ELSEVIER

European Journal of Pharmacology 427 (2001) 131–135

ejp

www.elsevier.com/locate/ejphar

Delayed cardioprotection by intestinal preconditioning is mediated by calcitonin gene-related peptide

Liang Xiao, Rong Lu, Chang-Ping Hu, Han-Wu Deng, Yuan-Jian Li *

Department of Pharmacology, Hunan Medical University, Changsha, Hunan, 410078 China

Received 23 April 2001; received in revised form 16 July 2001; accepted 20 July 2001

Abstract

Previous studies have shown that nitric oxide and calcitonin gene-related peptide (CGRP) are involved in mediation of the delayed cardioprotection of ischemic or pharmacological preconditioning, and nitric oxide can evoke the release of CGRP. In the present study, we examined the role of CGRP in nitric oxide-mediated delayed cardioprotection by brief intestinal ischemia in rats. The serum concentration of creatine kinase and infarct size were measured after 45-min coronary artery occlusion and 180-min reperfusion. Ischemic preconditioning was induced by six cycles of 4-min ischemia and 4-min reperfusion of the small intestine. Pretreatment with intestinal ischemic preconditioning for 24, 48, or 72 h significantly reduced infarct size and creatine kinase release, and the effects of ischemic preconditioning were completely abolished by L-nitroarginine methyl ester (L-NAME, 10 mg/kg, i.p.), an inhibitor of nitric oxide synthase, or by pretreatment with capsaicin (50 mg/kg, s.c.), which selectively depletes transmitters in capsaicin-sensitive sensory nerves. Intestinal preconditioning caused a significant increase in plasma concentrations of CGRP, and the effect was also abolished by L-NAME or capsaicin. These results suggest that the delayed cardioprotection afforded by intestinal ischemic preconditioning is mediated by endogenous CGRP via the nitric oxide pathway. © 2001 Published by Elsevier Science B.V.

Keywords: CGRP (calcitonin gene-related peptide); Intestinal ischemic preconditioning; Ischemia–reperfusion; Capsaicin; Nitric oxide (NO)

1. Introduction

The preconditioning of the heart induced by brief periods of ischemia can attenuate the injury due to ischemia–reperfusion (Murry et al., 1986), and the beneficial effects of ischemic preconditioning are also seen in a variety of tissues (Davis et al., 1999; Gurke et al., 2000). Recently, it has been found that repetitive short periods of ischemia in non-cardiac tissues such as the small intestine, kidney and skeletal muscle (Gho et al., 1996; Birnbaum et al., 1997) are also able to protect the myocardium against ischemia–reperfusion, and this phenomenon is defined as "remote organ preconditioning" (Yellon and Baxter, 1995). The mechanisms underlying preconditioning have not yet been elucidated but one suggestion is that the protective effects are mediated by endogenous active substances including neurotransmitters and autocoids. Nitric oxide has been shown to participate in the mediation of early cardioprotection of ischemic or pharmacological preconditioning and may be an important mediator of preconditioning (Vegh et al., 1992; Massoudy et al., 1995; Hu et al., 1999; Yao and Gross, 1993). It is not known, however, whether endogenous nitric oxide is also involved in the delayed cardioprotection afforded by remote organ preconditioning.

Calcitonin gene-related peptide (CGRP), a principal transmitter in capsaicin-sensitive sensory nerves, has been shown to participate in mediation of the cardioprotection induced by either cardiac preconditioning or remote organ preconditioning (Li et al., 1996; Ferdinandy et al., 1997; Lu et al., 1999; Tang et al., 1999). Ischemia can stimulate the release of multiple endogenous chemical mediators. It is likely that these endogenous substances mediate the protective effects of ischemic preconditioning via interactions with one another. Our recent work has shown that endogenous or exogenous nitric oxide-mediated preconditioning is related to stimulation of CGRP release (He et al., 2001; Hu et al., 1999). Therefore, in the present study, we examined interactions of CGRP and nitric oxide in media-

* Corresponding author. Tel.: +86-731-480-5441; fax: +86-731-447-1289.

E-mail address: liyj@public.cs.hn.cn (Y.-J. Li).

0014-2999/01/$ - see front matter © 2001 Published by Elsevier Science B.V.
PII: S0014-2999(01)01231-6

tion of the delayed cardioprotection provided by ischemia of the small intestine in rats.

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. Surgical preparation

Male Wistar rats weighing 260–300 g were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), and then mechanically ventilated with room air using a positive pressure ventilator. The ventilation rate was maintained at 30–35 strokes min^{-1} 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. A left thoracotomy was performed in the fourth intercostal space and the pericardium was opened to expose the heart. A 4–0 silk suture was passed around the left coronary artery and a snare was 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.

The anterior mesenteric artery was dissected free and a suture was placed around the artery to facilitate occlusion with a traumatic clamp. After the ischemic stimulus was applied, the abdomen was closed. The ischemia–reperfusion group underwent the same procedure but without clipping of the anterior mesenteric artery.

2.2. Experimental protocols

The experiment was done with the animals randomly divided into seven groups. All animals were subjected to 45 min of coronary artery occlusion followed by 180 min of reperfusion. In the intestinal ischemic preconditioning group, the rats were subjected to six cycles of 4-min anterior mesenteric artery occlusion followed by 4-min reperfusion 24, 48, or 72 h before coronary artery occlusion. For L-nitroarginine methyl ester (L-NAME) and capsaicin, the rats were pretreated with L-NAME (10 mg/kg, i.p.) or capsaicin (50 mg/kg, disolved in a vehicle containing 10% Tween 80, 10% ethanol and 80% saline, s.c.) for 30 min and 4 days, respectively, before anterior mesenteric artery occlusion.

The second series of experiments was designed to evaluate the effect of intestinal ischemic preconditioning on the release of CGRP. In order to rule out the effect of myocardial ischemia on CGRP levels, all groups were subjected to the same procedures as in the first series of experiments apart from coronary artery occlusion.

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 re-occluded, and 1 ml Evans blue (1%) was injected into the left ventricular cavity in vivo and was allowed to perfuse the non-ischemic portions of the heart. The entire heart was excised, frozen, and then sliced into 1-mm thick sections from apex to base. The slices were incubated in 1% triphenyl tetrazolium chloride (TTC) 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 were determined by planimetry of the tracings.

2.4. Creatine kinase assay

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

2.5. Measurement of CGRP-like immunoreactivity levels

Blood samples (3 ml) were collected from the carotid artery 24, 48, and 72 h after intestinal ischemic preconditioning, and placed in tubes containing 10% Na_2EDTA, 40 μl, and aprotinin 400 mU/l. Plasma was obtained by centrifugation at $1300 \times g$ for 20 min (4 °C). Plasma concentrations of CGRP-like immunoreactivity were determined with radioimmuoassay kits and antisera raised against rat CGRP, ^{125}I-labelled CGRP and rat CGRP standard.

2.6. Reagents

Capsaicin, L-nitroarginine methyl ester, triphenyl tetrazolium chloride and Evans blue were purchased from Sigma (St Louis, MO, USA). Creatine kinase assay kits were obtained from Zhongsheng Bioengineering (Beijing, P. R. China). Radioimmunoassay kits for measurement of CGRP were purchased from the Immunity Institute of Dongya (Beijing, P. R. China).

2.7. Statistical analysis

Data are expressed as means ± S.E.M. All values were analyzed with a one-way analysis of variance and the

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

Group	*n*	Heart wet weight (g)	Area at risk (cm^3)	Infarct size (cm^3)
Ischemia–reperfusion	7	0.75 ± 0.01	0.39 ± 0.01	0.24 ± 0.03
+ PC (24 h)	7	0.76 ± 0.01	0.39 ± 0.01	0.16 ± 0.01[a]
+ PC (48 h)	7	0.75 ± 0.01	0.38 ± 0.01	0.12 ± 0.01[a]
+ PC (72 h)	7	0.76 ± 0.01	0.37 ± 0.01	0.15 ± 0.02[a]
+ PC (48 h) and L-NAME	7	0.75 ± 0.01	0.40 ± 0.01	0.21 ± 0.01[b]
+ PC (48 h) and vehicle (Cap)	6	0.75 ± 0.01	0.40 ± 0.01	0.15 ± 0.02
+ PC (48 h) and Cap	6	0.76 ± 0.01	0.40 ± 0.01	0.22 ± 0.01[c]

Values are means ± S.E.M.; PC (24, 48, or 72 h): pretreatment with intestinal ischemic preconditioning for 24, 48, or 72 h; L-NAME: L-nitroarginine methyl ester (10 mg/kg, i.p.); Cap: capsaicin (50 mg/kg, s.c.).
[a] $P < 0.01$ vs. control.
[b] $P < 0.01$ vs. 48 h.
[c] $P < 0.05$ vs. vehicle.

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 weights and risk zone among groups, indicating that the size of the risk zone was comparable in all groups. Ischemia–reperfusion caused 62.0 ± 5.13% necrosis in the area at risk. Infarct size was reduced after pretreatment with intestinal ischemic preconditioning for 24, 48, or 72 h, and the maximal protective effect of ischemic preconditioning was at 48 h. L-NAME, an inhibitor of nitric oxide synthase, or pretreatment with capsaicin, which depletes transmitters in sensory nerves, abolished the decrease in infarct size by intestinal ischemic preconditioning. The vehicle of capsaicin had no effect on the protection provided by intestinal ischemic preconditioning (Fig. 1).

3.2. Creatine kinase release

Ischemia–reperfusion caused a significant increase in the serum level of creatine kinase. Similarly, the release of creatine kinase was reduced after pretreatment with intestinal ischemic preconditoning for 24, 48, or 72 h, and the effects were also abolished 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. Plasma concentrations of CGRP-like immunoreactivity

CGRP-like immunoreactivity levels in plasma were significantly elevated after pretreatment with intestinal is-

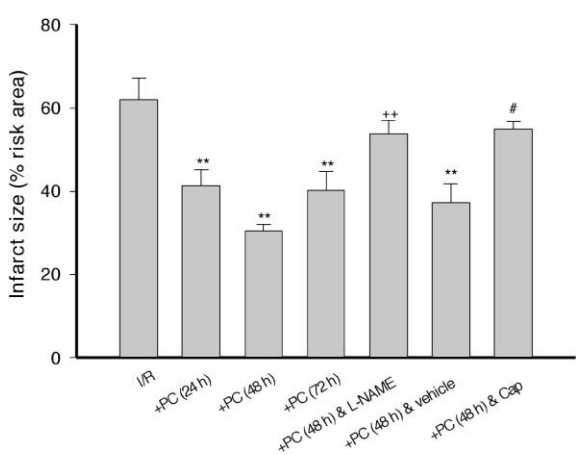

Fig. 1. Effect of intestinal ischemic preconditioning on creatine kinase activity. All values were expressed as means ± S.E.M. ($n = 6$). I/R: ischemia–reperfusion; PC (24, 48, or 72 h): pretreatment with intestinal ischemic preconditioning for 24, 48, or 72 h; L-NAME: L-nitroarginine methyl ester; Cap: capsaicin. $^{**}P < 0.01$ vs. I/R; $^{++}P < 0.01$ vs. PC (48 h); $^{\#\#}P < 0.01$ vs. PC and vehicle.

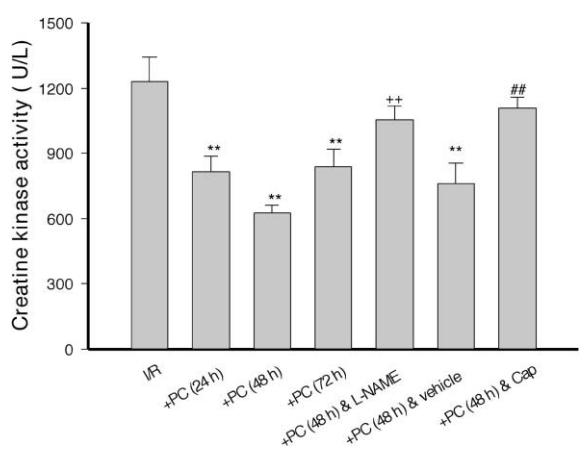

Fig. 2. Effect of intestinal preconditioning on myocardial infarct size, expressed as percentage of the area at risk (AAR). All values were expressed as means ± S.E.M. ($n = 6$–7). I/R: ischemia–reperfusion; PC (24, 48, or 72 h): pretreatment with intestinal ischemic preconditioning for 24, 48, or 72 h; L-NAME: L-nitroarginine methyl ester; Cap: capsaicin. $^{**}P < 0.01$ vs. I/R; $^{++}P < 0.01$ vs. PC (48 h); $^{\#}P < 0.05$ vs. PC and vehicle.

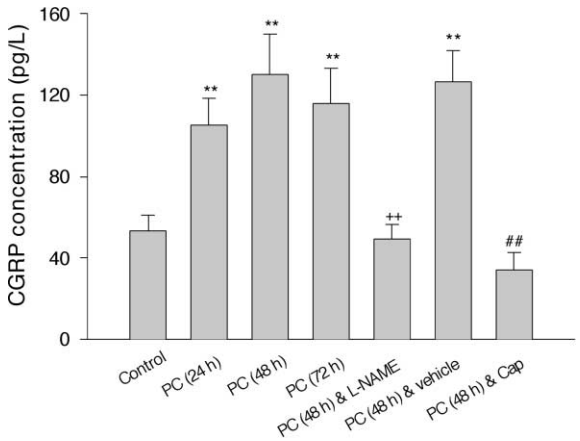

Fig. 3. Effects of intestinal ischemic preconditioning on plasma concentrations of CGRP-LI. All values were expressed as means ± S.E.M. ($n = 6$). I/R: ischemia–reperfusion; PC (24, 48, or 72 h): pretreatment with intestinal ischemic preconditioning for 24, 48, or 72 h; L-NAME: L-nitroarginine methyl ester; Cap: capsaicin. $^{**}P < 0.01$ vs. I/R; $^{++}P < 0.01$ vs. PC (48 h); $^{\#\#}P < 0.01$ vs. PC and vehicle.

chemic preconditioning for 24, 48, or 72 h. However, these increases in CGRP-like immunoreactivity by intestinal ischemic preconditioning were abolished by L-NAME or pretreatment with capsaicin (Fig. 3).

4. Discussion

It has been demonstrated that short-term ischemia not only triggers early preconditioning, but also induces delayed preconditioning or "second window of protection" (Kuzuya et al., 1993; Yellon and Baxter, 1995). It has been shown that the delayed cardioprotection provided by a pacing stimulus, or coronary occlusion, or drug pretreatment appears 24 h after preconditioning treatment and continues up to 72 h (Parratt and Szekeres, 1995; Baxter et al., 1997; Baxter and Yellon, 1997). In the present study, the cardioprotection was present at 24 h after ischemia of the small intestine, became manifest at 48 h, lasted for at least 3 days. The time course of this delayed protection afforded by brief ischemia of the small intestine is similar to that of the delayed protection of cardiac ischemic preconditioning.

There is substantial evidence to suggest that endogenous chemical mediators may play a pivotal role in the mediation of the protective effects of ischemic preconditioning. Previous investigations have shown that endogenous nitric oxide is involved in the early protection of heart or intestinal preconditioning (Vegh et al., 1992; Massoudy et al., 1995; He et al., 2001; Yao and Gross, 1993; Hotter et al., 1996). Recently, it has been shown that nitric oxide, endogenous or exogenous, is also involved in the delayed cardioprotection by ischemic preconditioning (Bolli et al., 1997; Jones et al., 1999). In the present study, the delayed cardioprotection provided by brief periods of ischemia in the small intestine was abolished by pretreatment with L-NAME, an inhibitor of nitric oxide synthase. These results support the hypothesis that nitric oxide may be an important mediator of the delayed cardioprotection induced by cardiac or remote organ preconditioning.

Neurogenic mechanisms have been suggested to be involved in the preconditioning induced by the intestinal preconditioning, and this is documented by previous observations that the cardioprotection by intestinal preconditioning is abolished by hexamethonium, a ganglion blocker (Gho et al., 1996). Previous studies have suggested that the beneficial effect of ischemic preconditioning is associated with stimulation of endogenous CGRP release, and CGRP also participates in mediation of the delayed preconditioning afforded by heat stress or drug pretreatment (Song et al., 1999; He et al., 2001). Our recent work has shown that CGRP is involved in the early and delayed cardioprotection afforded by brief ischemia of the small intestine in rabbits (Tang et al., 1999). In the present study, short-term ischemia of the small intestine in rats reduced infarct size and the release of creatine kinase concomitantly with an increase in plasma concentrations of CGRP, and the protective effects of intestinal preconditioning were abolished by pretreatment with capsaicin, which selectively deletes transmitters in capsaicin-sensitive sensory nerves. These findings suggest that the delayed cardioprotection induced by intestinal preconditioning is related to activation of capsaicin-sensitive sensory nerves.

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 effects of ischemic preconditioning. Among these peptides, CGRP has been shown to possess a beneficial effect on the myocardium and endothelial cells. Recently, it has been shown that the protection by ischemic preconditioning in vivo is abolished by CGRP antibodies (Ouyang et al., 1999). As mentioned above, in the present study, short-term ischemia of the small intestine caused an increase in concentrations of CGRP. These findings allow us to speculate that CGRP may play an important role in mediation of the delayed cardioprotection provided by intestinal preconditioning.

Previous investigations have suggested that endogenous substances mediate the protective effects of ischemic preconditioning via interactions with one another. Interactions of peptide with various autocoids, including nitric oxide, have been shown to occur pre-and postjunctionally (Wei et al., 1992; Booth et al., 2000). There is evidence that nitroglycerin, a nitric oxide donor, significantly evokes the release of CGRP in central and peripheral vessels (Bredt et al., 1990; Huges and Brain, 1994). The early protection by nitroglycerin-induced preconditioning has been shown to be related to stimulation of CGRP release. Recently, our work has shown that monophosphoryl lipid A-induced delayed preconditioning is mediated by CGRP through the nitric oxide pathway (He et al., 2001). In the present study,

the cardioprotection as well as the increased level of CGRP induced by intestinal preconditioning were abolished by pretreatment with L-NAME. This suggests that the delayed cardioprotection induced by intestinal preconditioning is also mediated by endogenous CGRP via the nitric oxide pathway.

In summary, the present results suggest that the delayed cardioprotection by intestinal ischemic preconditioning involves the release of CGRP via the nitric oxide pathway.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation China, No. 30070870.

References

Baxter, G.F., Yellon, D.M., 1997. Time course of delayed myocardial protection after transient adenosine A1-receptor activation in the rabbit. J. Cardiovasc. Pharmacol. 29, 631–638.

Baxter, G.F., Goma, F.M., Yellon, D.M., 1997. Characterization of the infarct-limiting effect of delayed preconditioning: timecourse and dose-dependency studies in rabbit myocardium. Basic Res. Cardiol. 92, 159–167.

Birnbaum, Y., Hale, S.L., Kloner, R.A., 1997. Ischemic preconditioning at a distance: reduction of myocardial infarct size by partial reduction of blood supply combined with rapid stimulation of the gastrocnemius muscle in the rabbit. Circulation 96, 1641–1646.

Bolli, R., Manchikalapudi, S., Tang, X.L., Takano, H., Qiu, Y., Guo, Y., Zhang, Q., Jadoon, A.K., 1997. The protective effect of late preconditioning against myocardial stunning in conscious rabbits is mediated by nitric oxide synthase. Evidence that nitric oxide acts both as a trigger and as a mediator of the late phase of ischemic preconditioning. Circ. Res. 81, 1094–1107.

Booth, B.P., Tabrizi-Fard, M.A., Fung, H., 2000. Calcitonin gene-related peptide-dependent vascular relaxation of rat aorta: an additional mechanism for nitroglycerin. Biochem. Pharmacol. 59, 1603–1609.

Bredt, D.S., Hwang, P.M., Snyder, S.H., 1990. Localization of nitric oxide synthase indicating a neural role for nitric oxide. Nature 347, 768–770.

Davis, J.M., Gute, D.C., Jones, S., Krsmanovic, A., Korthuis, R.J., 1999. Ischemic preconditioning prevents postischemic P-selectin expression in the rat small intestine. Am. J. Physiol. 277, H2476–H2481.

Ferdinandy, P., Csont, T., Csonka, C., Torok, M., Dux, M., Nemeth, J., Horvath, L.I., Dux, L., Szilvassy, Z., Jancso, G., 1997. Capsaicin-sensitive local sensory innervation is involved in pacing-induced preconditioning in rat heart: role of nitric oxide and CGRP? Naunyn-Schmiedeberg's Arch. Pharmacol. 356, 356–363.

Franco-Cereceda, A., 1988. Calcitonin gene-related peptide and tachykinin in relation to local sensory control of cardiac contractility and coronary vascular tone. Acta Physiol. Scand. 133 (Suppl. 596), 3–63.

Gho, B.C., Schoemaker, R.G., van den Doel, M.A., Duncker, D.J., Verdouw, P.D., 1996. Myocardial protection by brief ischemia in noncardiac tissue. Circulation 94, 2193–2200.

Gurke, L., Mattei, A., Chaloupka, K., Marx, A., Sutter, P.M., Stierli, P., Harder, F., Heberer, M., 2000. Mechanisms of ischemic preconditioning in skeletal muscle. J. Surg. Res. 94, 18–27.

He, S.Y., Deng, H.W., Li, Y.J., 2001. Monophosphoryl lipid A-induced delayed preconditioning is mediated by calcitonin gene-related peptide. Eur. J. Pharmacol. 420, 143–149.

Hotter, G., Closa, D., Prados, M., Fernandez-Cruz, L., Prats, N., Gelpi, E., Rosello-Catafau, J., 1996. Intestinal preconditioning is mediated by a transient increase in nitric oxide. Biochem. Biophys. Res. Commun. 222, 27–32.

Hu, C.P., Li, Y.J., Deng, H.W., 1999. The cardioprotective effects of nitroglycerin-induced preconditioning are mediated by calcitonin gene-related peptide. Eur. J. Pharmacol. 369, 189–194.

Huges, S.D., Brain, S.D., 1994. Nitric oxide-dependent release of vasodilator quantities of calcitonin gene-related peptide from capsaicin-sensitive nerves in rabbit skin. Br. J. Pharmacol. 111, 425–430.

Jones, W.K., Flaherty, M.P., Tang, X.L., Takano, H., Qiu, Y., Banerjee, S., Smith, T., Bolli, R., 1999. Ischemic preconditioning increases iNOS transcript levels in conscious rabbits via a nitric oxide-dependent mechanism. J. Mol. Cell. Cardiol. 31, 1469–1481.

Kuzuya, T., Hoshida, S., Yamashita, N., Fuji, H., Oe, H., Hori, M., Kamada, T., Tada, M., 1993. Related delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. Circ. Res. 72, 1293–1299.

Li, Y.J., Xiao, Z.S., Peng, C.F., Deng, H.W., 1996. Calcitonin gene-related-induced preconditioning protects against ischemia–reperfusion injury in isolated rat hearts. Eur. J. Pharmacol. 311, 163–167.

Lu, R., Li, Y.J., Deng, H.W., 1999. Evidence for calcitonin gene-related peptide-mediated ischemic preconditioning in the rat heart. Regul. Pept. 82, 53–57.

Massoudy, P., Becker, B.F., Gerlach, E., 1995. Nitric oxide accounts for postischemic cardioprotection resulting from angiotensin-converting enzyme inhibition: indirect evidence for a radical scavenger effect in isolated guinea pig heart. J. Cardiovasc. Pharmacol. 25, 440–447.

Murry, C.E., Jennings, R.B., Reimer, K.A., 1986. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 74, 1124–1136.

Ouyang, W., Qiang, X.X., Li, Z.L., Fu, X.Y., Wang, S.H., 1999. The roles of endogenous calcitonin gene-related peptide on myocardial ischemic preconditioning in intact rat. Chin. J. Arterioscler. (in Chinese) 7, 24–26.

Parratt, J.R., Szekeres, L., 1995. Delayed protection of the heart against ischaemia. Trends Pharmacol. Sci. 16, 351–355.

Song, Q.J., Li, Y.J., Deng, H.W., 1999. Early and delayed cardioprotection of heat stress is mediated by calcitonin gene-related peptide. Naunyn-Schmiedeberg's Arch. Pharmacol. 359, 477–483.

Tang, Z.L., Dai, W., Li, Y.J., Deng, H.W., 1999. Involvement of capsaicin-sensitive sensory nerves in early and delayed cardioprotection induced by a brief ischaemia of the small intestine. Naunyn-Schmiedeberg's Arch. Pharmacol. 359, 243–247.

Vegh, A., Szekeres, L., Parratt, J., 1992. Preconditioning of the ischaemic myocardium; involvement of the L-arginine nitric oxide pathway. Br. J. Pharmacol. 107, 648–652.

Wei, E.P., Moskowitz, M.A., Boccalini, P., Kontos, H.A., 1992. Calcitonin gene-related peptide mediates nitroglycerin and sodium nitroprusside-induced vasodilation in feline cerebral arterioles. Circ. Res. 70, 1313–1319.

Yao, Z., Gross, G.J., 1993. Acetylcholine mimics ischemic preconditioning via a glibenclamide-sensitive mechanism in dogs. Am. J. Physiol. 264, H2221–H2225.

Yellon, D.M., Baxter, G.F., 1995. A "second window of protection" or delayed preconditioning phenomenon: future horizons for myocardial protection? J. Mol. Cell. Cardiol. 27, 1023–1034.