

Calcium activated potassium channel and protein kinase C participate in the cardiac protection of remote post conditioning

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Abstract: The present study was designed to investigate the roles of Ca²⁺ activated K⁺ channel (K_{Ca}) and protein kinase C (PKC) in the protective mechanisms of remote ischemic post conditioning (RPostC) when rat heart was subjected to ischemia/reperfusion (I/R) injury in vivo. Rat heart was subjected to regional ischemia for 45 min and reperfusion for 180 min in vivo to mimic I/R injury. RPostC was induced by 5 min right femoral artery occlusion followed by 5 min reperfusion for 3 cycles (totally 30 min) after 15 min of cardiac ischemia. Delayed remote ischemic post conditioning (delayed RPostC) was induced after 10 min of cardiac reperfusion. The hemodynamic parameters including mean arterial blood pressure and heart rate (HR) were recorded, and lactate dehydrogenase (LDH) release in plasma and infarct size were determined, and arrhythmia scores were calculated. In contrast to I/R, RPostC reduced infarct size and LDH release during reperfusion, the occurrence of arrhythmia was decreased, but no changes in delayed RPostC. The specific inhibitor of K_{Ca} iberiotoxin and PKC inhibitor chelerythrine both attenuated the role of RPostC. The findings indicated that RPostC had a protective effect on myocardial ischemia/reperfusion injury. Opening of K_{Ca} and activating of PKC may be involved in the mechanisms of RPostC.

Keywords: Heart, Ca²⁺ activated K⁺ channel, protein kinase C, ischemia/reperfusion injury.

INTRODUCTION

Remote ischemic post conditioning (RPostC) refers to an ischemia fulfilled in a distant organ that can protect another organ which subjected to ischemia injury (Li *et al.*, 2006; Ren *et al.*, 2009; Wei *et al.*, 2011). Remote ischemic post conditioning is an important and potent remote preconditioning. The major merits include: (1) RPostC is used after heart ischemia, which intervention combined with the advantages of ischemic post conditioning and is adjacent to patients' consultation time when they are subjected to heart ischemia; (2) RPostC is put on different organs which is remote to heart, it is more safe in contrast to ischemic post conditioning which is put on heart directly, it maybe aggravate the injury degree of ischemia heart. So RPostC can be widely used and spread in clinical application. It has been reported that RPostC can play protective role in different organs such as brain, skeletal muscle, heart and so on (Li *et al.*, 2006; Liu *et al.*, 2007; Ren *et al.*, 2009; Eberlin *et al.*, 2009), and the cardioprotection is existed in different type animals such as rat, rabbit, swine, even in human. Since many organs remote to ischemia organ can play "remote protective role", RPostC maybe become a popular and effective method to protect ischemic organ. The mechanisms of RPostC are worth to study.

Ca²⁺ activated K⁺ (K_{Ca}) channel is on the inner

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mitochondrial membrane of cardiac myocytes (Xu *et al.*, 2002). Activation of K_{Ca} channel with NS1619 has demonstrated to play protective role in various cell injury models, including cardiac I/R, cardiomyocyte or neuronal cell anoxia, C2C12 myoblasts injured by oxidative stress or calcium overload (Chmielewska *et al.*, 2011). Protein kinase C (PKC) exists as a family of serine/threonine kinases. There had so many reports about PKCs signal pathway (Gerald *et al.*, 2010; Chen *et al.*, 2012; Tan *et al.*, 2012). PKC may be modulated by many mechanisms including phospholipase C activation, G-protein coupled receptors, growth factor receptors, adhesion receptors and so on. And PKCs were also involved in multiple physiological and pathological conditions (Belmonte *et al.*, 2011). We want to examine whether K_{Ca} channel and PKC were involved in the protective role of RPostC. So in our study, we duplicated RPostC through occlusion rat right femoral artery to observe the cardioprotective role of RPostC, and further to investigate the roles of K_{Ca} channel and PKC in RPostC.

METHODS

Animals and drugs

Male Sprague-Dawley rats (210-250 g) were obtained from Animal Center of Bengbu Medical College and all procedures were approved by the ethics committee for the use of experimental animals in Bengbu medical college, China.

Iberitoxin (IbTX), NS 1619, chelerythrine (Chel), 2, 3, 5-triphenyl-tetrazolium chloride (TTC) and Evans blue were purchased from Sigma Chemical Co (St Louis, Missouri, USA). Iberitoxin (IbTX) is a selective inhibitor of K_{Ca} channel while NS 1619 activate K_{Ca} channel opening. Chelerythrine (Chel) is an inhibitor of PKC.

Surgical procedures

Rats were injected with 0.4 g/kg chloral hydrate intraperitoneally for anesthesia, and deep anesthesia was kept with intermittent intraperitoneally injecting of 0.016 g/kg chloral hydrate. The tracheotomy was performed, and endotracheal intubation was performed with a cannula which connected to an animal respirator. The rats were ventilated with O_2 -enriched air at 70 breaths/min and maintained the tidal volume at 1.0 ml/100 mg body weight. Body temperature was sustained at 37°C using a heating lamp. Through Medlab biological signal collecting and processing system (Nanjing, China), hemodynamic parameters were measured continuously by intubation of left common carotid artery. The standard electrocardiogram was also recorded continuously.

Open heart surgery was performed at the fourth or fifth intercostal space in order to expose heart. A 5-0 suture was placed under the left descending coronary artery and its circumflex branch, and the suture ends were threaded through a polyethylene tubes to form snares for reversible coronary artery occlusion. Rat heart was underwent 45 min ligation of left anterior descending artery followed by 180 min of reperfusion to mimic ischemia/reperfusion (I/R) (Romero-Perez *et al.*, 2008; Tamareille *et al.*, 2009). Another suture was placed below the femoral artery of right hindlimb for ligation with an arterial clamp, and then loosening the clamp to recovery femoral artery blood. Remote post conditioning (RPostC) was induced through ligation of right femoral artery for 5 min, than loosening for 5 min, and repeated for 3 cycles (Xu *et al.*, 2009).

Plasma lactate dehydrogenase level measurement

Lactate dehydrogenase (LDH) level in plasma was determined spectrophotometrically after 10 min of reperfusion.

Myocardial infarct size measurement

At the end of reperfusion, the heart was taken out and the blood was washed out by saline. After left coronary artery was re-occluded in isolated Langendorff-perfused equipment, hearts were perfused with 1% Evans blue to delineate the non-ischemic area, the risk and infarct areas were undyed. Then the tissues were frozen at -20°C for several hours and sliced transversely into 2-3 mm sections. The slices were incubated in 1% 2, 3, 5-triphenyl-tetrazolium chloride (TTC) buffer solution (pH 7.4) for 10 - 15 min at 37°C (Zhang *et al.*, 2008; Zhu *et al.*, 2008). Infarct (pale) and risk (red) areas were

calculated by planimetry using Image/J software from NIH. Infarct size was expressed as the percentage of risk area.

Arrhythmia scoring

The arrhythmia scoring system (Walker *et al.*, 1988) was used to do quantitative comparisons (table 1). According to the system, ventricular fibrillation is the most serious injury, then ventricular tachycardia, the injury of premature ventricular contraction is mild.

Table 1: Arrhythmia scoring system

Arrhythmia score	Type of arrhythmia
0	No arrhythmia
1	Atrial arrhythmia or occasional PVC
2	Frequent PVC
3	VT (1-2 episodes)
4	VT (>3 episodes) or VF

PVC: premature ventricular contraction; VT: ventricular tachycardia; VF: ventricular fibrillation.

Experimental protocols

The rats were assigned to one of the following 7 groups (n = 6/group) randomly: (1) ischemia/reperfusion (I/R) group; (2) remote ischemic post conditioning (RPostC) group: after 15 min of heart ischemia, RPostC was induced; (3) delayed RPostC: after 10 min of heart reperfusion, RPostC was induced; (4) NS 1619 group: rats were administered with 1 mg/kg NS 1619 (the specific activator of K_{Ca} channel) intravenously at the end 5 min of heart ischemia (Redel *et al.*, 2008) during I/R; (5) RPostC+NS 1619 group: rats were administered with 1 mg/kg NS 1619 intravenously at the end 5 min of heart ischemia during RPostC intervention; (6) RPostC+IbTX group: rats were administered with 0.05 mg/kg Iberitoxin (IbTX, the selective inhibitor of K_{Ca} channel) intravenously at the end 5 min of heart ischemia (Redel *et al.*, 2008) during RPostC intervention; (7) RPostC+Chel group: rats were administered with 5 mg/kg chelerythrine (Chel, the inhibitor of PKC) intravenously at the end 5 min of heart ischemia (Weinbrenner *et al.*, 2002) during RPostC intervention.

STATISTICAL ANALYSIS

All data were expressed as mean \pm SD. One-way analysis of variance with Student-Newman-Keuls (SNK) post hoc test was used to test the statistical significance. P values <0.05 was considered significant.

RESULTS

Blood hemodynamics

The hemodynamical parameters including mean arterial blood pressure and heart rate were attenuated in all groups

Table 2: Hemodynamic data in rats

Group	n	Baseline	CAO 45 min	Reperfusion 60 min	Reperfusion 120 min	Reperfusion 180 min
MAP (mmHg)						
I/R	6	100±5	85±6**	86±5**	82±4**	80±6**
RPostC	6	102±7	86±7*	90±8	85±9**	84±12**
Delayed RPostC	6	101±5	86±8**	89±7*	85±8**	83±10**
NS 1619	6	100±6	85±7**	89±6*	84±5**	84±7**
RPostC+NS 1619	6	102±4	87±8**	90±7*	86±8**	84±8**
RPostC+IbTX	6	104±6	88±11**	92±9*	86±5**	82±6**
RPostC+Chel	6	102±4	87±13*	90±10	84±7**	83±9**
HR (beats/min)						
I/R	6	398±15	337±16**	358±32*	351±34*	334±24**
RPostC	6	392±27	362±17	348±18*	330±25**	316±31**
Delayed RPostC	6	396±24	358±18*	350±21**	328±24**	310±29**
NS 1619	6	400±28	362±21*	351±26**	332±26**	312±25**
RPostC+NS 1619	6	398±27	360±19*	348±23**	329±30**	310±31**
RPostC+IbTX	6	404±25	364±25*	376±22	357±19**	326±23**
RPostC+Chel	6	395±34	350±19*	355±26*	344±25**	317±21**

Mean arterial blood pressure (MAP) and heart rate (HR) during the experimental protocols. CAO: coronary artery occlusion; I/R: ischemia/reperfusion; RPostC: remote ischemic post conditioning (mean ± SD, n=6). * $P < 0.05$, ** $P < 0.01$ vs Baseline.

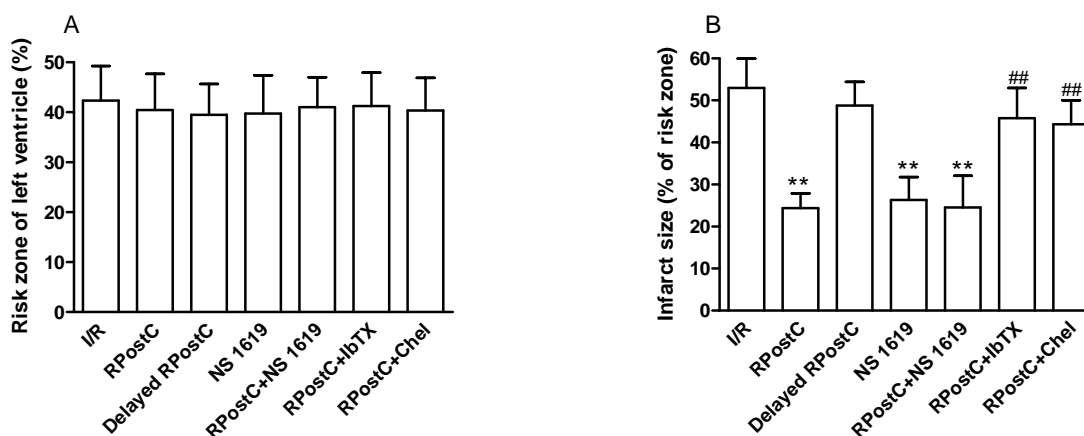


Fig. 1: Effects of ischemia/reperfusion (I/R) and remote ischemic post conditioning (RPostC) on risk zone (A) and infarct size (B) after 180 min of reperfusion following 45 min of ischemia *in vivo*. mean ± SD, ** $P < 0.01$ vs I/R; ## $P < 0.01$ vs RPostC, n=6.

after ischemia and resumed to varying extents after 180 min reperfusion. But there were no differences among the different groups (table 2).

Effect of remote ischemic post conditioning on ischemia/reperfusion injury

In all groups, the risk zones of left ventricle were almost same. In contrast to I/R group, RPostC decreased myocardial infarct size, LDH release and the occurrence of arrhythmia were attenuated. It suggested RPostC can play cardioprotective effect. But delayed RPostC had no significant difference to I/R (figs. 1, 2 and table 3).

The role of Ca^{2+} activated K^+ channel in remote ischemic post conditioning

In RPostC group, intravenous administration of IbTX at 0.05 mg/kg, a specific inhibitor of K_{Ca} channel produced an infarct size, LDH release and arrhythmia occurrence similar to those in I/R rats (figs. 1, 2 and table 3). It suggested activating K_{Ca} channel was involved in the mechanisms of RPostC cardioprotective role.

NS 1619, the specific activator of K_{Ca} channel, decreased infarct size and LDH release and the occurrence of arrhythmia. But no significant differences were observed

among NS 1619, RPostC and NS 1619 + RPostC groups (figs. 1, 2 and table 3).

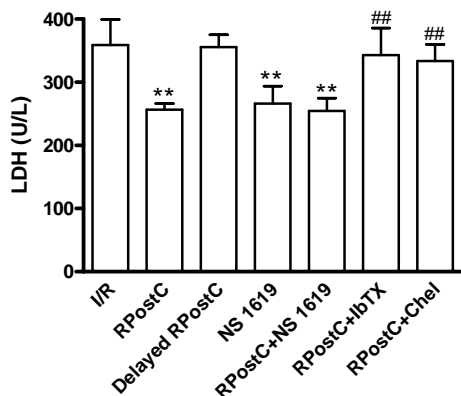


Fig. 2: Effects of ischemia/reperfusion (I/R) and remote ischemic post conditioning (RPostC) on plasma lactate dehydrogenase (LDH) level at 10 min of reperfusion. mean \pm SD, ** $P < 0.01$ vs I/R; ### $P < 0.01$ vs RPostC, n=6

The role of protein kinase C in remote ischemic post conditioning

In RPostC+Chel group, PKC inhibitor Chel (5 mg/kg) increased infarct size, LDH release and arrhythmia occurrence in contrast to rats in RPostC group (figs. 1, 2 and table 3). It suggested activating PKC was involved in the mechanisms of RPostC cardioprotective role.

DISCUSSION

We demonstrated that 5 min of right femoral artery occlusion with 5 min of reperfusion for 3 cycles to mimic RPostC after 15 min of regional ischemia in heart decreased infarct size, LDH release and arrhythmia occurrence, it suggested RPostC played a cardioprotective effect. We further showed that blockage of K_{Ca} channel with IbTX and blockage of PKC with Chel both attenuated the protective effect of RPostC, suggesting that activation of K_{Ca} channel and PKC were involved in the myocardial protective role afforded by RPostC. Interestingly, delayed RPostC can not play

cardioprotective role, suggesting that the protective role of remote conditioning occurs at the beginning of reperfusion.

RPostC can play cardioprotective role in different types of animals, also be fulfilled in different organs. The mechanisms are complex. Kerendi (Kerendi *et al.*, 2005) firstly reported that a single 5 minute episode of renal artery occlusion and reperfusion before the onset of coronary artery reperfusion played the cardiac protective effect, endogenous adenosine receptor activation was involved in the mechanism. In rabbit model, occlusion of another coronary artery or carotid artery at the end of index ischemia both decreased cardiac infarct size, and these interventions conferred a more obvious protective role than classic post conditioning (Gritsopoulos *et al.*, 2009). RPostC applied to the lower limb decreased infarct size and release of creatinine kinase in swine (Andreka *et al.*, 2007). Especially, RPostC was also applied in human (Loukogeorgakis *et al.*, 2007). Five minutes of ischemia and reperfusion on the contralateral arm or leg during ischemic phase was used in human arm of 20 min ischemia followed by reperfusion. RPostC prevented endothelial I/R injury both in healthy volunteers and atherosclerosis patients, and the protective role was depended on activation of K_{ATP} channel. These results suggested that RPostC stimuli could play the protective effect in patients with acute ischemia when undergoing therapeutic reperfusion.

In our study, we selected occlusion of right femoral artery to duplicated RPostC, the operation is simple and propitious to apply to clinic. The protective role has been recognized widely but the concrete mechanisms are unclear. Activation of K_{Ca} channel participated in the protective role against I/R injury. This channel had involved in the protective role of puerarin, TNF α and other pharmacological preconditioning as well as IPC (Gao *et al.*, 2005; Cao *et al.*, 2005; Gao *et al.*, 2007). In cardiac myocytes, K_{Ca} channel is expressed in mitochondria (Xu *et al.*, 2002). When mitochondrial K_{Ca} channel was activated, K^+ entered into mitochondrial matrix during cardiac ischemia, which increased matrix

Table 3: The arrhythmia scores in the groups of hearts subjected to 45 min of ischemia and 180 min of reperfusion

	Baseline	Reperfusion (min.)		
		0-60	61-120	121-180
I/R	0 \pm 0	3.50 \pm 0.84	1.50 \pm 0.92	1.00 \pm 0.71
RPostC	0 \pm 0	1.80 \pm 0.86**	1.42 \pm 0.71	0.81 \pm 0.45
Delayed RPostC	0 \pm 0	3.12 \pm 0.77#	1.60 \pm 0.54	0.80 \pm 0.62
NS 1619	0 \pm 0	1.91 \pm 0.67**	1.52 \pm 0.44	0.75 \pm 0.40
RPostC+NS 1619	0 \pm 0	1.76 \pm 0.93**	1.33 \pm 0.60	0.68 \pm 0.55
RPostC+IbTX	0 \pm 0	3.15 \pm 0.75#	1.25 \pm 0.50	0.62 \pm 0.58
RPostC+Chel	0 \pm 0	3.23 \pm 0.92#	1.20 \pm 0.55	0.60 \pm 0.54

I/R: ischemia and reperfusion; RPostC: remote ischemic post conditioning. (mean \pm SD, n = 6) ** $P < 0.01$ vs I/R; # $P < 0.05$ vs RPostC

volume and preserves ATP synthesis. It also can inhibit the opening of mitochondrial permeability transition pore and lead to mild uncoupling of the electron transport chain. This is useful to reduce the burst of ROS at the beginning of reperfusion (Skulachev., 1998; Dos Santos *et al.*, 2004). Whether the channel participates in the role of RPostC? In our study, we observed that the specific K_{Ca} channel inhibitor IbTX attenuated the effect of RPostC, it suggested that K_{Ca} channel was involved in the mechanisms of RPostC protective role.

It is mentioned that when we combined the specific activator of K_{Ca} channel NS 1619 and RPostC in rat heart, it was not enhance the protective effect of RPostC. We speculated these two interventions could play the role through same pathway: activating K_{Ca} channel. NS 1619 alone can directly open K_{Ca} channel, maybe RPostC alone also can open K_{Ca} channel through some signaling transductions, it is worth to do further research.

Protein kinase C (PKC) medicates the injury of cardiac ischemia and reperfusion. PKC was first identified in 1977, and PKC was involved in numerous signal transduction processes including differentiation and proliferation, tumor progression, secretion, apoptosis and so on. Activation of PKC was reported to participate in the protective role of ischemic preconditioning (Okamura *et al.*, 1999). We observed that chelerythrine, the inhibitor of PKC attenuated the effect of RPostC, suggesting activation of PKC was also involved in the mechanisms of RPostC.

It is controversial about the relationship of PKC and K_{Ca} channel, it has been recognized that PKC can regulate the activity of K_{Ca} channel (Cao *et al.*, 2005). But others reported that PKA activation is necessary for the K_{Ca} channel opening (Sato *et al.*, 2005; Redel *et al.*, 2008). In RPostC intervention, it needs more evidences to verify whether PKC plays the cardioprotective role through modulating the activity of mito K_{Ca} channel.

CONCLUSION

The present study demonstrated that activation of K_{Ca} channel and protein kinase C both participate in remote ischemic post conditioning-induced cardioprotective role in rat heart subjected to ischemia/reperfusion injury *in vivo*.

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