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Myocardial capillary angiogenesis and coronary flow in ischemia tolerance rat by adaptation to intermittent high altitude hypoxia[1]

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KEY WORDS altitude; anoxia; ischemia; reperfusion injury; coronary circulation; capillaries; angiogenesis

ABSTRACT

AIM: To determine the effects of simulated intermittent high altitude hypoxia adaptation (IHA) on coronary capillary and coronary flow (CF) in rat hearts. **METHODS**: Model of Langendorf-perfused isolated rat hearts were used to measure CF during ischemia-reperfusion, and immunoperoxidase staining assay and computer-aid morphometry analysis were conducted to determine the myocardial capillary densities. Cyclic GMP (cGMP) level in myocardium was measured by radio-immunoassay. **RESULTS**: Pre-ischemia level of CF in IHA rats was higher (IHA28 13.4 mL/min ± 1.5 mL/min, IHA42 15.4 mL/min ± 2.0 mL/min, $P < 0.01$) than that of normoxic rats (11.0 ± 0.8) mL/min, and the recovery of CF after ischemia-reperfusion was better in IHA rats. As an adaptive result, the myocardial capillary densities of the left ventricular myocardium in IHA rats were 1.5 times of those in normoxic control rats, but there was no apparent ventricular hypertrophy in IHA rats. Myocardial cGMP content (1.8 ± 0.7) nmol/g in IHA rats were increased significantly compared with control rats (1.1 ± 0.4) nmol/g, but cGMP level was not altered before and after ischemia-reperfusion in either group. It was also revealed that in isolated rat hearts perfused, myocardial function recovered better in IHA rats than that in normoxic control rats. **CONCLUSION**: IHA adaptation increased the tolerance of rat hearts against subsequent ischemia-reperfusion injury, and increase in coronary circulation and angiogenesis might be the mechanisms of myocardium protected by IHA.

INTRODUCTION

Adaptation to intermittent high altitude hypoxia (IHA) results in increased resistance to subsequent severe myocardial ischemia. The incidence and severity of ischemia/reperfusion induced ventricular arrhythmia were significantly reduced in anesthetized rats adapted to IHA hypoxia training[1]. Though it has potent cardioprotective effects, the mechanism is far from clear. In general, the cardioprotection against ischemia-reperfusion injury can be realized by two ways: 1) increase tolerance of cardiomyocytes; 2) increase oxygen supply[2]. Our previous study indicated that IHA increased myocardial heat shock protein expression and antioxidant enzymes (SOD) activity[1,3,4], and IHA prevented mtDNA deletion and mitochondrial structure damage induced by ischemia-reperfusion injury[5]. Those results represented the stimulated endogenous tolerant process of IHA adapted hearts. But there is far more less evidence concentrated on the coronary circulation and adjustment of myocardial oxygen supply during ischemia-reperfusion in IHA hearts. It has been speculated that, in the face of hypoxia stress, tissue oxygenation is threatened, and compensatory angiogenesis might be a mechanism of preserved myocardial blood supply. Although the effect of chronic hypoxia on proliferation of coronary vessels has been examined in a great number of studies, the results were not consistent, partial due to adverse effects, such as ventricular hypertrophy induced by long-term exposure to hypoxia. Besides hypoxia induced angiogentic effects, preserved coronary circulation may also balance the mismatch between the myocardial oxygen demand and supply in ischemia. There has been no comprehensive morphological study quantitating the cardiac adaptations to

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IHA hypoxia. Therefore we designed to identify the changes in microvasculature of rat hearts subjected to IHA hypoxia, and the myocardial functional responses, especially coronary flow (CF), to acute ischemia-reperfusion were examined in by Langendorff method.

MATERIALS 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), and were grouped at random. Intermittent hypoxia exposure group: rats were put in a hypobaric chamber (simulating 5 km altitude over sea level, 11.1 % O_2, p_{O_2} 11.3 kPa) for 6 h a day. The exposure lasted for 28 d (IHA28), and 42 d (IHA42), respectively. The temperature in the chamber was maintained at 22 – 24 ℃. Rats of normoxic (control) groups were kept in the same circumstances as described above. All animals were maintained on a 12 h light/dark cycle.

Isolated rat heart perfusion The rats were anaesthetized with intraperitoneal pentobarbitone sodium (45 mg/kg). After opening the chest, heart was isolated and perfused in the Langendorf apparatus under constant pressure of 70 mmHg. A collapsed latex balloon was placed in the left ventricular cavity via left atrial incision, and initial intra-balloon pressure was adjusted to 4 – 6 mmHg. Left ventricular pressure was monitored via a pressure transducer (MPU-0.5A, Gold, USA) connected to a Bridge Amplifier (PowerLab, AD Instrument Ltd, Australia). Both pressure parameters (LVDP: left ventricular development pressure; LVEDP: left ventricular end-diastolic pressure; ± dp/dt: the maximum rate of rise or fall of LVDP) and heart rate (HR) were continually recorded, signals were acquired by PowLab/8 s data acquisition system (AD Instrument Ltd, Australia) and stored in a computer. Data were analyzed off-line with Software Chart v 3.04 (AD Instrument Ltd, Australia).

The heart was perfused with Krebs-Henseleit's (K-H) solution (in mmol/L, NaCl 120, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 1.8, $NaHCO_3$ 25, glucose 11; aerated with 95 % O_2 + 5 % CO_2, pH 7.4 at 37 ℃ ± 0.5 ℃). After initiation of coronary perfusion, a 15-min stabilizing period was then followed by 30 min global non-flow ischemia by clamping the perfusion line. The reperfusion was achieved by restored the perfusion of K-H solution for 30 min. At the end of the ischemia-reperfusion protocol, hearts were rapidly dissected to separate each ventricle, and ventricular masses were weighted. Then the ventricles were frozen and stored in liquid nitrogen until used for radio-immmunoassay of cGMP measurements.

cGMP measurement Frozen samples of myocardium (50 mg) from ischemic region was weighed and homogenized in ice-cold acetic acid [50 mmol/L, containing Na_2 ethylenediamine tetraacetic acid (EDTA) 4 mmol/L] at 4 ℃, and the supernatant was extracted 2 times with 75 % ethanol 2 mL. Residual traces of ethanol were evaporated by heat to 60 ℃ for 10 min and the sample was stored at −20 ℃ until use. The samples were diluted 1 to 50 with 0.05 mol/L Tris-EDTA buffer (pH 7.5 containing EDTA 4 mmol/L). Aliquots (50 μL) were assayed in duplicate with a ^{125}I-labeled scGMPTME radio-immunoassay kit (obtained from Shanghai Traditional Chinese Medicine University).

Myocardial capillary densities Rat hearts from different groups (without ischemia-reperfusion damage) were fixed and embedded in paraffin. Histological sections were prepared perpendicularly to the spectrum in the region about 4 mm below the atrio-ventricular border followed by standard deparaffinization procedure. Rabbit anti-rat vWF antiserum (DAKO, Denmark), ultra vision stretavidin-peroxidase (SP)-plus Kit (Maxim Biotech Inc USA), and DAB (3 – 3′-diaminobenzidine tetrahydrochloride dihydrate)-chromagen (MBI, USA) were used for immunoperoxidase staining assay. The capillaries' densities were counted in the lateral part of the walls of both ventricles near cavity. The counting was achieved by a computer morphometric system (Leica Q550 IW, Germany) and analyzed by Software Qwin v 1.0 (Leica Microsystems, Germany).

Statistics Data were expressed as $\bar{x} \pm s$. Comparisons between groups were assessed by two-way ANOVA with Post Hoc analysis using the Student-Newman-Keuls test. Statistical significance was defined as $P < 0.05$.

RESULTS

IHA increased coronary flow and preserved the cardiac function Except CF, there were no much difference in other pre-ischemia function parameters of perfused rat hearts between the IHA and nomoxic rats (Fig 1, 2). Basal CF was significantly increased in

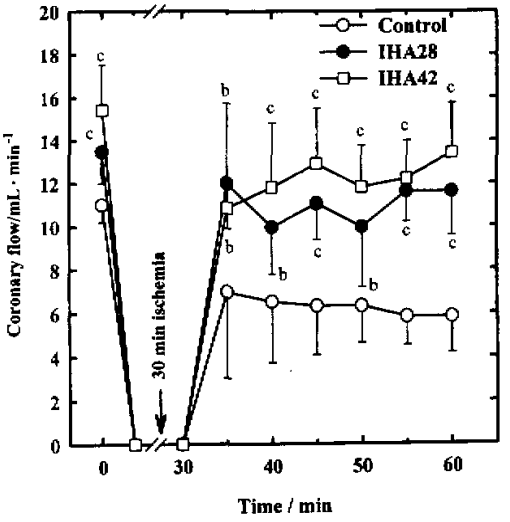

Fig 1. Effects of IHA on CF in rat hearts subjected to ischemia-reperfusion. (○) **Control** (n = 9); (●) **IHA28** (n = 10); (□) **IHA42** (n = 9). $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

IHA rats (IHA28 13.4 mL/min ± 1.5 mL/min, IHA42 15.4 mL/min ± 2.0 mL/min, $P < 0.01$) compared to 11.0 mL/min ± 0.8 mL/min in normoxia. In normoxic rats, CF recovered to only 59 % of pre-ischemia level after 10 min reperfusion, and 52 % after 30 min reperfusion. While, CF recovered much better in IHA rat hearts ($P < 0.01$ compared with control rats). It reached to 74 % (IHA28), 79 % (IHA42) at 10 min reperfusion, and 85 % (IHA28), 86 % (IHA42) at 30 min reperfusion, respectively.

After 30 min reperfusion, LVDP, $+dp/dt$, and $-dp/dt$ recovered to 62 %, 61 %, and 55 % of pre-ischemia level in normaxic rats, respectively. LVEDP (mmHg) elevated from 6.2 ± 0.5 (pre-ischemia) to 28 ± 5 (30 min reperfusion). The recovery of all myocardial function parameters (LVDP, $+dp/dt$, and $-dp/dt$) in IHA rats was much better than that in normaxic rats (> 80 % compared with control rats, Fig 2), while increase of LVEDP in IHA rats was less than that in normaxic rats ($P < 0.05$, Fig 2).

IHA increased myocardial cGMP content No significant change on myocardial cGMP content before and after the rats subjected to *in vitro* ischemia/reperfusion in either normoxic or IHA rats, however the myocardial cGMP level in the IHA rats increased significantly compared to that in control normoxia rats (Tab 1). The results indicated that the formation of cGMP was not due to ischemia stress, but to intermittent hypoxia adaptation.

Quantitative measurement of capillary densities The capillary densities increased after rats exposed to intermittent hypoxia for 28 d and 42 d (Tab 1). Such effects were more prominent in left ventricles than those in right ventricles. The capillary densities increased by 39 % (IHA28) and 48 % (IHA42) in left ventricle of preadapted rats compared with normoxia control, while in right ventricle, it only increased 12 % and 23 % for 28 d and 42 d hypoxia exposure.

The IHA exposure for 28 d and 42 d did not induce apparent ventricular hypertrophy. Ratios of ventricular weight over body weight (IHA28 0.32 ± 0.04, IHA42 0.334 ± 0.021, $P > 0.05$ *vs* 0.31 ± 0.04 in control group), the ratios of right or left ventricular weight over heart weight (Tab 1), and the ratio of right ventricular weight over left (IHA28 0.27 ± 0.06 and IHA42 0.28 ± 0.08 *vs* 0.26 ± 0.06 in control, $P > 0.05$) showed no difference between IHA rat hearts and normoxic rat hearts.

Tab 1. Myocardial cGMP content (nmol/g) and quantitative morphometry measures of weight ratio of right or left ventricle over that of whole heart (WH), and capillary density was expressed as counts of capillary numbers per mm². $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control. **Animal numbers used in experiments: $n = 9$ for cGMP measurement and ratio of RV/WH and LV/WH, $n = 4$ for capillary densities (control); $n = 10$ for cGMP measurement and ratio of RV/WH and LV/WH, $n = 3$ for capillary densities (IHA28); $n = 9$ for cGMP measurement and ratio of RV/WH and LV/WH, $n = 3$ for capillary densities (IHA42).**

	cGMP/nmol·g^{-1}		RV/WH	LV/WH	Capillary density/mm^2	
	Non-ischemia	Ischemia			Left ventricle	Right ventricle
Control	1.1 ± 0.4	1.05 ± 0.24	0.21 ± 0.03	0.72 ± 0.03	2099 ± 54	2083 ± 79
IHA28	1.8 ± 0.7^c	1.7 ± 0.5^c	0.22 ± 0.03	0.71 ± 0.06	2918 ± 66^c	2324 ± 76^b
IHA42	1.7 ± 0.4^c	1.6 ± 0.5^c	0.23 ± 0.04	0.71 ± 0.04	3114 ± 55^c	2585 ± 92^b

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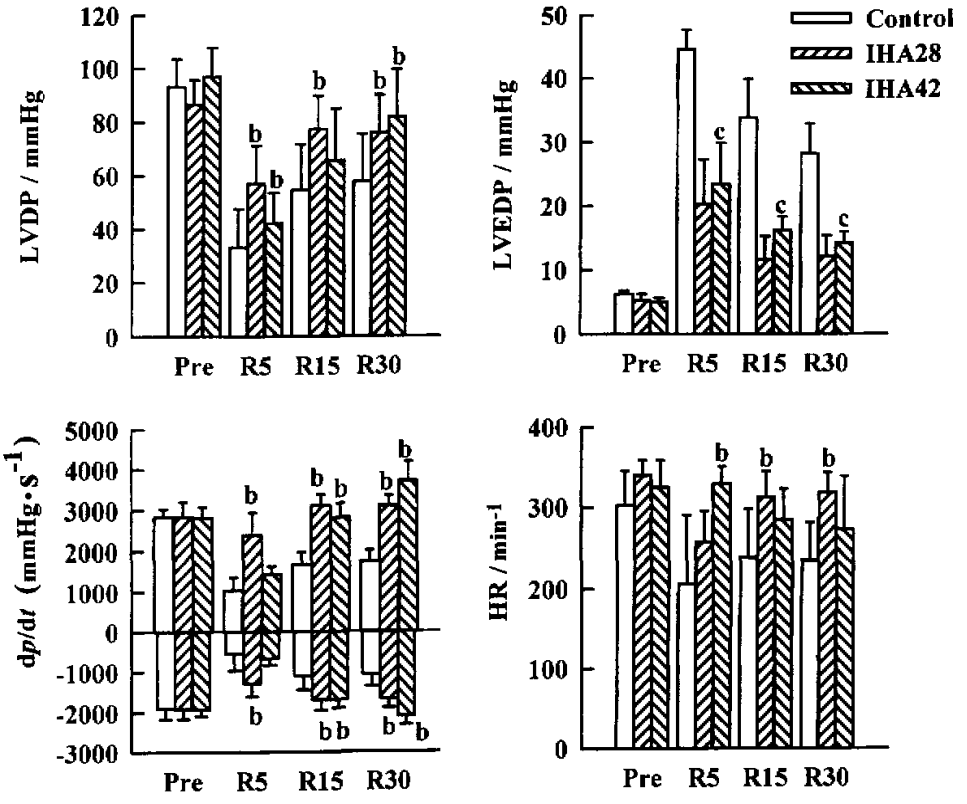

Fig 2. Effects of IHA adaptation on cardiac function recovery in post-ischemia reperfused rat hearts. Pre: pre-ischemia; R5, R15, R30: 5, 15, 30 min after reperfusion. $n = 28$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

DISCUSSION

Our results presented increased coronary blood flow and capillary densities in rat hearts after a month exposure to simulated intermittent hypoxia. Such adaptation changes contributed to a better functional recovery when isolated rat hearts subjected to a severe ischemia-reperfusion injury, and the adaptation did not cause ventricular hypertrophy.

The blood and tissue oxygen transport are two major aspects in achieving the demand of oxygen consumption. Chen and Zhou found that the concentration of myoglobin increased in IHA rat myocardium[4], which might improve the blood oxygen supply. Intact coronary circulation plays a major role in accomplishing sufficient myocardial tissue oxygen transport, which was regulated by both diffusion distance between capillaries and myocardium, and coronary artery resistance.

Results showed that intermittent hypoxia exposure significantly increased the myocardial cGMP content. Baker *et al* found that increased tolerance to ischemia in rabbit hearts adapted to chronic hypoxia was associated with increased expression of constitutive nitric oxide synthase. It showed that myocardial release of nitrite/nitrate, and cGMP were increased two to three times in hypoxic hearts, and also it was noted an increase in myocardial cGMP content[6]. Increased NO production had been ensured in rats subjected to intermittent hypobaric hypoxia[7]. During adaptation, the nitrite/nitrate level progressively increased and was correlated with the increase in NO stores. Meanwhile, IHA prevented impairment of endothelium-dependent relaxation[8]. cGMP is revealed as a link between NO and ATP-sensitive potassium channel (K_{ATP}) activation. The adaptation to hypoxia results in elevation of NO, which activates soluble guanylyl cyclase causing cGMP accumulation and possible activation of cGMP-dependent protein kinase. Then the activated protein kinase

phosphorylates and activates K_{ATP} channels[9]. The NO-cGMP pathway mediated K_{ATP} activation contributes to coronary vasodilatation, and then reduces the coronary circulation resistance. Besides the beneficial vasiodilatory effects of NO-cGMP, other possible NO-dependent protective mechanisms in adaptation to intermittent hypoxia are also illustrated, which includes activation of local defense systems such as HSP70, antioxidant, *etc*[10].

Besides coronary vasodilatation, the myocardial capillary capacity is another important compensatory aspect in augmenting cardiac oxygen supply. Turek *et al* observed a greater coronary flow during ventilation of hypoxic gas mixture in rats acclimatized to simulated chronically high altitude hypoxia than in normoxia rats. The results indicated that an improving vascular capacity of the heart, which was achieved by stimulated angiogenesis and presented in both left and right ventricles[11]. Hypoxia/reoxygenation promoted myocardial angiogenesis was due to increased expression of angiogenic factor vascular endothelial growth factor (VEGF)[12,13]. The increased capillary densities decreased the intercapillary distance (reduced from 17 μm in normoxia to 11 μm). The intercapillary distance was a main determinant of diffusion distance, which reduced from 8.5 μm (normal) to 5.5 μm (exposed to hypoxia)[14]. Such adaptation might improve the efficiency of oxygen transport which was also associated with a lower coronary resistance after adaptation to hypoxia[15]. The increased CF in present study is a combined outcome of decreased coronary resistance and increased capillary densities.

Compared to widely known temporal cardiac protection by ischemia-preconditioning, the mechanisms of hypoxia adaptation may be different. Tajima's observation indicated that hypoxia training (rats exposed to 10 % oxygen for 3 weeks) increased myocardial tolerance to ischemia, and subsequent acute ischemia preconditioning increased the tolerance further. Such additive protective effects by ischemia preconditioning and by adaptation of chronic hypoxia may suggest that these two phenomena might be independent and with different mechanism[16]. Our previous study also revealed that the cardioprotective effects of IHA hypoxia adaptation could last for at least 3 weeks after the rats stopped the hypoxia training[1], while ischemia-preconditioning only lasted less than 72 h. The IHA hypoxia training not only induces functional (CF increases) but also morphological adaptation (myocardial capillarity). Such adaptive alteration increases both oxygen supply and removal of metabolic waste, which contributes to a well-preserved cardiac function during ischemia-reperfusion.

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间歇性高海拔低氧适应对大鼠缺血耐受的心肌毛细血管生成以及冠脉血流的影响[1]

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关键词 高海拔; 低氧; 局部缺血; 再灌注损伤; 冠状动脉循环; 毛细血管; 血管生成

目的: 为了阐述间歇性高海拔低氧对于大鼠心脏冠脉毛细血管以及冠脉血流的影响. **方法**: 利用离体大鼠心脏 Langendorff 灌流模型来检测缺血复灌期的冠脉流量变化, 利用免疫过氧化酶染色测定法和计算机辅助形态计量分析法来测定心脏毛细血管密度. 利用放免方法检测心肌中 cGMP 的水平. **结果**: 缺血前, 间歇性低氧适应(IHA)的大鼠的冠脉流量水平(IHA28 13.4 mL/min ± 1.5 mL/min, IHA42 15.4 mL/min ± 2.0 mL/min, $P < 0.01$)要比常氧下的大鼠(11.0 ± 0.8) mL/min 高, IHA 大鼠在缺血复灌后冠脉流量的恢复也较好. 作为适应的结果, IHA 大鼠左心室心肌毛细血管密度约为常氧大鼠的 1.5 倍, 但没有发现明显的心室肥大. 缺血复灌前后这两组的心肌 cGMP 的水平都没有改变. 然而与常氧大鼠组比较, IHA 训练大鼠的 cGMP 水平明显增加. 在离体心脏灌流中, IHA 大鼠缺血复灌后心功能的恢复较常氧组好. **结论**: IHA 增加了大鼠心脏对缺血复灌损伤的耐受, IHA 诱导的冠脉循环增加以及血管生长可能是 IHA 心肌保护效应的机制.

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