[Article ID] 1000 - 4718(2006)11 - 2127 - 05

Delayed protection of HO - 1 in the exercise preconditioning from the myocardial relative ischemic reperfusion injury

ZHAI Qing - feng, LIU Hong - tao, MA qiang
(*Institute of Health and Environment Medicine, Academy of Military Medical Sciences, Tianjin* 300050, *China. E - mail:sdzzqqff@ yahoo. com. cn*)

[**ABSTRACT**] **AIM**: To explore the delayed protection of heme oxygenase - 1 (HO - 1) in the exercise preconditioning (EP) from the myocardial relative ischemia reperfusion injury (rI/R). **METHODS**: 40 Wistar Rats were divided into 5 groups randomly: control group (CN), rI/R group (IR), EP + rI/R group (EI), HO - 1 inductor hemin + rI/R group (HE) and HO - 1 inhibitor ZnPP + EP + rI/R group (EZ). The following indexes were detected, including the HO - 1activity in myocardium, the cardiac function parameter - pressure - rate product (heart rate × left ventricular developed pressure, PRP) and the content of MDA in coronary effluent. **RESULTS**: After myocardial rI/R, HO - 1 activity increased significantly. Moreover, EP or HO - 1 inductor could enhance this effect manifestly. Nevertheless, when the HO - 1 inhibitor was administered before EP, HO - 1 activity decreased. In addition, there was no distinct difference in the HO - 1 activity between EI group and HE group. At the 30 min point of reperfusion, the PRP recovery rate of EI group was higher clearly than that of IR group. However, there was reverse effect between the EZ group and the EI group. The MDA in coronary effluent of EI group, EZ group and HE group were lower obviously than that of IR group and there was significant difference between EI group and EZ group. **CONCLUSION**: EP could protect the heart from the rI/R injury occurring 24 hours later, which might be performed through activating the HO - 1.

[**KEY WORDS**] Heme oxygenase; Exercise preconditioning; Delayed preconditioning; Myocardial reperfusion injury

[**CLC number**] R363 [**Document code**] A

In 1993, Marber et al[1] found that the protection of ischemic preconditioning (IPC) for the heart disappeared one to two hours later, but reappeared 24 hours later. Based on this discovery, they put forward that IPC consisted of two phases: the early preconditioning (EPC) and the delayed preconditioning (DPC). Since the protection phase of DPC performed longer, it had a great application value. At present, most researches support that the mechanism of DPC involved the active of the cell signal transduction. That was to say, brief ischemia caused the myocardium releasing many kinds of endogenous bioactive compound, which combined with the corresponding receptors and then activated the cell signal transduction. At last, many cell - protector proteins were produced[2]. Hangaishi et al[3] had discovered that the HO - 1 and HO - 1 mRNA rose significantly after myocardial ischemic reperfusion (I/R). This meant that HO - 1 could protect the myocardium from the I/R injury. However, there was no report whether HO - 1 was an effective protective protein in the DPC. Our previous study had verified that the excessive physical exercise could induce the myocardial relative ischemic reperfusion injury (rI/R), but the exercise preconditioning (EP), induced by the 4 - times short - time excessive physical exercise and performed 24 hours before the rI/R, could reduce the rI/R injury and improve the heart function. In light of the research above and our previous findings, we hypothesized that HO - 1 might play an important role in the EP. In order to test this hypothesis and elucidate the delayed protective mechanisms of the EP, the study as following was carried out.

MATERIALS AND METHODS

1 Materials

Hemin chloride, protoporphyrin IX zinc (ZnPP -

[**Received date**] 2005 - 02 - 02 [**Accepted**] 2005 - 06 - 16
Tel: 022 - 84655196; E - mail:sdzzqqff@ yahoo. com. cn

IX), G-6-P, G-6-PD, NADP, BSA V and CB-BG-250 were purchased from Sigma (Germany).

Male Wistar rats weighing 180-220 g were obtained from the Animal Center of the Academy of Military Medical Sciences. All animals received humane care in compliance with "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and Published by the National Institute of Health (NIH Publication No. 85-23, revised 1985).

2 Animal model

2.1 rI/R model induced by suprathreshold electric stimulation[4] The rat was anaesthetized with 10% ethylcarbamate (1 mL/100 g BW), and then the rat heart was excised rapidly into Krebs-Henseleit (K-H) buffer solution (mmol/L: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.2, glucose 11.1, pH 7.3-7.4) at 4 ℃. Immediately, the heart was perfused retrogradely with K-H buffer solution at 37 ℃, which was saturated with 95% O_2 and 5% CO_2, at a constant perfusion pressure of 100 cmH_2O in the Langendorff setting. In the same time, the cardiac functional parameters were collected with a Pclab Bio-signal recorder. When the heart beat rhythmically in the Langendorff setting, the suprathreshold electric stimulation (pulse duration 2 ms, voltage 100 mV, frequency 10Hz) was given to speed up the heart rate for 30 min, and then the heart was reperfused for 60min. By this way, we simulated the rI/R model induced by the excessive physical exercise.

2.2 EP model Referring to Zhang,et al.[5] description, the rats had an adaptive running for 30 minutes in the small animal treadmill (gradient: 10°; speed: 5 m/min) one day before the experiment. In the experiment, the rats run for 10 min (gradient: 10°; speed: 25 m/min) and then rested for 10 min. This treatment was repeated for 4 times.

3 Experiment protocol

Male Wistar rats (8 weeks old, 180-220 g, n = 40) were randomly assigned to 5 groups: (1) control group (CN): the rats were placed in the static treadmill for 24 h, then the rat hearts were separated and perfused in the Langendorff setting without the electric stimulation. (2) rI/R group (IR): the rats were placed in the static treadmill for 24 h, and then received the rI/R. (3) EP + rI/R group (EI): first, the rats received the EP, and 24 h later they suffered the rI/R injury. (4) Hemin (HO-1 inductor) + rI/R group (HE): the rats received intraperitoneal injection with hemin (10 μmol/kg BW), and 24 h later, they were treated as rI/R model. (5) ZnPP (HO-1 inhibitor) + EP + rI/R group (EZ): firstly, the rats received intraperitoneal injection with ZnPP (10 μmol/kg BW). And then, they were treated as the EP model one hour later and the rI/R model 24 h later.

4 Methods

4.1 The cardiac function parameter recording The cardiac function parameters [the heart rate (HR), the left ventricular developed pressure (LVDP)] were recorded with a Pclab Bio-signal recorder constantly, and the cardiac function recovery rate was reflected synthetically by the recovery rate of the pressure-rate product [PRP, rate = (PRP before rI/R)/(PRP of every time point) × 100%].

4.2 Assay of the malondialdehyde (MDA) At 10 min before rI/R and 20 min after rI/R, about 1 mL of the coronary effluent was collected, and then frozen as soon as possible at -20 ℃. Later, the content of MDA in coronary effluent was assayed by the improved barbituric acid detection as described previously[6].

4.3 Determination of HO-1 activity Heme oxygenase activity in myocardium was represented by the rate of bilirubin generation. Firstly, the HO-1 microsomes and the biliverdin reductase were prepared as previously described, and then the concentration of them was detected with Coomassie brilliant blue[7]. The reaction mixture (final volume 2 mL) contained HO-1 1 mg, biliverdin reductase 1 mg, G-6-P 1 mmol/L, NADP 0.8 mmol/L, G-6-PD 0.2 U, bovine serum albumin 0.3 mg, hemin 20 μmol/L. Finally, the formed bilirubin was extracted with chloroform after the reaction mixture reacted for 1 h at 37 ℃ in dark, and its concentration was determined spectrophotometrically using the difference in absorbance at wavelength from λ 463 nm to λ 530 nm with an absorption coefficient of 40 $mmol \cdot L^{-1} \cdot cm^{-1}$. Accordingly, HO-1 activity was expressed as mmol of bilirubin formed / g of HO-1 protein /1 h.

5 Statistical analyses

All values are expressed as $\bar{x} \pm s$. Statistical analysis was carried out by analysis of variance and the q-test.

RESULTS

1 HO – 1 activity

As shown in Fig 1, there were significant differences in HO – 1 activity in 5 groups. Compared among the 5 groups, the HO – 1 activity of EI group [(25.48 ± 9.47) mmol · g^{-1} protein · h^{-1}] and HE group [(18.50 ± 7.53) mmol · g^{-1} protein · h^{-1}] were higher than that of IR group [(10.22 ± 2.84) mmol · g^{-1} protein · h^{-1}] significantly ($P < 0.01$, $P < 0.05$), while EZ group [(3.19 ± 2.01) mmol · g^{-1} protein · h^{-1}] had the reverse effect ($P < 0.05$). Between EZ group and EI group, the former was lower distinctly than the later ($P = 0.001$). There was no clearly difference between the EI group and HE group. According to the results, it could be concluded that EP could active the HO – 1 and this effect would be blocked by ZnPP. Moreover, hemin could affect the HO – 1 activity like the EP.

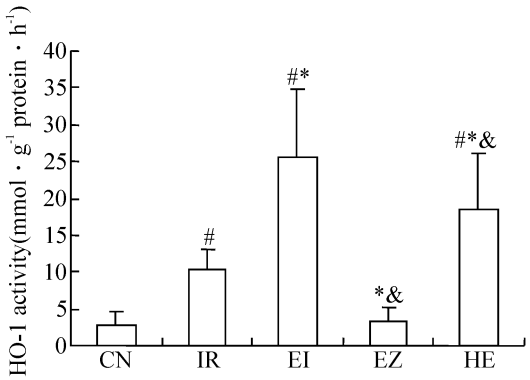

Fig 1 HO – 1 activity after the rI/R. $\bar{x} \pm s$. $n = 8$. $^{\#}P < 0.05$ *vs* CN; $^{*}P < 0.05$ *vs* IR; $^{\&}P < 0.05$ *vs* EI. CN: control group; IR: rI/R group; EI: EP + rI/R group; EZ: ZnPP + EP + rI/R group; HE: hemin + rI/R group.

2 The effects of HO – 1 on PRP during the reperfusion (Fig 2)

As shown in Fig 2, the PRP of EI group and HE group rose highly during the reperfusion, and at the point of 30 min, they reached the peak and almost as fine as that before the rI/R. But the PRP of IR group and EZ group recovered slightly. There were significant differences in 5 groups at the point of 30 min. Compared among the 5 groups, the rate of PRP in EI group (101.19% ± 7.78%) and HE group (74.95% ± 20.39%) were higher obviously than that in IR group (59.93% ± 12.21%) ($P < 0.01$, $P < 0.01$), and EZ group (59.83% ± 13.66%) decreased significantly compared to EI group ($P = 0.001$). It indicated that EP could promote the recovery of cardiac function injured by rI/R while ZnPP could block this protection. Furthermore, hemin could amend the cardiac function just like EP.

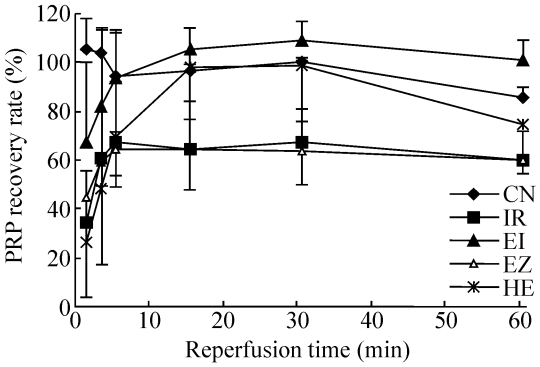

Fig 2 The effects of HO – 1 on PRP during the reperfusion. $\bar{x} \pm s$. $n = 8$. CN: control group; IR: rI/R group; EI: EP + rI/R group; EZ: ZnPP + EP + rI/R group; HE: hemin + rI/R group.

3 The effects of HO – 1 on MDA content in coronary effluent after rI/R

As shown in Fig 3, analyzing the difference between MDA content before rI/R and after rI/R in each group, we found there were significant differences in 5 groups. Compared among the 5 groups, the MDA of EI group [$\triangle = (0.29 \pm 0.24)$ μmol/L] and HE group [$\triangle = (0.41 \pm 0.26)$ μmol/L] were lower significantly than that of IR group [($\triangle = 1.27 \pm 0.52$) μmol/L] while EZ group [$\triangle = (0.79 \pm 0.26)$ μmol/L] was higher than EI group ($P < 0.01$). There was no obvious difference between EI group and HE group. Thus, it signed that EP could protect the heart from the injury of lipid peroxidation induced by rI/R while ZnPP could block this protection. In addition, hemin could affect the MDA as EP did.

DISCUSSION

HO was the first and rate – limiting enzyme in heme breakdown to generate equimolar quantities of biliverdin, free ferrous iron and CO. Subsequently, biliverdin was rapidly converted to bilirubin by biliverdin reducatase. Among the three reported HO isoforms (HO – 1, – 2, – 3), HO – 1 was highly inducible and expressed in many cell types in response to numerous stimuli such as heme and other oxidants, endotoxin, inflammation, hypoxia, hyperoxia, and heavy metals et al. Contrarily, HO – 2 and HO – 3 were constitutively synthesized[8]. Since HO – 1 could protect the myocar-

dium from I/R injury and DPC was one of the most effective endogenous protective measures from myocardial I/R injury, we hypothesized that HO - 1 might play an important role in EP which had the delayed protection to the heart as DPC did. To test this hypothesis, the 4 - times short - time excessive physical exercise was performed to set up the EP model. 24 h later, the heart suffered the suprathreshold electrical stimulation to speed up the heart rate and resulted in the relative myocardial ischemia[9].

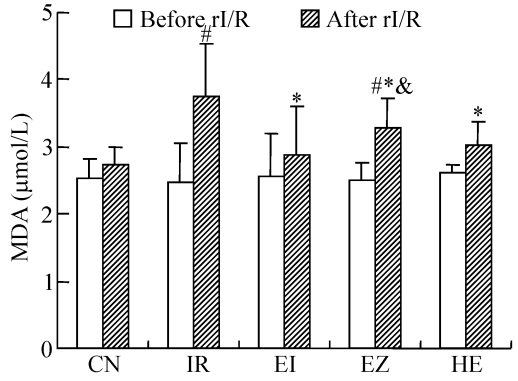

Fig 3 The effects of HO - 1 on MDA in coronary effluent after rI/R. $\bar{x} \pm s$. $n = 8$. $^{\#}P < 0.05$ *vs* CN; $^{*}P < 0.05$ *vs* IR; $^{\&}P < 0.05$ *vs* EI. CN: control group; IR: rI/R group; EI: EP + rI/R group; EZ: ZnPP + EP + rI/R group; HE: hemin + rI/R group.

The result of HO - 1 activity indicated that rI/R could activate HO - 1, which was correspond to the Hangaishi et al[3] finding, and when EP was performed 24 h before rI/R, HO - 1 could be activated further. However, when the HO - 1 inhibitor ZnPP was administrated before EP, HO - 1 activity was decreased significantly. In conclusion, EP had a close relation with HO - 1 and could active HO - 1 powerfully.

In order to make clear the delayed protection mechanism of HO - 1 in EP from myocardial rI/R injury, the PRP recovery rate and the MDA content in coronary effluent were detected. When EP was performed 24 hours before rI/R, the recovery rate of cardiac function increased markedly, while the MDA in serum and in coronary effluent decreased obviously compared with rI/R groups. Since the recovery rate PRP reflected synthetically the cardiac function recovery rate and MDA was a sensitive index to reflect the degree of myocardial lipid peroxidation injury[6], these results showed that EP could decrease the injury of myocardial lipid peroxidation and improve the cardiac function. That was to say, EP had delayed protection to the heart from rI/R injury. This conclusion was coincided with our previous research[10]. Nevertheless, when HO - 1 inhibitor (ZnPP) was performed before EP, the protection of EP disappeared. Otherwise, when the HO - 1 inductor (hemin) was injected 24 h before rI/R, HO - 1 activity increased significantly. Accordingly, PRP recovery rate and the MDA content in coronary effluent had no clearly difference to EI group. These results above directly indicated HO - 1 possessed the delayed protection in EP from myocardial rI/R injury. There had been reported that bilirubin (one of the catalytic product of HO - 1) was a powerful endogenous antioxidant. Llesuy F et al[11] had found the protection of HO - 1 from myocardial I/R injury and elucidated the mechanism which was mainly due to the antioxidation of bilirubin. In addition, CO (another catalytic product of HO - 1) was one of the intracellular signal transduction factors and mediated many endogenous protections. In 2003, Masini et al[12] discovered that hemin injected 18 h before I/R could decrease the myocardial infarct size, the content of MDA in tissue and calcium overload. Thus they found the protective mechanism of HO - 1/CO. In light of these researches, it could be concluded that the delayed protection mechanism of EP involved the active of the endogenous HO - 1/CO reaction system, whose products such as CO, bilirubin and the Fe protein could ameliorate the lipid peroxidation insult[13].

Interestingly, Rong et al[14] had found that performing heat preconditioning 24 h before I/R, HO - 1 and HO - 1 mRNA were significantly higher than those of I/R group, while the myocardial infarct size and the serum creatine kinase decreased significantly. Considering of the researches above, we supposed that the delayed protection of EP might also due to the increased synthesis of HO - 1 and the enhancement of HO - 1 activity which could improve the cardiac function[12,15]. Owing to this, we planed to check whether the synthesis of HO - 1 was enhanced by EP from the molecular level.

Finally, based on our experiment and the research above by others, the mechanisms of delayed protection of EP from myocardium rI/R injury might be that EP induced the increased synthesis of HO - 1 and enhanced the activity of HO - 1. Subsequently, the catalysate of HO - 1 bilirubin and CO protected the myocardium from the injury of rI/R occurring 24 h later.

[REFERENCES]

[1] Marber MS, Latchman DS, Walker JM, et al. Cardial stress protein elevation 24 hours after brief ischemia or heat stress in associated with resistance to myocardial infarction [J]. Circulation, 1993, 88 (3): 1264 - 1272.

[2] Kong YJ, Liu YX, Lou JS. The progress of delayed protection of ischemic preconditioning [J]. Chinese Journal of Pharmacology and Toxicology, 2002, 16(3): 235 - 240.

[3] Hangaishi M, Ishizaka N, Aizawa T, et al. Induction of heme oxygenase - 1 can act protectively against cardiac ischemia/reperfusion *in vivo* [J]. Biochem Biophys Res Commun, 2000, 279 (2): 582 - 588.

[4] Zhu B, Min S, Long C. The IPC can't protect the heart of rabbit[J]. Chinese Journal of Anesthesiology, 2001, 21 (2): 97 - 99.

[5] Zhang M, Pu JZ, Zhu Q. The effect of exercise preconditioning to the express of CuZn - SOD gene in rats myocardium[J]. Chinese Journal of Sports Medicine, 2001, 20 (3): 239 - 309.

[6] Maulik N, Engelman DT, Engelman RM, et al. Nitric oxide/carbon monoxide - A molecular switch for myocardial preservation during ischemia [J]. Supplement Ⅱ Circulation, 1996, 94(9): Ⅱ 398 - Ⅱ406.

[7] Huang XY, Wang LX, Chen SX, et al. The regulation of nitric - oxide synthase/nitric - oxide system by endogenous carbon monoxide in rats with pulmonary hypertension [J]. Chinese Journal of Pathophysiology, 2003, 19 (10): 1357 - 1360.

[8] Mark AP, Shaw FY. Role of heme oxygenase - 1 in cardiovascular function [J]. Current Pharmaceutical Design, 2003, 9(30):2479 - 2487.

[9] Guo BS, Liu HT, Dou LJ, et al. The role of NO in myocardial I/R injury [J]. Chinese Journal of Applied Physiology, 2001, 17(4): 370 - 372.

[10] Zhai QF, Liu HT, Wang TH. The delayed protection of exercise preconditioning from the relative myocardial ischemia - reperfusion injury [J]. Chin J Ind Hyg Occup Dis, 2005, 23(1): 38 - 41.

[11] Llesuy SF, Tomaro ML. Heme oxygenase and oxidative stress. Evidence of involvement of bilirubin as physiological protector against oxidative damage [J]. Biochim Biophys Acta, 1994, 1223 (1): 9 - 14.

[12] Masini E, Vannacci A, Marzocca C, et al. Heme oxygenase - 1 and the ischemia - reperfusion injury in the rat heart [J]. Exp Biol Med, 2003, 228 (5): 546 - 549.

[13] Zhao TC, Kukreja RC. Late Preconditioning elicited by activation of adenosine A3 receptor in heart: Role of NF - κB, iNOS and mitochondrial K_{ATP} channel [J]. J Mol Cell Cardiol, 2002, 34 (3): 263 - 277.

[14] Rong L, Peng J, Liang X, et al. Heme oxygenase - 1 pathway is involved in delayed protection induced by heat stress against cardiac ischemia/reperfusion injury [J]. Int J Cardiol, 2002, 82(2): 133 - 140.

[15] Takeda K, Lin J, Okubo S, et al. Transient glucose deprivation causes upregulation of heme oxygenase - 1 and cyclooxygenase - 1 expression in cardiac fibroblasts [J]. J Mol Cell Cardiol, 2004, 36(6): 821 - 830.

血红素氧合酶 -1 对心肌相对缺血再灌注损伤的延迟保护作用

翟庆峰， 刘洪涛， 马 强

（军事医学科学院卫生学环境医学研究所，天津 300050）

[摘 要] **目的**：探讨血红素氧合酶 - 1(HO - 1)在运动预适应(EP)中对大鼠心肌相对缺血再灌注(rI/R)损伤的延迟保护作用及机制。**方法**：40 只 Wistar 大鼠随机分为 5 组：正常对照组(CN)、相对缺血再灌注组(IR)、运动预适应 + 相对缺血再灌注组(EI)、Hemin(HO - 1 诱导剂) + 相对缺血再灌注组(HE)和运动预适应 + ZnPP(HO - 1 抑制剂) + 相对缺血再灌注组(EZ)。测定大鼠再灌注期心率脉压乘积(PRP)、冠脉流出液 MDA 含量、HO - 1 活性等。**结果**：心肌 HO - 1 活性：EI 组和 HE 组较 IR 组显著升高，EZ 组则显著降低。EZ 组较 EI 组显著降低，EI 组和 HE 组间亦有显著差异。再灌注后 60 min 时点 PRP 恢复率：EI 组较 IR 组显著增高；IR 组与 HE 组未见显著差异，但 30 min 时点 HE 组显著增高；EZ 组较 EI 组显著降低。冠脉流出液 MDA 含量：EI 组、EZ 组和 HE 组 MDA 含量较 IR 组皆显著降低；EZ 组较 EI 组显著升高。**结论**：EP 可以诱导 HO - 1 合成，进而通过 HO - 1 对 24 h 后发生的 rI/R 损伤产生延迟保护作用。

[关键词] 血红素氧合酶；运动预适应；延迟预适应；心肌再灌注损伤

[中图分类号] R363 [文献标识码] A