ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 May；21 (5)：467－472
http：//www.chinaphar.com

Intermittent hypoxia exposure-induced heat-shock protein 70 expression increases resistance of rat heart to ischemic injury[1]

ZHONG Ning[2], ZHANG Yi, FANG Qi-Zhi, ZHOU Zhao-Nian (*Physiological Laboratory of Hypoxia, Shanghai Institute of Physiology, Chinese Academy of Sciences, Shanghai 200031, China*)

KEY WORDS heat-shock proteins 70；anoxia；reverse transcriptase polymerase chain reaction；arrhythmia；reperfusion injury

ABSTRACT

AIM：To quantify the levels of HSP70 induced by different durations of intermittent (high altitude) hypoxia and to correlate them with the degree of protection of the rat heart from ischemic injury. **METHODS**：Reverse transcriptase polymerase chain reaction (RT-PCR) was used to detect the level of HSP70 mRNA expression in rat myocardium. Ischemia/reperfusion injury was presented as severity of arrhythmias induced by occlusion and reperfusion of the left anterior descending coronary artery of rat heart. **RESULTS**：The level of HSP70 mRNA expression increased progressively along with the duration of intermittent hypoxia training. It was 2.6, 3.6, and 3.8 folds after 14-, 28-, and 42-d exposures compared to that of normoxia. The tolerance of rat heart to ischemia/reperfusion injury increased with hypoxia pretreatment. Such an effect was significant after rat were exposed to a 28-d intermittent hypoxia (IH). The scores for ischemia and reperfusion inducing arrhythmia for 28- and 42-d IH were 1.2 ± 0.5, 1.0 ± 0.5 and 1.0 ± 0.5, 0.9 ± 0.5 ($P<0.01$ compared with 4.0 ± 0.7, 3.3 ± 0.6 in normoxia rats). The overexpression of HSP70 and the increased tolerance to subsequent acute ischemia/reperfusion injury could last for 2 wk after the rats (subjected to 28 d IH) returned to normoxia. Furthermore, there was a reverse correlation between the amount of HSP70 induced and the arrhythmia occurrence ($r=-0.98$, -0.92 for ischemia and reperfusion induced arrhythmia, $P<0.01$). **CONCLUSION**：These results suggest that increased resistance of rat heart to ischemia/reperfusion injury after intermittent hypoxia exposure may be related to the amount of HSP70 induced.

INTRODUCTION

The whole body heat stress mechanism has been shown to reduce infarct size *in vivo* and *in vitro*, and to enhance postischemic contractile function *in vitro*. The protective effects of thermal pretreatment are connected with intracardiac heat shock protein (HSP) accumulation (so-called cross tolerance)[1]. Marber *et al* demonstrated that the increase in HSP70 was associated with resistance to myocardial stunning 48 and 72 h after repetitive ischemia[2]. The best evidence of cytoprotective effects of HSP accumulation have been provided in the transgenic cell line and transgenic mice overexpressing a foreign inducible HSP70[3,4]. These findings suggest that increased myocardial HSP70 expression results in a protection against subsequent ischemic injury.

In addition to the heat stress and ischemic preconditioning, a number of other stimuli such as a brief hypoxia episode, restraint stress, and pretreatment with cytokines induce tolerance to myocardial ischemia. Previous data from our research suggests that acute hypoxia (mimicing high altitude hypoxia) increases HSP70 synthesis in rat heart *in vivo* and *in vitro*[5,6]. Engelmen *et al* have observed augmented expression of catalase and HSP70 in hypoxically preconditioned rat hearts which exhibit improved post-ischemic function. It is hypothesized that HSP induction may be one of the adaptive mechanism mediating hypoxic preconditioning of myocardium[7]. In a study by Meerson *et al*, it was shown that acclimatization of rats to intermittent hypoxic exposure (40 d at high altitude of 4000 m, 5 h per day) increased two HSP70 isoforms but the resistance of the isolated perfused heart to postischemic contractile dysfunction and creatine kinase release was not improved[8]. Therefore, the effects of stress proteins on the myocardial protection induced by in-

[1] Project supported by National Natural Science Foundation of China (Grant № 3950052).
[2] Correspondence to Dr ZHONG Ning.
Phn 86-21-6431-3251, ext 8564. Fax 86-21-6474-6305.
E-mail nolannz@sunm.shcnc.ac.cn
Received 1999-10-14 Accepted 2000-02-20

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 May；21 (5)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

termittent hypoxia need to be further studied.

Our present study was undertaken to quantify the levels of HSP70 induction by different duration of intermittent hypoxia and to correlate the myocardial HSP70 induction with the degree of protection from ischemic injury.

MATERIAL AND METHODS

Intermittent hypoxia training Male Sprague-Dawley (150 – 180 g) rats were supplied by Shanghai Animal Center, Chinese Academy of Sciences (Grade Ⅱ, Certificate № 005). They were grouped at random. Intermittent hypoxia exposure group: rats were put in the hypobaric chamber (simulating 5000 m altitude over sea level, 11.1 % O_2, pO_2 11.3 kPa) for 6 h a day. The exposure lasted for 14 ($n = 8$), 28 ($n = 10$), and 42 d ($n = 9$) respectively. The temperature in the chamber was maintained at 22 – 24 ℃. Two other normoxic groups were kept in the same circumstances as described above. One was for myocardial ischemia and reperfusion injury (IR, $n = 14$), and the other was the normal control ($n = 6$). All animals were maintained on a 12-h light/dark cycle.

Animal model of acute myocardial ischemia and reperfusion Intermittent hypoxia adapted and IR rats were subjected to a protocol of ischemia and reperfusion. Rats were anesthetized with 45 mg·kg^{-1} ip injection of pentobarbital sodium. After tracheotomy, they were ventilated with room air (stroke volume of 15 mL/kg, at 55 strokes·min^{-1}). Body temperature was maintained at (37 ± 0.5) ℃. ECG lead Ⅱ, together with the blood pressure at the carotid artery, was continuously monitored and recorded by using a data acquisition system (PowerLab/8 s, ADInstrument, Australia). Both BP and ECG signals were analyzed with software Chart 3.4 (ADInstrument).

Regional ischemia was obtained by occlusion of left anterior descending coronary artery (LAD) for 15 min, followed by reperfusion for 120 min. Myocardial ischemia was verified visually by the appearance of regional cyanosis, ST segment elevation on the ECG, and by the occurrence of ventricular arrhythmia. Ventricular arrhythmias during ischemia and reperfusion were defined in accordance with the guideline of the Lambeth Conventions for analysis of experimental arrhythmias[9], and quantified with Arrhythmia Score (AS) according to Johnston standard[10]. After the experiment, rat heart was frozen and stored in liquid nitrogen until used for RNA isolation.

RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR) Total RNA was extracted from the left ventricle of rat heart with TRIzol reagent (Life Technologies). The purity of isolated RNA was identified by OD_{260}/OD_{280} ratio and electrophoresis. No DNA and protein contamination was noted. The RNA was stored at – 70 ℃ until prepared for first strand cDNA synthesis. Oligo (dT) primed synthesis of cDNA was performed using Superscript Reverse Transcriptase (Gibco, BRL).

Amplification of 3 μL of the reverse transcription mixture was carried out with primers for the inducible rat HSP70, sense primer 5′- TGCTGACCAAGATGAAG-3′ (amino acid region 124 – 129, base pair region 545 – 561), and antisense primer 5′-AGAGTCGATCTCCAGGC-3′ (amino acid region 282 – 286, base pair region 1019 – 1035). The amplification results in expected 491-bp product. As an internal standard, GADPH was used with the sense primer 5′-TGGGAAGCTGGTCATCAACG and antisense primer 5′-GCAGTGATGGCATGGACTGT to get a 357-bp product. The GADPH and HSP70 gene were amplified simultaneously in multiplex comparative PCR. All PCR were done in a 50 μL volume. The PCR mixture contained 25 pmol of each primer, 100 μmol·L^{-1} of each dNTPs, 1 × PCR buffer (KCl 50, TrisHCl 10, $MgCl_2$ 2.5 mmol·L^{-1}, pH 8.4) and 2 U Ampli Taq DNA polymerase (Perkin Elmer Cetus). Thirty-five cycles of 30 s at 95 ℃, 30 s at 55 ℃, and 45 s at 72 ℃ were performed in a Perkin Elmer 9600 Thermal Cycler. The PCR products were electrophoresised on 2 % agarose gel, visualized by ethidum bromide staining, and analyzed by an autogel analysis system (Tanon UV-2000, China).

Statistics All values were expressed as $\bar{x} \pm s$. Comparisons between groups were assessed for significance by one way ANOVA with pos doc analysis using the Student-Newman-Keuls test. Statistical significance was defined as $P < 0.05$.

RESULTS

Heat shock protein expression By using a semi-quantitative RT-PCR and the auto gel analysis system, the HSP70 PCR products were normalized by relative optical density of HSP mRNA over that of GADPH. The transcripts for HSP70 were barely detectable in normoxic control hearts, while after acute ischemia/reperfusion, there was a modest increase in HSP70 mRNA expression, with the levels increasing from (18 ± 4) % to

(24 ± 4) %. On increasing the duration of hypoxia exposure, there was a progressive increase in the expression of myocardial HSP70. The HSP70 level was 2.6, 3.6, and 3.8 folds after 14-, 28-, and 42-d intermittent hypoxic exposures compared to normoxic rats subjected to LAD occlusion and reperfusion (Fig 1A, 1B). The elevated HSP70 mRNA expression lasted for 3 wk after the rats (28 d hypoxia-preadapted) returned to the normoxia. The levels remained high, (87 ± 8) % for 1 wk, (75 ± 2) % for 2 wk, and (45 ± 2) % for 3 wk after a 28-d hypoxia exposure (Fig 2A, 2B).

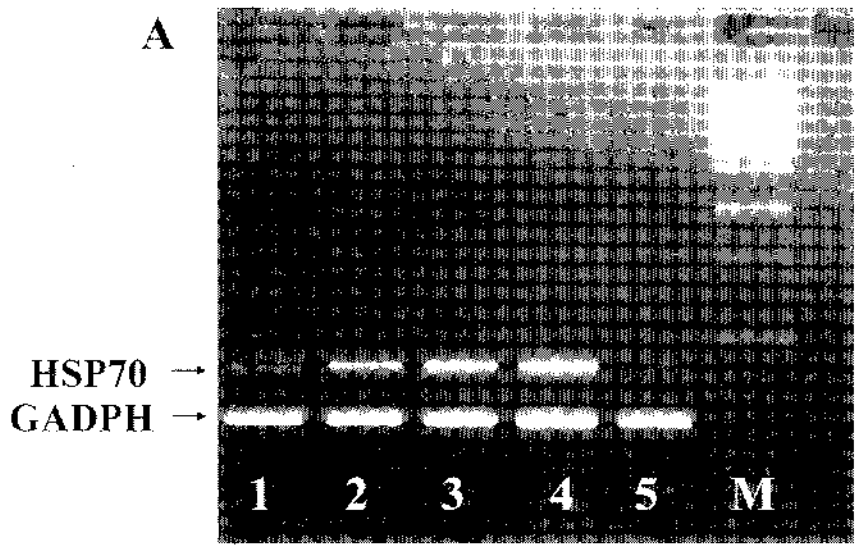

Fig 1. HSP70 mRNA expression in rat hearts subjected to different durations of intermittent hypoxic exposure. A) RT-PCR amplification 491-bp fragment of inducible HSP70 mRNA. 1) normoxic rats subjected to ischemia reperfusion injury. 2) 14-, 3) 28-, 4) 42-d exposure to intermittent hypoxia. 5) normoxic control. M) DNA molecular marker. B) Level of normalized HSP70 mRNA (expressed as optical density ratio of HSP70 over GADPH) in different groups (at least 4 samples in each group were amplified). $^{c}P < 0.01$ *vs* normoxia. $^{f}P < 0.01$ *vs* ischemia.

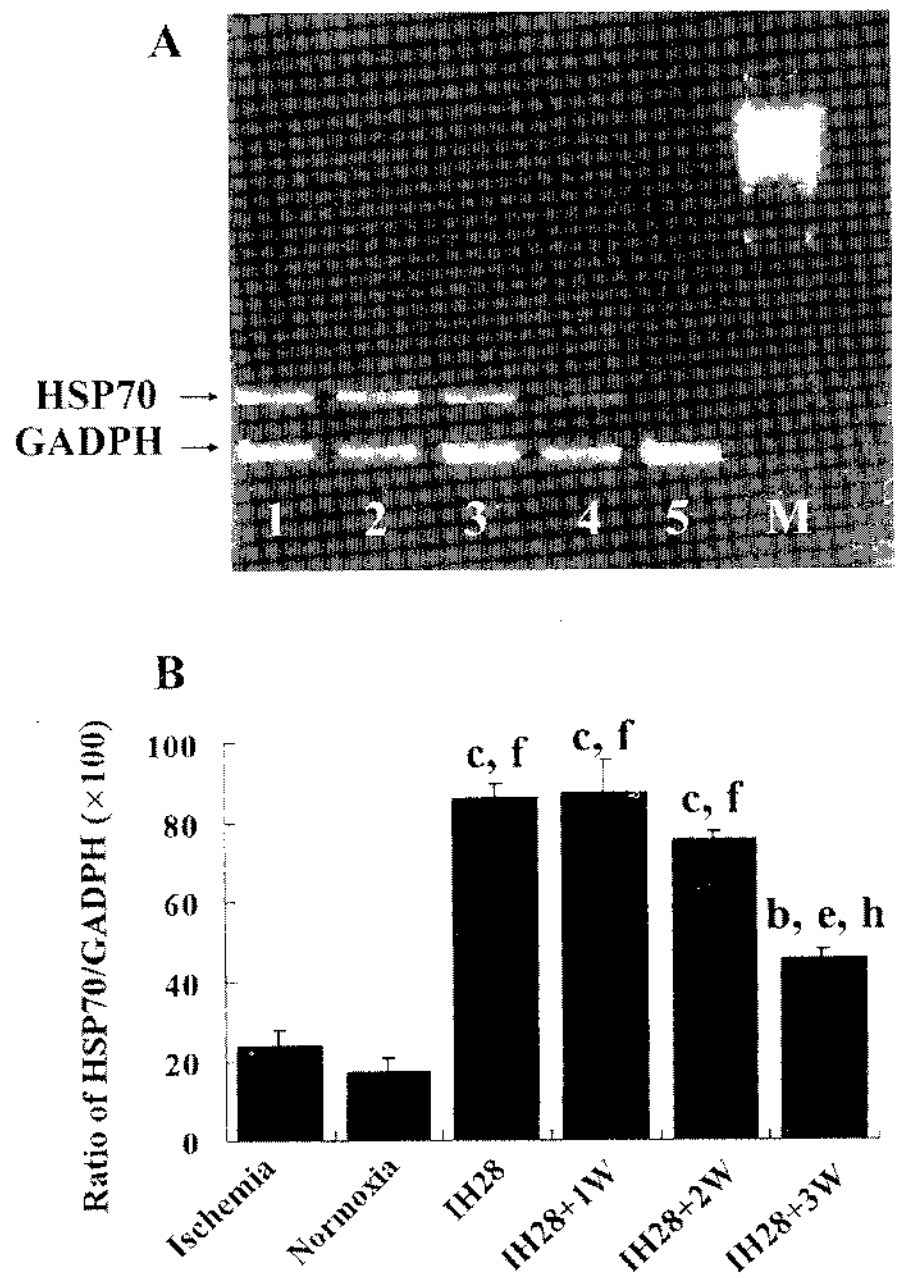

Fig 2. HSP70 mRNA expression in rat hearts subjected to a 28-d intermittent hypoxia exposure and in those returned to normoxia for 1, 2, and 3 wk. A) RT-PCR amplification 491-bp fragment of inducible HSP70 mRNA. 1) a 28-d intermittent hypoxic exposure. 2) 1, 3) 2, 4) 3 wk after the rats (28 d exposure to intermittent hypoxia) returned to normoxia. 5) normoxic control. M) DNA molecular marker. B) Level of normalized HSP70 mRNA in different groups (at least 4 samples in each group were amplified). $^{b}P < 0.05$, $^{c}P < 0.01$ *vs* normoxia. $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* ischemia. $^{h}P < 0.05$ *vs* IH28.

Arrhythmia score The severity of ischemia/reperfusion induced injury was represented by the occurrence of severe ventricular arrhythmias. The AS was 4 ± 0.7 (ischemia induced) and 3.3 ± 0.6 (reperfusion induced) for the normoxic rats subjected to LAD occlusion and reperfusion. In IH14 rats, the AS was slightly reduced, but with no statistic significance. After a 28-d exposure the tolerance of rat heart (IH28, 42 group) increased remarkably. Such protective effect could also last for 2 wk after the rats returned to sea level conditions (Fig 3A, 3B).

Correlation between the HSP70 induction and severity of myocardial injury As demonstrated, there was an inverse correlation between the amount of cardiac HSP70 induced by intermittent hypoxia and ventricular arrhythmia induced by LAD occlusion and

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 May；21 (5)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

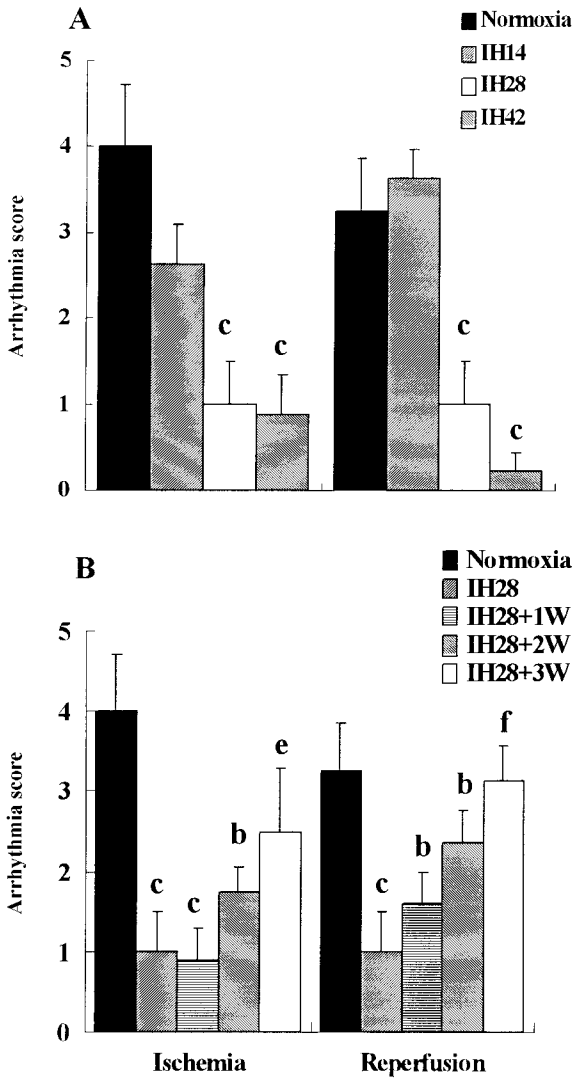

Fig 3. Arrhythmia score of LAD occlusion and reperfusion in rats exposed to different durations of intermittent hypoxia (A) and in those returned to normoxic state for several weeks (B). $^{b}P < 0.05$, $^{c}P < 0.01$ *vs* normoxia. $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* IH28.

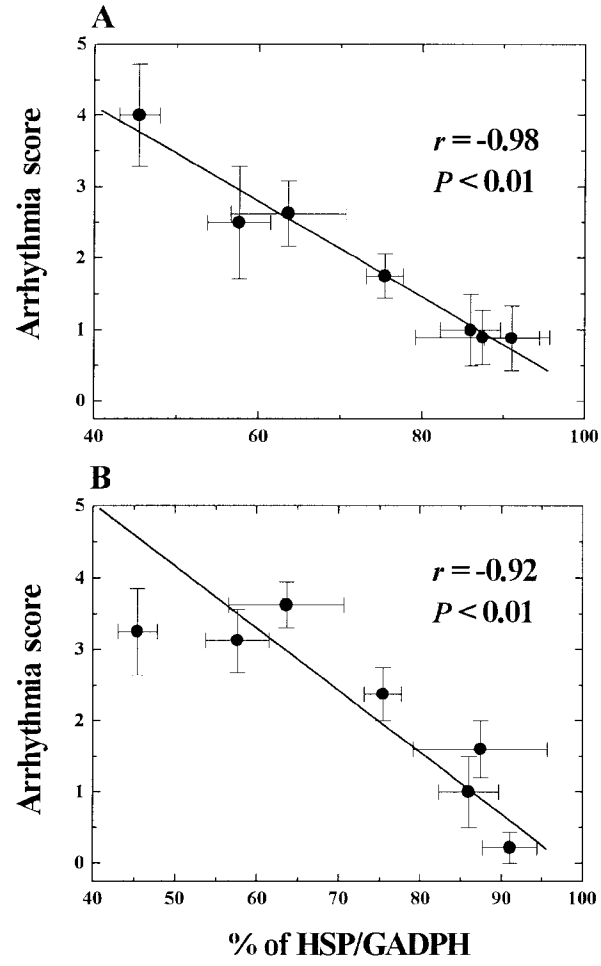

Fig 4. Liner correlation between the amount of HSP70 induced and arrhythmia score induced by LAD occlusion (A) and reperfusion (B).

reperfusion. This correlation was linear with $r = -0.98$ and $P < 0.001$ (ischemia induced arrhythmia); $r = -0.92$ and $P = 0.0038$ (reperfusion induced arrhythmia) (Fig 4).

DISCUSSION

The evidence regarding protective effects of intermittent hypoxia adaptation seems to be sufficient. Also its importance for clinical application is apparent, especially in the preconditioning of ischemic hearts. But there is limited data on the mechanisms underlying cardioprotective effects mediated by exposure to intermittent hypoxia.

A substantial literature reports the induction of HSP70 by ischemia, or hypoxia, and HSP70 has been reported as an intrinsic protective factor in ischemia preconditioning or hypoxia stress. We firstly reported the correlation between myocardial expression of HSP70 mRNA and cardioprotection in response to intermittent hypoxia exposure in rats. We observed that an increased duration of intermittent hypoxia exposure resulted in greater expression of HSP70, the elevated levels of HSP70 mRNA still remained high until 2 wk after the pretreated rats returned to normoxic state. Furthermore, there was a direct relation between the amount of HSP70 expressed and the degree of protection from subsequent acute ischemia/reperfusion injury. These results were not consistent with those reported by Meerson *et al*[8]. Such difference may be due to the selection of the animal models. Meerson *et al* have observed that the antiarrhythmic action due to adaptation to intermittent hypoxia in acute ischemia

was most pronounced in conscious animals, fairly prominent in anesthetized animals, and almost absent in isolated heart[11].

It still remains to be known why intermittent hypoxia causes an intracardiac increase in HSP70. Meerson *et al* have previously suggested a possible role of the secondary messengers and protein kinases in the adaptive stabilization of the heart. The accumulation of inducible HSP70 and the activation of the inostiol triphosphate (IP3)-diacylglycerol (DAG) circuit in hearts of rats adapted to immobilization stress were equally necessary for the development of cardiotolerance to reperfusion injury[12]. However, it is yet unclear whether the HSP70- and IP3-, DAG-mediated cardioprotective mechanisms act independently of one another or whether they are interrelated.

Stress proteins are thought to mediate cardioprotection through their biological functions as molecular chaperones. The major functions of HSP as molecular chaperones are twofold: (1) mediating correct protein-following, and (2) repairing denatured proteins or promoting their degradation[13]. It is still unclear whether the protein itself is responsible for the protection or that the protein activates secondary mechanisms yielding the protective effect. Besides the induction of HSP70, our previous study showed that level of antioxidant enzymes, superoxide dismutase (SOD), and catalase, were increased in rat myocardium after the rats were subjected to 3500 m altitude hypoxia ($6\ h \cdot d^{-1}$, 4 – 6 weeks)[14]. Mersson *et al* have also indicated that increased antioxidant activity induced by intermittent hypoxic adaptation seemed to be involved in the protection[15]. Apart from the mechanisms discussed above, there can be several additional factors that play possible roles in the beneficial effects of intermittent hypoxic adaptation. The capacity of blood oxygen transport, tissue oxygen transportation, altered energetic metabolism, and some neurohumoral regulation may also play important role in such protective effects[16, 17]. As oxygen depletion affects most systems, and as the cardioprotective effects of intermittent hypoxic adaptation employs multifold mechanisms, their clear elucidation needs more efforts. The proper duration and procedure of hypoxic adaptation should be further studied and defined before its clinical application.

REFERENCES

1 Currie RW, Karmazyn M, Kloe M, Mailer K. Heat shock response is associated with enhanced post-ischemic ventricular recovery. Circ Res 1988; 63: 543 – 9.
2 Marber MS, Latchman DS, Walker JM, Yellon DM. Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. Circulation 1993; 88: 1264 – 72.
3 Heads RJ, Yellon DM, Latchman DS. Differential cytoprotection against heat stress protein genes in myogenic cells. J Mol Cell Cardiol 1995; 27: 1669 – 78.
4 Marber MS, Mestril R, Chi SH, Sayen MR, Yellon DM. Overexpression of the rat inducible 70-KD heat shock protein in a transgenic mouse increase the resistance of the heart to ischemic injury. J Clin Invest 1995; 95: 1446 – 56.
5 Ren HM, Zhou ZN. Hypoxia-induced stress proteins in heart of rats. Chin Sci Bulletin 1993; 38: 1811 – 5.
6 Ren HM, Xiao Y, Zhou ZN. Induction of stress proteins in hypoxic and normoxic perfused isolated rat hearts. Chin J Phytophysiol 1998; 14: 491 – 5.
7 Engelman DT, Chen CZ, Watanabe M, Engelman RM, Rousou JA, Flack JE 3rd, *et al*. Improved 4- and 6-hour myocardial preservation by hypoxic preconditioning. Circulation 1995; 92: II417 – 22.
8 Meerson FZ, Malyshev IYU, Zamotinsky AV. Differences in adaptive stabilization of structures in response to stress and hypoxia relate with the accumulation of hsp70 isoforms. Mol Cell Biochem 1992; 111: 87 – 95.
9 Walker MJA, Curtis MJ, Hearse DJ, Campbell RW, Janse MJ, Yellon DM, *et al*. The Lambeth Conventions: guidelines for the study of arrhythmias in ischemia, infarction, and reperfusion. Cardiovasc Res 1988; 22: 447 – 55.
10 Johnston KM, Macleod BA, Walker JA. Responses to ligation of a coronary artery in conscious rats and the actions of antiarrhythmics. Can J Physiol Pharmacol 1983; 61: 1340 – 53.
11 Meerson FZ, Ustinova EE, Orlova EH. Prevention and elimination of heart arrhythmias by adaptation to intermittent high altitude hypoxia. Clin Cardiol 1987; 10: 783 – 9.
12 Meerson FZ, Malyshev IYU, Zamotinsky AV. The role of hsp70 and IP3-DAG mechanism in the adaptive stabilization of structures and heart protection. J Mol Cell Cardiol 1996; 28: 835 – 43.
13 Benjamin IJ, McMillan DR. Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. Circ Res 1998; 83: 117 – 32.
14 Chen M, Zhou ZN. Effects of hypoxia on myoglobin and antioxidant enzymes in rat myocardium. Exp Clin Cardiol 1997; 2: 179 – 83.
15 Merrson FZ. Adaptive protection of the heart. Moscow: CRC Press, 1991: 249 – 78.
16 Ostadal B, Ostadalova I, Dhalia NS. Development of cardiac sensitivity to oxygen deficiency: comparative and ontogenetic aspects. Physiol Rev 1999; 79: 635 – 59.
17 Zhuan JG, Zhou ZN. Protective effects of intermittent hypoxia adaptation on myocardium and its mechanism. Biol Signals Recept 1999; 8: 316 – 22.

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 May；21 (5)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

间歇性低氧诱导的心肌热休克蛋白 70 表达增加大鼠心脏对缺血损伤的耐受[1]

钟 宁[2]，张 翼，方奇志，周兆年
（中国科学院上海生理研究所，上海 200031，中国）

关键词 热休克蛋白 70；低氧；逆转录聚合酶链反应；心律失常；再灌注损伤

目的：定量检测暴露于间歇性低氧不同时程后心肌热休克蛋白（HSP70）表达量并探讨 HSP70 与大鼠心脏对缺血再灌注损伤的耐受性之间的关系. 方法：结扎冠脉左前降支造成心肌缺血及再灌注模型；并以逆转录 PCR 方法检测大鼠心肌 HSP70 mRNA 的表达量. 结果：间歇性低氧暴露 14，28，42 天后 HSP70 表达量分别增加 2.6，3.6，3.8 倍；低氧训练 28 天后大鼠心脏对缺血-再灌注损伤的耐受性明显增加，缺血和再灌注心律失常诱发评分（AS）显著降低；大鼠脱离低氧环境后，上述作用能够维持 2 周. 而且 HSP70 的表达量与心肌耐受性的增加存在明显相关（$r = 0.98$，0.92；$P < 0.01$）. 结论：间歇性低氧暴露后心肌对缺血-再灌注损伤耐受性的增加与 HSP70 的表达量有关.

（责任编辑 李 颖）