately filtering through glass fiber filter. Each filter was washed two times with 5 mL of cold Tris-HCl buffer, and the bound radioactivity was determined with a beta counter with 50% efficiency. Specific binding was defined as the difference between total binding and binding inhibited by 1 μmol/L propranolol. The maximal binding capacity (Bmax) and dissociation constant (Kd) were calculated by the use of Scatchard program.

2.2.6 AC activity

AC was assayed by the method of Zhang.[4] We used non-radiolabeled ATP instead of radiolabeled ATP as the reaction substrate to produce non-radiolabled cAMP by AC catalysis, then checked cAMP by radioimmunoassay (RIA) kit. The AC activity is expressed in the content of cAMP per gram dry weight per 15 min.

2.2.7 cAMP assay

The RIA procedure used for cAMP determination was performed according to the directions of the kit. The cAMP measurements were expressed in pmol per gram dry weight.

2.2.8 Statistical analysis

All data are expressed as mean±SEM. Statistical significance was taken as $p < 0.05$.

3 RESULTS

3.1 Serum myocardium enzymogram assay

The results of CK, CK-MB, LDH of each group were listed in Table 1. Three enzymes in IP were significantly lower than in I/R group ($p < 0.001$).

Table 1 Serum myocardium enzymogram assay

Groups	CK(U/L)	CK-MB(U/L)	LDH(U/L)
I/R	6234.6±359	3350.7±425.9	2985±231
IP	2718.6±178	1598.1±256.5	1476±201
CON	2102.4±365.8	1079.6±118.5	1317.6±218.8
I/R vs IP	$p < 0.001$	$p < 0.001$	$p < 0.001$
IP vs CON	$p < 0.005$	$p < 0.002$	$p > 0.2$

3.2 Infarct size analysis of IP and I/R groups

We used Matamorph system to quantify the infarct size. Infarct size of I/R group was 703711±223437. Infarct size of IP group was 187305±52645. There was significant difference between the two groups (p <0.001).

3.3 β–AR density and affinity

The changes of β–AR density (Bmax) and affinity (Kd) were summarized in Table 2. The Bmax of β–AR in IP group were much higher than in I/R group (p <0.001). No difference of Kd could be seen between the two groups. In addition, it was shown that sustained myocardial ischemia and reperfusion did not change the density and affinity of β–AR.

Table 2 The changes of β–AR density and affinity

Groups	Bmax(fmol/mg protein)	Kd(nmol/L)
I/R	0.465±0.012	2.421±0.157
IP	0.608±0.048	2.412±0.314
CON	0.364±0.117	3.368±0.988
I/R vs IP	p <0.001	p >0.5
IP vs CON	p <0.01	p >0.05
I/R vs CON	p >0.05	p >0.01

3.4 Determination of AC activity and cAMP content

As shown in Table 3, the activity of AC and the cAMP content were much higher in IP than in I/R (p <0.05 and 0.001, respectively).

Table 3 Changes of the activity of AC and myocardial cAMP content

Group	AC activity (pmol cAMP/mg protein)	cAMP (pmol/g)
I/R	2.375±0.509	0.362±0.047
IP	3.6±0.818	0.624±0.093
CON	1.59±0.194	0.359±0.072
I/R vs IP	p <0.05	p <0.001
IP vs CON	p <0.01	p <0.01

4 DISCUSSION

In the present study we used rats' left major coronary artery occlusion and reperfusion to make the models of myocardial ischemic preconditioning *in vivo*. We analyzed the infarct size by TTC staining and measured serum myocardial enzymogram, and

found that IP can lessen necrosis, reduce enzyme leakage and provide protection against ischemic damage.

Rats are the frequently used animals to make the model of ischemia/reperfusion. Rats have some special characteristics in the cardiovascular study, for example, little variability in cardiovascular system, anatomic structure of cardiovascular system and the areas of coronary artery domination similar to human. But there are dark sides when using rats, such as high difficulty in making models, especially the surgery of high ligation of left major coronary artery. In the present study, the rate of success in making models is only about 70%.

Many reports indicate that acute myocardial ischemia will increases the number of β–AR, but the affinity has no change. The report of Maisel, using guinea pigs and rabbits to make the acute myocardial ischemia, showed that β–AR increased after 15 min of ischemia and it was time–dependent, and after reperfusion, β–AR slowly recovered to normal. This finding is similar to ours. In addition, our study show that the density of β-AR in IP group is much higher than I/R group and no difference is found in Kd between the two groups. It is demonstrated that β-AR can maintain higher situation during IP. *Therefore, it is possible that up–regulation of β–AR plays an important cardioprotective* role in IP.

The possible mechanisms of up–regulation of β–AR that may be involved are: (1) *Endogenous catecholamine increases dramatically, and its high consistency makes β-AR* desensitized. Catecholamine at a relatively high dose is capable of using a large amount of high energy phosphates consumption and thereby inducing metabolic imbalance between energy consumption and production, which lead to a reduction of tissue glycogen levels. The partial depletion of tissue glycogen is considered to be a possible mechanism for IP.[5] (2) Externalization of β–AR makes the content of β–AR changed. β–AR in the myocardial cell transfers to cytomembrane. (3) The mechanism of internalization of β–AR were damaged, which destroyed the balance of receptors in cells.

Classical theory tells us that β–AR couples to Gs–adenyl cyclase signal transduction pathway. Activating β–AR can increase the content of cAMP in cells, and cAMP acts as the second messenger and can produce various responses in hearts. Our data show that the content of cAMP are much higher in IP group than those in I/R and CON groups (p <0.001 and 0.01, respectively). The activity of AC, which was used as a catalytic enzyme for the decomposition of ATP to cAMP, increased obviously (p <0.001). This demonstrates that higher activity of AC is one of the reasons for the high content of cAMP in IP hearts. In the experiments of Tomoyuki *et al.*[6], who used rabbits in their tests, it was found that GppNHp and sodium fluoride stimulated maximal AC activities are preserved in the IP hearts. In this situation, it is possible that the β–adrenergic stimulus is amplified substantially at the level distal to the receptors in the preconditioned heart.

This conclusion is similar to ours.

High content of cAMP can activate protein kinase A (PKA). PKA can increase Ca^{2+} flow and Ca^{2+} releasing from sarcoplasmic reticulum via phosphorylation L type Ca^{2+}. In addition, it is well recognized that β–stimulation can increase the intracellular concentration of Ca^{2+} via a cAMP–dependent pathway. The increase in the intercellular concentration of Ca^{2+} can stimulate phospholipase C, which may lead to activation of PKC via formation of diacylglycerol.

However, some experiments have failed to reveal similar results. Sandhu *et al*[7] found that cAMP levels were increased nearly twofold during regional myocardial ischemia in rabbit hearts. This increase in cAMP levels was attenuated when ischemia was preceded by a signal cycle of transient ischemia and reperfusion, and was prevented when ischemia was preceded by three cycles of transient ischemia and reperfusion. The reason for the discrepancy in the response of cAMP to IP is not clear. It may be ascribed to differences in the duration of ischemia, whether reperfusion intervened, the use of conscious versus anesthetized animals, and perhaps species differences. In addition, we cannot ignore the function of phosphodiesterase (PDE), which acts as an enzyme decomposition and inactivation cAMP. Some reports[8] showed that the change of cAMP in the procedure of IP was opposite to the change of PDE activity. This suggests that the fluctuation of cAMP in IP is partly caused by the change of PDE.

5 CONCLUSION

Ischemic preconditioning can protect the hearts from necrosis and reduce endoenzyme leakage. β–adrenergic stimulation during the ischemia can increase the cAMP production via raising the activity of AC in IP. β–adrenergic receptor–adenyl cyclase system might potentially play an important cardioprotective role in preconditioning.

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