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Delayed preconditioning by cardiac ischemia involves endogenous calcitonin gene-related peptide via the nitric oxide pathway

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Abstract

Previous investigations have shown separately that calcitonin gene-related peptide (CGRP) or nitric oxide (NO) is involved in mediation of ischemic preconditioning. In the present study, we tested interactions of CGRP with NO in mediation of delayed preconditioning. In Sprague–Dawley rats, ischemia–reperfusion injury was induced by 45-min occlusion followed by 3-h reperfusion of coronary artery, and preconditioning was induced by four cycles of 3-min ischemia and 5-min reperfusion. Infarct size, plasma creatine kinase activity, the plasma level of NO and CGRP, and the expression of CGRP mRNA in dorsal root ganglion were measured. Pretreatment with preconditioning significantly reduced infarct size and the release of creatine kinase during reperfusion, and caused a significant increase in the expression of CGRP mRNA, concomitantly with an elevation in the plasma level of CGRP and NO. The effects of preconditioning were completely abolished by administration of L-nitroarginine methyl ester (L-NAME, 10 mg/kg, i.p.), an inhibitor of NO synthase. Pretreatment with capsaicin (50 mg/kg, s.c.), which depletes transmitters in capsaicin-sensitive sensory nerves, also blocked the cardioprotection of preconditioning and reduced the synthesis and release of CGRP, but did not affect the concentration of NO. The present results suggest the delayed protection afforded by ischemic preconditioning is also mediated by endogenous CGRP via the NO pathway in rat heart.

Keywords: Calcitonin gene-related peptide (CGRP); Ischemic preconditioning; Ischemia–reperfusion; Capsaicin; Nitric oxide (NO)

1. Introduction

Brief episodes of sublethal ischemia enhance the resistance of the myocardium to subsequent ischemic insults (Murry et al., 1986). This phenomenon, termed ischemic preconditioning, was found to consist of two phases: an early phase, which occurs immediately and lasts for 1–2 h, and a late phase, which becomes apparent 12–24 h later and lasts for 3–4 days (Baxter and Yellon, 1997). Recently, more interest has focused on the latter, because of its sustained duration and effective cardioprotection against both myocardial infarction and stunning (Bolli, 1996). The mechanisms responsible for the protective effect of ischemic preconditioning have not been fully elucidated. Abundance of evidence has suggested that endogenous active substances including neurotransmitters and autacoids are involved in the mediation of ischemic preconditioning (Parratt, 1993).

Calcitonin gene-related peptide (CGRP), a principal transmitter in capsaicin-sensitive sensory nerves, has been proved to participate in the early phase of ischemic preconditioning in rat heart (Xiao et al., 1996; Lu et al., 1999). Our recent study found that CGRP was also involved in the mediation of delayed preconditioning induced by intestinal ischemia (Tang et al., 1999), heat stress (Song et al., 1999) and some drugs (He et al., 2001; Zhou et al., 2002).

Nitric oxide (NO) plays a critical role on the late phase of ischemic preconditioning (Bolli, 2001) and it has been shown that there are interactions of NO with other endogenous substances such as prostacyclin in mediation of ischemic preconditioning (Shinmura et al., 2002). More recently, our work found that delayed preconditioning induced by intestinal ischemia and some drugs was related to stimulation of the synthesis and release of CGRP via the NO pathway (Xiao et al., 2001; Zhou et al., 2002; Peng et al., 2002). In the

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present study, therefore, we tested whether delayed cardioprotection afforded by cardiac ischemic preconditioning involves endogenous CGRP via the NO pathway.

2. Materials and methods

All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 86-23, revised 1986).

2.1. Experimental protocols

Male Sprague–Dawley rats weighing 220–280 g was randomly divided into six groups. All rats were subjected to 45 min of coronary artery occlusion followed by 180 min of reperfusion. I/R; in the ischemic preconditioning group, rats were subjected to four cycles of 3-min left coronary artery occlusion followed by 5-min reperfusion 24 h before I/R; the sham group underwent the same procedure but without occlusion of coronary artery 24 h before I/R; in the L-nitroarginine methyl ester (L-NAME) plus ischemic preconditioning group, rats were pretreated with L-NAME (10 mg/kg, i.p.) 30 min before preconditioning; in the capsaicin treated group, rats were treated with capsaicin (50 mg/kg, dissolved in a vehicle containing 10% Tween 80, 10% ethanol and 80% saline, s.c.) 4 days before experiment.

The second series of experiments was designed to evaluate the effect of cardiac ischemic preconditioning on the synthesis and release of CGRP, and the role of NO. Rats were randomly divided into four groups: sham group, ischemic preconditioning, L-NAME and ischemic preconditioning, capsaicin and ischemic preconditioning. In order to rule out the effect of myocardial ischemia on the levels of CGRP and NO, all groups were subjected to the same procedures as in the first series of experiments apart from coronary artery occlusion.

2.2. Surgical preparation

After anesthetized with sodium pentobarbital (60 mg/kg, i.p.), rats were intubated with an endotracheal tube through glottis and mechanically ventilated with room air using a positive pressure ventilator. The ventilation rate was maintained at 30–35 strokes/min with a tidal volume of approximately 15 ml. Electrocardiograph (ECG) leads were connected to the chest and limbs for continuous ECG monitoring throughout the experiment. After administration of antibiotics, a left thoracotomy was performed in the fourth intercostal space and the pericardium opened to expose the heart. A 4-0 silk suture was passed around the left coronary artery and a snare formed by passing both ends of the suture through a piece of polyethylene tubing. Occlusion of the coronary artery, by clamping the snare against the surface of the heart, caused an area of epicardial cyanosis with regional hypokinesis and ECG changes. Reperfusion was achieved by releasing the snare and was confirmed by conspicuous hyperaemic blushing of the previously ischemic myocardium and gradual resolution of the changes in the ECG signal. After ischemic preconditioning was applied, the thoracic cavity was closed. Twenty-four hours later, the thoracic cavity was opened again, this time the animals experienced a long-term ischemia–reperfusion.

2.3. Infarct size and risk area

At the end of 3-h reperfusion, blood samples were collected from the carotid artery. The left coronary was reoccluded, and 1 ml Evans blue (1%) was injected into the left ventricular cavity in vivo and allowed to perfuse the nonischemic portions of the heart. The entire heart was excised, frozen, and then sliced into 1-mm-thick sections from the apex to base. The slices were incubated in 1% triphenyl tetrazolium chloride solution at 37 °C for 20 min to stain the viable myocardium brick red. The samples were then fixed in a 10% formalin solution for 24 h. Sections were traced onto acetate sheets. The area of infarct and risk zone was determined by planimetry of the tracings.

2.4. Creatine kinase assay

At the end of 3-h reperfusion, the serum creatine kinase activity was measured spectrophotometrically.

2.5. In situ hybridization

After pretreatment with cardiac ischemic preconditioning for 24 h, blood samples were collected from the carotid artery for measurement of CGRP and NO concentration, and then thoracic T1 dorsal root ganglion were rapidly dissected and snap frozen in liquid nitrogen. The samples were serially cut in a cryostat (20 μm) and mounted onto poly-L-lysine slides, fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, PH 7.5) for 15 min, and then stored at −70 °C until used.

After several washes, the sections were digested with pepsin in 3% citromalic acid (1:10 dilution) for 2 min, and then preincubated with hybridization buffer for 2 h at 37 °C. The probe we used is GreenStar* DIG-labeled GeneDect™ oligonucleotide probe, which was complementary to nucleotides 60–103 of rat CGRP/calcitonin gene (44-mer, ACAACCAGGAAAGGGGAGAACTTCAGAAAGCC-CATGATGCCTCC). Each slide was overlaid with 25 μl of probe in hybridization buffer at a concentration of 400 ng/ml, covered with paraffin and incubated for hybridization in humidified containers at 37 °C for 20 h. After hybridization, the sections were washed in 1× SSC (standard saline citrate) and subsequently in 0.5× SSC, each for 2× 15 min at 55 °C and then for 15 min at room temperature. After being incubated with blocking solution for 30 min, the sections were immunoreacted with sheep anti-Digoxigenin-AP at a

Table 1
Heart wet weight, area at risk, infarct size of each group

Group	*n*	Heart wet weight (g)	Area at risk (cm^2)	Infarct size (cm^2)
I/R	7	0.81±0.01	0.40±0.01	0.18±0.01
+Sham	8	0.82±0.03	0.40±0.02	0.19±0.02
+IPC	8	0.82±0.03	0.41±0.02	0.11±0.03[a]
+L-NAME and IPC	7	0.80±0.04	0.38±0.03	0.17±0.04[b]
+Cap and IPC	7	0.79±0.03	0.40±0.01	0.18±0.02[b]
+Vehicle and IPC	8	0.81±0.02	0.41±0.02	0.10±0.02

Values are means±S.E.M.; I/R: ischemia–reperfusion; IPC: pretreatment with cardiac ischemic preconditioning; L-NAME: L-nitroarginine methyl ester; Cap: capsaicin; vehicle: the vehicle of capsaicin.
[a] $P<0.01$ vs. I/R.
[b] $P<0.01$ vs. ischemic preconditioning.

dilution of 1:200 for 4 h at room temperature. The colour development was carried out over a 12-h period using BCIP/NBT at 37 °C. Finally, the slides were dehydrated through an alcohol series, cleared with xylene and cover-slipped. In negative control, the sections were hybridized with sense CGRP probe or in the absence of probe.

The semiquantitative analysis was according to the previous researches (Schicho and Donnerer, 1999). Three sections of the T1 dorsal root ganglion from each animal were viewed with microscope and photographed by a digital camera coupled to a computer. The pictures were processed and evaluated by an image analysis program. A total of 300–400 neurons/ganglion with visible nucleus from sections 60 μm apart to avoid measurement of the same neuron twice were counted. Background grain densities were determined by averaging grain counts of cells clearly not expressing. Only the neurons with densities five times more than background were considered positive for expression. The percentage of CGRP mRNA expressing neurons was determined by dividing the number of positive neurons by the number of all neurons in a section.

2.6. Measurement of CGRP-LI levels

Blood samples (3 ml) were placed in tubes containing 10% Na_2EDTA 40 μl and aprotinin 400 mU/l. Plasma was obtained by centrifugation at 1300×*g* for 20 min (4 °C). Plasma concentrations of CGRP-like immunoreactivity (CGRP-LI) were determined by radioimmuoassay kits using antisera raised against rat CGRP, ^{125}I-labeled CGRP and rat CGRP standard.

2.7. Measurement of the plasma NO concentration

The plasma level of nitric oxide was determined indirectly as the content of nitrite and nitrate. The plasma level of nitrite/nitrate was measured as previously described (Feng et al., 2001). Briefly, nitrite was converted to nitrate with aspergillus nitrite reductase, and the total nitrate was measured with the Griess reagent. The absorbance was determined at 540 nm with a spectrophotometer.

2.8. Reagents

Capsaicin, L-nitroarginine methyl ester, triphenyl tetrazolium chloride and Evans blue were purchased from Sigma (St. Lous, MO, USA). Creatine Kinase assay kits were obtained from Zhongsheng Bioengineering (Beijing, China). Radioimmunoassay kits for measurement of CGRP were purchased from Immunity Institute of Dongya (Beijing, China). NO assay kits were provided by Ju-Li Biological Medical Engineering Institute (Nanjing, China). GreenStar* DIG-labeled GeneDect™ oligonucleotide probes (sense and antisense) were purchased from GeneDect (New Zealand). Hybridization buffer was provided by DAKO (Denmark). Sheep anti-Digoxigenin-AP was obtained from Roche Molecular Biochemicals (Germany). Pepsin and BCIP/NBT kits were provided by Boster Biological Technology (Wuhan, China).

2.9. Statistical analysis

Data are expressed as means±S.E.M. All values were analyzed by using one-way analysis of variance and the Student–Newman–Keuls test. $P<0.05$ was regarded as significant.

3. Results

3.1. Infarct size

As shown in Table 1, there were no significant differences in heart weight and risk zone among groups, indicating that the size of the risk zone was comparable in all groups. Ischemia–reperfusion caused 44.66±2.66% necrosis in the area at risk. Pretreatment with cardiac preconditioning

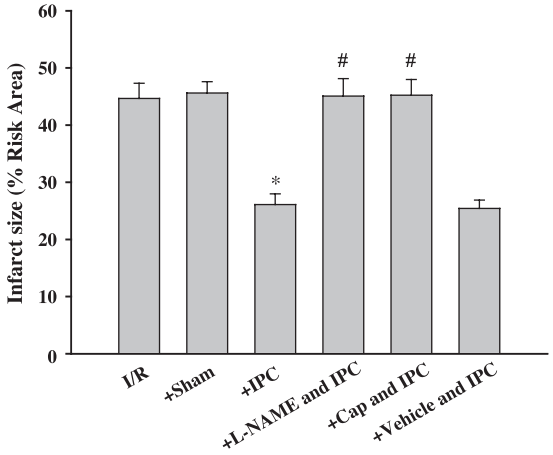

Fig. 1. Effect of cardiac ischemic preconditioning on myocardial infarct size, expressed as percentage of the area at risk (AAR). All values were expressed as means±S.E.M. (*n*=7–8). I/R: ischemia–reperfusion; ischemic preconditioning: pretreatment with cardiac ischemic preconditioning; L-NAME: L-nitroarginine methyl ester (10 mg/kg, i.p.); Cap: capsaicin (50 mg/kg, s.c.).*$P<0.01$ vs. I/R; #$P<0.01$ vs. ischemic preconditioning.

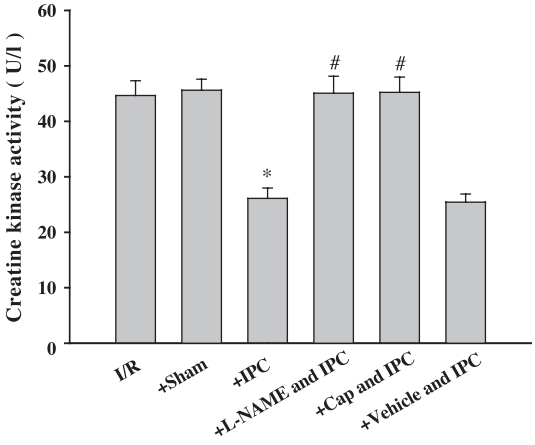

Fig. 2. Effect of cardiac ischemic preconditioning on creatine kinase activity. All values were expressed as means±S.E.M. (n=7–8). I/R: ischemia–reperfusion; ischemic preconditioning: pretreatment with cardiac ischemic preconditioning; L-NAME: L-nitroarginine methyl ester (10 mg/kg, i.p.); Cap: capsaicin (50 mg/kg, s.c.). *P<0.01 vs. I/R; $^{\#}P$<0.01 vs. ischemic preconditioning.

significantly reduced infarct size (26.11±1.85%). L-NAME, an inhibitor of nitric oxide synthase, or pretreatment with capsaicin, which depletes transmitters in sensory nerves, reduced the decreased infract size by preconditioning. The vehicle of capsaicin had no effect on the cardioprotection provided by preconditioning (Fig. 1).

3.2. Creatine kinase release

Ischemia–reperfusion caused a significant increase in the serum level of creatine kinase. The release of creatine kinase was remarkably reduced after pretreatment with preconditioning, and the effect was also attenuated by L-NAME or capsaicin pretreatment. The vehicle of capsaicin also had no effect on the release of creatine kinase during reperfusion (Fig. 2).

3.3. CGRP mRNA expression

CGRP signals were distributed in the cytoplasm of neurons. Most of the signals were found in small- and medium-sized cells, but also in the large-sized cell population (Fig. 3). The percentage of neurons with expression of CGRP mRNA in ischemic preconditioning group was significantly increased to 50.34±2.61% compared with the sham group (36.03±1.92%), which was abolished by pretreatment with L-NAME and capsaicin (Fig. 4). In both negative controls, there is no detectable CGRP signal.

3.4. Plasma concentrations of CGRP-LI

Pretreatment with cardiac ischemic preconditioning markedly elevated CGRP-LI level in plasma. The increased release of CGRP by ischemic preconditioning was abolished by L-NAME or pretreatment with capsaicin (Fig. 5).

3.5. Serum concentrations of nitrite/nitrate

Serum concentrations of nitrite/nitrate in the rats pretreated with preconditioning were remarkably increased compared with the sham group. Pretreatment with L-NAME blocked the elevated level of nitrite/nitrate by ischemic

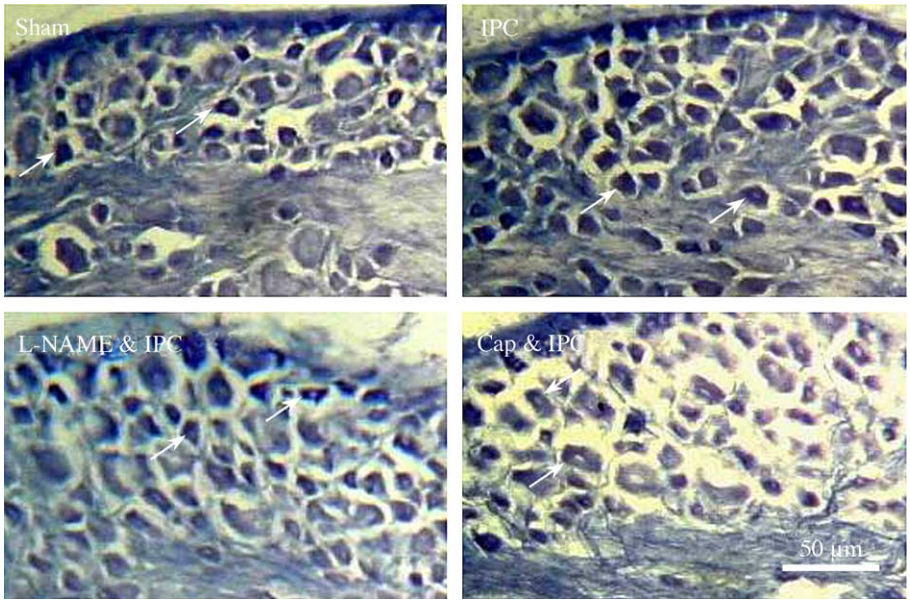

Fig. 3. Detection of CGRP mRNA of T1 dorsal root ganglion with in situ hybridization. There are many CGRP mRNA-positive neurons in the sections of dorsal root ganglion (arrowed). CGRP mRNA signals are distributed in the cytoplasm of neuron. Compared with sham group, cardiac ischemia preconditioning markedly increased the numbers of CGRP mRNA-positive cells, while pretreatment of L-NAME and capsaicin abolished this effect.

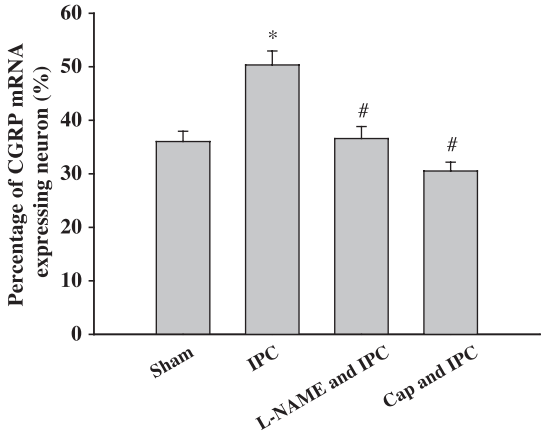

Fig. 4. Effect of cardiac ischemic preconditioning on the level of CGRP mRNA in T1 dorsal root ganglion. Values are expressed as percentage of CGRP mRNA-positive neurons and as means±S.E.M. (n=5–6). Ischemic preconditioning: pretreatment with cardiac ischemic preconditioning; L-NAME: L-nitroarginine methyl ester (10 mg/kg, i.p.); Cap: capsaicin (50 mg/kg, s.c.). *P<0.01 vs. sham; $^{\#}P$<0.01 vs. ischemic preconditioning.

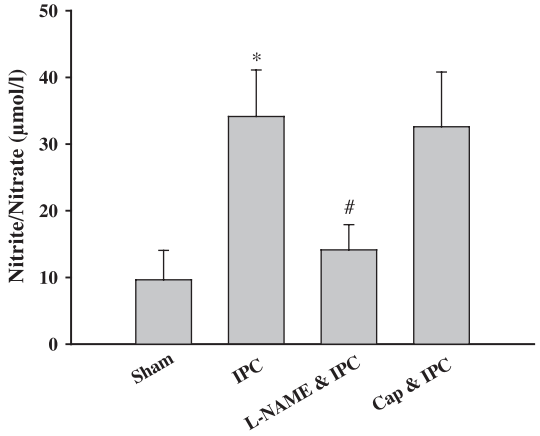

Fig. 6. Effect of cardiac ischemic preconditioning on plasma concentration of nitrite/nitrate. All values were expressed as means±S.E.M. (n=6). Ischemic preconditioning: pretreatment with cardiac ischemic preconditioning; L-NAME: L-nitroarginine methyl ester (10 mg/kg, i.p.); Cap: capsaicin (50 mg/, s.c.).*P<0.05 vs. sham; $^{\#}P$<0.05 vs. ischemic preconditioning.

preconditioning. Pretreatment with capsaicin had no effect on nitrite/nitrate level (Fig. 6).

4. Discussion

CGRP, the principal transmitter in capsaicin-sensitive sensory nerves, is widely distributed in cardiovascular tissues (Franco-Cereceda, 1988). CGRP has protective effects on the ischemic myocardium (Ren et al., 1993; Kallner and Franco-Cereceda, 1998) and endothelial cells (Peng et al., 1995), and it has been considered as an important endogenous myocardial protective substance. An extensive series of studies has shown that CGRP is not only a important mediator in early protection of cardiac ischemic preconditioning (Ouyang et al., 1999; Lu et al., 1999), but also participates in mediation of delayed cardioprotection afforded by other stimulus, such as heat stress (Song et al., 1999), intestinal ischemia (Tang et al., 1999) and some drugs (He et al., 2001; Zhou et al., 2002). In the cardioprotection of preconditioning, early protection is ascribed to stimulation of CGRP release, whereas delayed protection is related to elevation of the synthesis and release of CGRP. In the present study, cardiac ischemic preconditioning remarkably reduced infarct size and decreased CK release during ischemia–reperfusion, concomitantly with an increase in concentration of CGRP in plasma and the level of CGRP mRNA in dorsal root ganglion, a major site of CGRP synthesis. The effects of preconditioning were abolished by capsaicin, which depletes neurotransmitter in sensory nerves. Taken together, these results add to the existing evidence that delayed cardioprotection induced by various factors is related to the synthesis and release of CGRP.

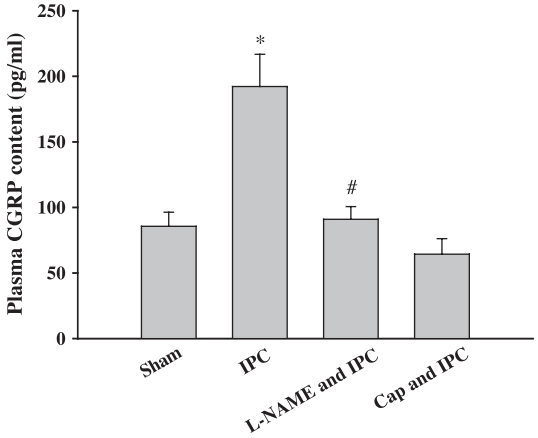

Fig. 5. Effect of cardiac ischemic preconditioning on plasma concentrations of CGRP-LI. All values were expressed as means±S.E.M. (n=6). Ischemic preconditioning: pretreatment with cardiac ischemic preconditioning; L-NAME: L-nitroarginine methyl ester (10 mg/kg, i.p.); Cap: capsaicin (50 mg/kg, s.c.).*P<0.01 vs. sham; $^{\#}P$<0.01 vs. ischemic preconditioning.

It is noteworthy that capsaicin-sensitive sensory nerves contain a number of peptides, including CGRP, substance P and neurokinin A (Franco-Cereceda, 1988). Besides CGRP, substance P and neurokinin A could also mediate the effect of capsaicin. Among these peptides, CGRP has been shown to exert a beneficial effect on the myocardium. Although substance P and neurokinin A were shown to cause only vasodilation and contraction of smooth muscles in bronchi (Maggie, 1988; Pernow, 1983), we cannot exclude the possibility that either substance P or neurokinin A is beneficial to the myocardium, although direct protection by either has not yet been reported.

Nitric oxide, an ubiquitous air molecule, has been proved to be an essential mediator in preconditioning (Guo et al., 1999). Ischemia can induced NO generation, and it has been proposed as initially the trigger and subsequently the mediator of delayed preconditioning (Bolli, 2001). Nitroglycerin, a donor of NO, induced delayed preconditioning-

like protection (Takano et al., 1998). Others have shown that monophosphoryl lipid A, a detoxified derivative of endotoxin, induced delayed cardioprotection by stimulation of NO production (Tosaki et al., 1998; Xi et al., 1999). NO was also involved in mediation of delayed preconditioning of intestine ischemia (Tang et al., 1999). Our present results confirm the previous observations that the blood concentration of NO was increased in the rats pretreated with preconditioning, and the cardioprotection of preconditioning was completely abolished by pretreatment with L-NAME, the inhibitor of NO synthase.

It has been shown that the protective effects of ischemic preconditioning involves multiple endogenous active substances, such as NO, CGRP, bradykinin, noradrenalin, prostaglandin and opioids, which may mediate the cardioprotection via interactions with one another (Yellon et al., 1998). For example, the beneficial effect of NO is mediated by prostaglandin pathway (Shinmura et al., 2001). Delayed preconditioning induced by MLA is related to stimulation of the synthesis and release of CGRP via increasing NO production (Peng et al., 2002); nitroglycerin-induced delayed protection is mediated by endogenous CGRP via the NO-cGMP pathway (Zhou et al., 2002). Additionally, delayed cardioprotection by intestinal ischemic preconditioning also involved the release of CGRP via stimulation NO generation (Xiao et al., 2001). In the present study, pretreatment with L-NAME abolished the elevation of CGRP mRNA expression in dorsal root ganglion, and the CGRP level in plasma. However, the increase in level of NO by preconditioning has not been affected by capsaicin. Our recent work has shown that protective effects of preconditioning involve co-mediation of NO and CGRP. These findings allow us to speculate that ischemic preconditioning induces NO production, with a subsequent stimulation of the synthesis and release of CGRP, resulting in cardioprotection.

In conclusion, the present study suggest that delayed cardioprotection afforded by ischemic preconditioning is mediated by endogenous CGRP via the NO pathway.

Acknowledgements

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