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Role of calcitonin gene-related peptide in nitric oxide-mediated myocardial delayed preconditioning induced by heat stress[1]

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KEY WORDS myocardial reperfusion injury; calcitonin gene-related peptide; heat stress; nitric oxide

ABSTRACT

AIM: To study the role of calcitonin gene-related peptide (CGRP) in nitric oxide (NO)-mediated myocardial delayed preconditioning induced by heat stress. **METHODS**: The isolated rat heart was perfused in a Langendorff model. Hearts for all groups were subjected to 4 h hypothermia (4 ℃) and 40 min reperfusion (37 ℃). In the hyperthemia-treated group, rats were subjected to whole-body hyperthermia (rectal 42 ℃, 15 min) 24 h before the experiment. Heart rate, coronary flow, left ventricular pressure, and its derivative ($\pm dp/dt_{max}$) were recorded, and calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) in plasma and the activity of creatine kinase (CK) in the coronary effluent were measured. **RESULTS**: Pretreatment with hyperthermia significantly imporved the recovery of cardiac protection, reduced the release of CK, and increased plasma concentrations of CGRP. Pretreatment with *L*-NAME, an inhibitor of NOS, or capsaicin, which selectively depleted sensory neurotransmitter content, abolished the protective effects and the increased level of CGRP elicited by hyperthermia. **CONCLUSION**: Endogenous NO is involved in the cardioprotection afforded by heat stress, and the beneficial effects of NO are mediated by CGRP in the rat.

INTRODUCTION

It has been suggested that endogenous chemical substances including transmitters and autacoids play an important role in the mediation of ischemic, hyperthermic, or pharmacological preconditioning[1-5]. For example, endogenous calcitonin gene-related peptide (CGRP), a principal transmitter in capsaicin-sensitive sensory nerves, has been shown to participate in the mediation of early and delayed preconditioning induced by ischemia, hyperthermia, or some drugs such as nitroglycerin[6-10], and endogenous nitric oxide (NO) may also relate to the preconditioning induced by ischemia or some drugs such as monophosphoryl lipid A[11,12]. The cardioprotection of ischemic preconditioning may involve multiple endogenous substances including neurotransmitters and autacoids[13]. There is evidence to suggest that hyperthermia can stimulate release of multiple endogenous substances. We postulate that a similar protection afforded by sublethal hyperthermia may be due to co-mediation of endogenous substances. Our recent work has shown that preconditioning of the heart with nitroglycerin, a donor of NO, is related to stimulation of CGRP release[10]. Therefore, in the present study we examined whether the cardioprotection afforded by heat stress-induced delayed preconditioning is mediated by endogenous CGRP via activation of the NO pathway.

MATERIALS AND METHODS

Reagents Capsaicin and *L*-nitroarginine methyl ester (*L*-NAME) were purchased from Sigma (St louis, MO, USA). Radioimmunoassay kits for measurement of CGRP were obtained from Dongya Immunity Institute (Beijing, China). Creatine kinase assay kits were obtained from Zhongshen Bioengineering Co (Beijing, China).

Preparation of the isolated heart Male Sprague-Dawley rats weighting 180-220 g (Grade Ⅱ, Certificate № 20-011) were obtained from Hunan Medical University Animal Center. Animals were anaesthetized with sodium pentobarbital (60 mg·kg^{-1}, ip). The heart was excised rapidly into Krebs-Henseleit (K-H)

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buffer solution at 4 ℃, and then perfused retrogradely in a non-recirculating system in a Langendorff model, at constant perfusion pressure of 100 cm H_2O. The heart was perfused with K-H buffer saturated with 95 % O_2 and 5 % CO_2, maitained at 37 ℃ and pH 7.4. The K-H buffer had the following composition (mmol · L^{-1}): NaCl 119.0, $NaHCO_3$ 25.5, KCl 4.3, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5 and glucose 11.0. A water-filled latex balloon was inserted into the left ventricular and adjusted to a left-ventricular (LV) end-diastolic pressure of 3 – 4 mmHg. The LV pressure, its derivatives ($\pm dp/dt$) and heart rate were monitored continuously. The resulting electrical signals were digitized by a Maclab analogue-to-digital converter and recorded on a Power Macintosh 7200 computer. Coronary flow was measured by timed collection of the coronary effluent and samples of coronary effluent at 5 min of reperfusion were collected for measurement of creatine kinase.

Creatine kinase assay Myocardial injury was monitored by assaying creatine kinase (CK) released from the heart. The activity of CK in the coronary effluent at 5 min of reperfusion was measured spectrophotometrically.

Determination of plasma CGRP concentrations Blood sample (3 mL) was collected from carotid artery into tubes containing 10 % Na_2 edetic acid 30 μL and aprotinin 400 mU · L^{-1}. The plasma was obtained by centrifugation at 1300 × g for 20 min (4 ℃). CGRP-like immunoreactivity (CGRP-LI) in plasma was measured using anti-sera raised against rat CGRP, ^{125}I-labelled CGRP, and rat CGRP standard.

Experimental protocols Thirty-six animals were randomly divided to six groups. In the hyperthermia-treated group, the rat was pretreated with whole-body hyperthermia (rectal 42 ℃, 15 min) 24 h before the experiment. For studies on the effect of *L*-NAME on protective effects of heat stress, rats were pretreated with *L*-NAME (10 mg · kg^{-1}, ip) 30 min before hyperthermia. In the capsaicin plus hyperthermia group, rats were pretreated with capsaicin 4 d before hyperthermia. Capsaicin (dissolved in a vehicle containing 10 % Tween 80, 10 % ethanol and 80 % saline, 50 mg · kg^{-1}) was administrated by sc injection.

All hearts had an initial stabilization period (37 ℃) for 20 min, and then infused with St Thomas cardioplegia solution (4 ℃) for 2 min through a sidearm of the cannula. The St Thomas cardioplegia solution had the following composition (mmol · L^{-1}): NaCl 110, KCl 16, $MgCl_2$ 16, $CaCl_2$ 1.2, and $NaHCO_3$ 10. The hearts were immersed in cardioplegic solution, maintained (4 ℃) for 4 h, and then reperfused with K-H solution (37 ℃) for 40 min.

Statistics All values are expressed as $\bar{x} \pm s$. Statistical analysis was carried out by analysis of variance and the Newman-Keuls test. The level of significance was chosen as $P < 0.05$.

RESULTS

There were no significant differences in the basic values of LVP and $\pm dp/dt_{max}$, coronary flow, and heart rate before hypothermic ischemia. A decline in LVP, $\pm dp/dt_{max}$, coronary flow, and an increase in the release of CK were shown during reperfusion after 4 h of ischemia. Pretreatment with hyperthermia caused a significant improvement of cardiac function (Tab 1 – 5) and a decrease in the release of CK (Tab 6). The protective effects of heat stress were abolished by *L*-NAME, an inhibitor of NO synthase.

In order to test the possible contribution of endogenous CGRP in the preconditioning with heat stress,

Tab 1. The effect of heat stress on left ventricular pressure (mmHg). $n = 6$ rats. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* ischemia/reperfusion. $^dP > 0.05$, $^fP < 0.01$ *vs* heat stress (HS). $^hP < 0.05$, $^iP < 0.01$ *vs* vehicle & HS.

	Preischemia	Reperfusion/min 5	10	20	30	40
Ischemia/reperfusion	119 ± 22	49 ± 10	51 ± 5	58 ± 12	62 ± 10	62 ± 10
+ Heat stress (HS)	114 ± 16	87 ± 9^c	90 ± 7^c	93 ± 10^c	96 ± 8^c	96 ± 8^c
+ *L*-NAME	123 ± 22	58 ± 14^a	60 ± 14^a	57 ± 12^a	55 ± 5^a	55 ± 11^a
+ *L*-NAME & HS	116 ± 33	56 ± 12^f	58 ± 11^f	58 ± 15^f	55 ± 16^f	56 ± 16^f
+ Vehicle & HS	112 ± 22	69 ± 18^d	75 ± 15^d	81 ± 15^d	78 ± 14^d	78 ± 14^d
+ Capsaicin & HS	101 ± 14	48 ± 9^h	48 ± 9^i	51 ± 5^i	50 ± 3^i	50 ± 8^i

Tab 2. The effect of heat stress on $+dp/dt_{max}$ (mmHg/s). $n = 6$ rats. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* ischemia/reperfusion. $^dP > 0.05$, $^fP < 0.01$ *vs* heat stress (HS). $^iP < 0.01$ *vs* vehicle & HS.

	Preischemia	Reperfusion/min 5	10	20	30	40
Ischemia/reperfusion	4162 ± 1005	1508 ± 495	1503 ± 593	1883 ± 503	1943 ± 353	1988 ± 353
+ Heat stress (HS)	4050 ± 308	2700 ± 383^c	2858 ± 353^c	2993 ± 450^c	3255 ± 443^c	3188 ± 443^c
+ *L*-NAME	4140 ± 690	1868 ± 480^a	1913 ± 480^a	1920 ± 495^a	1943 ± 345^a	1920 ± 345^a
+ *L*-NAME & HS	3998 ± 1110	1688 ± 405^f	1853 ± 450^f	1995 ± 555^f	1995 ± 645^f	1950 ± 615^f
+ Vehicle & HS	4125 ± 420	2775 ± 510^d	2918 ± 360^d	3053 ± 225^d	3158 ± 285^d	3045 ± 285^d
+ Capsaicin & HS	3713 ± 503	1538 ± 360^i	1560 ± 480^i	1733 ± 510^i	1740 ± 525^i	1718 ± 510^i

Tab 3. The effect of heat stress on $-dp/dt_{max}$ (mmHg/s). $n = 6$ rats. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* ischemia/reperfusion. $^dP > 0.05$, $^fP < 0.01$ *vs* heat stress (HS). $^iP < 0.01$ *vs* vehicle & HS.

	Preischemia	Reperfusion/min 5	10	20	30	40
Ischemia/reperfusion	2963 ± 818	1155 ± 428	1140 ± 465	1328 ± 525	1268 ± 270	1350 ± 323
+ Heat stress (HS)	2813 ± 300	1838 ± 218^c	1980 ± 173^c	2115 ± 263^c	2280 ± 210^c	2235 ± 203^c
+ *L*-NAME	3080 ± 360	1425 ± 533^a	1170 ± 255^a	1328 ± 298^a	1290 ± 233^a	1275 ± 255^a
+ *L*-NAME & HS	2813 ± 908	1140 ± 210^f	1238 ± 270^f	1275 ± 368^f	1328 ± 465^f	1350 ± 510^f
+ Vehicle & HS	2708 ± 713	2040 ± 443^d	2108 ± 398^d	2063 ± 420^d	2295 ± 278^d	2213 ± 285^d
+ Capsaicin & HS	2873 ± 398	1148 ± 248^i	1335 ± 345^i	1455 ± 278^i	1538 ± 218^i	1440 ± 285^i

Tab 4. The effect of heat stress on coronary flow (mL/min). $n = 6$ rats. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* ischemia/reperfusion. $^dP > 0.05$, $^fP < 0.01$ *vs* heat stress (HS). $^iP < 0.01$ *vs* vehicle & HS.

	Preischemia	Reperfusion/min 5	10	20	30	40
Ischemia/reperfusion	10.4 ± 1.1	6.7 ± 0.6	6.6 ± 0.8	6.7 ± 0.8	6.8 ± 0.7	6.5 ± 1.0
+ Heat stress (HS)	10.9 ± 1.6	10.4 ± 1.6^c	10.5 ± 1.7^c	10.4 ± 1.5^c	10.5 ± 1.7^c	10.5 ± 1.7^c
+ *L*-NAME	11.0 ± 2.3	6.5 ± 1.5^a	6.4 ± 1.5^a	6.6 ± 1.6^a	6.7 ± 1.6^a	6.2 ± 1.5^a
+ *L*-NAME & HS	11.4 ± 2.4	7.0 ± 0.8^f	6.8 ± 0.9^f	7.1 ± 0.7^f	6.9 ± 0.6^f	6.7 ± 0.9^f
+ Vehicle & HS	10.3 ± 1.3	9.6 ± 2.0^d	9.7 ± 2.1^d	9.7 ± 2.1^d	9.9 ± 1.9^d	9.7 ± 2.0^d
+ Capsaicin & HS	10.7 ± 2.0	6.0 ± 0.4^i	6.2 ± 0.5^i	6.3 ± 0.3^i	6.3 ± 0.4^i	6.3 ± 0.4^i

Tab 5. The effect of heat stress on heart rate (beats/min). $n = 6$ rats. $\bar{x} \pm s$. $^aP > 0.05$ *vs* ischemia/reperfusion. $^dP > 0.05$ *vs* heat stress (HS). $^gP > 0.05$ *vs* vehicle & HS.

	Preischemia	Reperfusion/min 5	10	20	30	40
Ischemia/reperfusion	287 ± 17	264 ± 50	258 ± 57	259 ± 49	272 ± 51	256 ± 51
+ Heat stress (HS)	306 ± 26	320 ± 52^a	295 ± 21^a	292 ± 25^a	290 ± 11^a	283 ± 14^a
+ *L*-NAME	296 ± 24	262 ± 20	275 ± 36	296 ± 25	295 ± 21	294 ± 22
+ *L*-NAME & HS	326 ± 41	265 ± 71^d	262 ± 68^d	262 ± 72^d	272 ± 78^d	269 ± 86^d
+ Vehicle & HS	296 ± 29	306 ± 26	320 ± 21	324 ± 28	327 ± 20	325 ± 22
+ Capsaicin & HS	321 ± 35	249 ± 24^g	284 ± 38^g	294 ± 48^g	306 ± 50^g	311 ± 46^g

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Tab 6. The effects of heat stress on the activity of creatine kinase (CK) in coronary effluent and the plasma concentrations of CGRP. $n = 6$ rats. $\bar{x} \pm s$. [a] $P > 0.05$, [c] $P < 0.01$ *vs* ischemia/reperfusion. [d] $P > 0.05$, [f] $P < 0.01$ *vs* heat stress (HS). [i] $P < 0.01$ *vs* vehicle & HS.

	CK/U·min^{-1}·g^{-1} wet wt	CGRP-LI/ ng·L^{-1}
Ischemia/reperfusion	0.94 ± 0.18	72 ± 13
+ Heat stress (HS)	0.38 ± 0.14[c]	129 ± 35[c]
+ *L*-NAME	0.89 ± 0.24[a]	71 ± 22[a]
+ *L*-NAME & HS	0.97 ± 0.21[f]	70 ± 22[f]
+ Vehicle & HS	0.52 ± 0.15[d]	112 ± 28[d]
+ Capsaicin & HS	0.97 ± 0.17[i]	65 ± 16[i]

capsaicin, which selectively depleted neurotransmitters in sensory nerves, was used. Pretreatment with capsaicin also abolished the protective effects of heat stress. Pretreatment with heat stress significantly increased concentrations of CGRP-LI, which was abrogated by *L*-NAME or capsaicin (Tab 6). Capsaicin vehicle had no effect on the cardioprotection afforded by heat stress.

DISCUSSION

Many methods have been used to strengthen the protective effect of St Thomas solution in the storage of heart transplant and cardiac-bypass surgery. It has been reported that pharmacological[14] or hypoxic preconditioning[15] protects against myocardial damages after prolonged cardioplegic arrest, and the protective effects of preconditioning have been suggested to be mediated by endogenous chemical mediators. Recently, early preconditioning induced by heat stress is also capable of enhancing preservation with cardioplegia[16]. In the present study, the delayed preconditioning induced by heat stress also significantly improved preservation with cardioplegia in the isolated rat heart, as shown by improvement of the recovery of cardiac function and reduction of creatine kinase release. These results suggest that heat stress-induced preconditioning, early or delayed, improves preservation with cardioplegia.

CGRP, a 37-amino acid peptide, is a principal transmitter in capsaicin-sensitive sensory nerves and widely distributed in cardiovascular tissues[17]. CGRP, besides regulating vascular tone, has a protective effect on the ischemic myocardium and endothelial cells, which is documented by previous observations that exogenous administration of CGRP protects the myocardium against damages due to ischemia-reperfusion[6]. Recently, we and others have shown that endogenous CGRP may play an important role in the mediation of ischemic preconditioning. The preconditioning of the heart with brief periods of ischemia is abolished by $CGRP_{8-37}$, the selective CGRP receptor antagonist, or by CGRP antibody, or by capsaicin which selectively depletes transmitters in sensory nerves. Studies in clinic have also shown that myocardial outflow of CGRP is increased during coronary artery bypass grafting without cardiopulmonary bypass[18]. Furthermore, pretreatment with capsaicin aggravates myocardial infarction in the porcine heart[19]. These findings suggest that CGRP may be an important mediator in the cardioprotection of ischemic preconditioning.

As mentioned above, hyperthermia is also capable of inducing myocardial adaptation including early and delayed protection. However, the mechanism responsible for the beneficial effect of heat stress has not yet been fully understood. Early studies have found that a stress, cold or heat, is also capable of activating capsaicin-sensitive sensory nerves and stimulating the release of neurotransmitters from their peripheral terminals[20]. The present study confirmed previous observations that hyperthermic treatment caused a significant increase in plasma concentrations of CGRP concomitantly with an improvement of cardiac function and inhibition of CK release, and that the protection afforded by heat stress was abolished by pretreatment with capsaicin. A similar effect has been seen in the retrograde perfused hearts, and this early preconditioning by hyperthermia was abolished by $CGRP_{8-37}$, the selective CGRP receptor antagonist, in further support of the hypothesis that endogenous CGRP may play a pivotal role in the mediation of heat stress-induced delayed preconditioning.

Previous investigations have shown that endogenous NO may be involved in the mediation of preconditioning in the rabbit[21,22]. Recently, it has been reported that the delayed preconditioning induced by some drugs such as monophosphoryl lipid A[3,23], angiotensin-converting enzyme inhibitors[24], and adenosine[4] is related to stimulation of NO production. There is evidence that NO is involved in heat shock reaction[25]. In the present study, pretreatment with hyperthermia caused a significant improvement of cardiac function, which was abolished by *L*-NAME, suggesting that the delayed protection afforded by heat stress also involved endogenous NO.

As mentioned above, ischemia or hyperthermia can

stimulate the release of multiple endogenous chemical substances. It is likely that these endogenous substances mediate the protection of preconditioning via interactions among them. There is evidence to suggest that NO is capable of modulating neurotransmission in central and peripheral nerves[26,27]. Recently, it has been found that nitroglycerin, a NO donor, significantly evokes the release of CGRP in the central and peripheral vessels[28,29]. In the present study, pretreatment with hyperthermia caused an increase in the content of plasma CGRP concomitantly with an improvement of cardiac function and inhibition of the release of CK. The elevated level of CGRP and protection induced by heat stress were abolished by *L*-NAME. These results support the hypothesis that the beneficial effect of heat stress is related to the stimulation of endogenous CGRP via the activation of NO pathway in rats.

The mechanisms responsible for the protective effects of CGRP remain unclear. The cardioprotection of CGRP-mediated preconditioning is related to the activation of protein kinase C[30], but not K_{ATP} channels in the rat heart[31]. Recently, our work has shown that the cardioprotective effects afforded by CGRP-mediated ischemic preconditioning are related to inhibition of cardiac TNF-α production[31], an ultimate effector in signal transduction pathways of ischemic preconditioning[32].

In summary, the present study suggests that endogenous NO is involved in the cardioprotection afforded by heat stress, and the beneficial effects of NO are mediated by CGRP in the rat.

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降钙素基因相关肽在一氧化氮介导热应激诱导心肌延迟预适应中的作用[1]

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关键词 心肌再灌注损伤; 降钙素基因相关肽; 热应激; 一氧化氮

目的: 研究一氧化氮-降钙素基因相关肽途径是否参与热应激诱导的心肌延迟预适应. **方法**: 采用Langendorff装置灌注离体心脏. 心脏低温(4 ℃)保存4 h后, 再灌注40 min (37 ℃). 实验前24 h大鼠进行高温处理(直肠温度42 ℃, 15 min). 记录心率, 冠脉流量、左室内压以及最大变化速率, 并测定血浆降钙素基因相关肽(CGRP)浓度和冠脉流出液中肌酸激酶(CK)释放量. **结果**: 热应激能显著增强心肌停搏液的保护作用, 减少CK释放量, 并升高血浆CGRP浓度. 这些作用能被预先给予亚硝基精氨酸甲酯及辣椒素所取消. **结论**: 一氧化氮参与了对大鼠心脏的延迟保护, 其作用是由内源性CGRP所介导.

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