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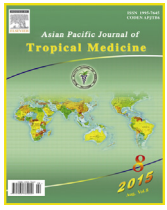

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Late cardioprotection of exercise preconditioning against exhaustive exercise-induced myocardial injury by up-regulatation of connexin 43 expression in rat hearts

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ABSTRACT

Objective: To investigate the expression of myocardium connexin 43 (Cx43) in late exercise preconditioning (LEP) cardioprotection.

Methods: Eight-week-old adult male Sprague Dawley rats were randomly assigned into four groups (n = 8). Myocardial injury was judged in accordance with serum levels of cTnI and NT-proBNP as well as hematoxylin basicfuchsin picric acid staining of myocardium. *Cx43* mRNA was detected by *in situ* hybridization and qualified by real-time fluorescence quantitative PCR. Cx43 protein was localized by immunohistochemistry and its expression level was determined by western blotting.

Results: The LEP obviously attenuated the myocardial ischemia/hypoxia injury caused by exhaustive exercise. There was no significant difference of *Cx43* mRNA level between the four groups. Cx43 protein level was decreased significantly in group EE ($P < 0.05$). However, LEP produced a significant increase in Cx43 protein level ($P < 0.05$), and the decreased Cx43 protein level in exhaustive exercise was significantly up-regulated by LEP ($P < 0.05$).

Conclusions: LEP protects rat heart against exhaustive exercise-induced myocardial injury by up-regulating the expression of myocardial Cx43.

1. Introduction

Cardiovascular disease, which is induced by ischemic, is the major cause of death in the industrialized world. Repeated transient ischemia, which is called ischemic preconditioning (IP), have been shown to protect myocardium against a subsequent more sustained ischemic insult [1–3]. Until now, many studies have evidenced that IP shows cardioprotection by enhancing myocardial tolerance to ischemia-reperfusion (I/R) injury and improving myocardial function [4,5]. Myocardium connexin 43 (Cx43) is a predominant protein forming gap junctions and non-junctional hemichannels in ventricular myocardium, through which ions and small molecules diffuse between cells [6]. It plays a key role in IP cardioprotection against I/R injury and IP have been indeed documented to affect the phosphorylation status of Cx43. Jain *et al.* showed that IP attenuates ischemia-induced dephosphorylation of myocardial Cx43 and the resulting electrical uncoupling [7]. The preserved phosphorylation of Cx43 during IP may be related to the enhanced association of Cx43 with PKC and p38 mitogen activated protein kinases [8]. Furthermore, IP cardioprotection disappears in heterozygous Cx43-deficient mice [9,10], indicating that Cx43 is very important in relieving IP cardioprotection against I/R injury. Like IP cardioprotection, exercise preconditioning (EP), which is brief episode regular exercises, is also widely demonstrated to protect heart during an I/R insult [11–15]. At present, many researches about myocardial protection have focused especially on EP, which includes a biphasic protection manner, *i.e.*, early cardioprotection of exercise preconditioning (EEP) occurring immediately after the exercise and late cardioprotection of exercise preconditioning (LEP) developing 24 h post exercise. For example, EP can improve myocardial function and enhance myocardial tolerance to I/R injury. Its endogenous myocardial protection mechanisms may be closely related to channels

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opening, attenuation of apoptosis, and proteins activation closely [12,15–17]. EP was also found to markedly attenuate exhaustive exercise-induced myocardial injury, and PKC is probably involved in EP cardioprotection [18].

Although many studies have demonstrated the protective effect of EP against exhaustive exercise-induced myocardial injury, its mechanisms are still unclear. Considering the same powerful cardioprotective effects of both EP and IP as well as the key role of increased expression of Cx43 in IP cardioprotection, we hypothesized that Cx43 may involve in cardioprotective signaling transduction of EP. It has been observed that endurance exercise training preserves a higher expression level of Cx43 in heart under resting conditions [19]. Moreover, the expression level of Cx43 is down-regulated in trained mice during acute exercise [20]. However, to our knowledge, no research has been done to study the regulatory effects of Cx43 on EP cardioprotection against exhaustive exercise-induced myocardial injury. Therefore, this study aimed to determine the relationship between Cx43 and LEP cardioprotection against exhaustive exercise-induced myocardial injury.

2. Materials and methods

2.1. Rats

Eight-week-old adult male Sprague Dawley rats weighting 220–300 g were purchased from Chinese Academy of Sciences (Shanghai, China). All rats were housed in standard rat cages and maintained at a constant temperature and humidity with a 12 h: 12 h light–dark cycle. They were fed and watered *ad libitum*. All animal care and experimental procedures were conducted in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences and approved by the Ethics Committee for Science Research of the Qufu Normal University.

2.2. Experimental protocol

All rats were given an adaptive training on a treadmill for 5 d, with an adaptive training velocity of 15 m/min and a time course of 10–20 min/d. After 1-day rest, all rats were randomly divided into four groups, eight in each group: (i) sedentary control group (group C), the rats of which were placed on the treadmill without any treadmill exercise; (ii) exhaustive exercise group (group EE), rats of which run to exhaustion on the 0% grade treadmill at a speed of 35 m/min to induce myocardial injury; (iii) late exercise preconditioning group (group LEP), rats of which were allowed to run on the treadmill for four periods of 10 min, each at 30 m/min, with intervening periods of rest of 10 min at 0% grade. Exercise began and ended with 5-min 'warm up' and 'cool down' periods at 15 m/min and 0% grade. The rats were sacrificed 24 h after the exercise. (iv) late exercise preconditioning plus exhaustive exercise group (group LEP + EE), rats of which were treated as those in the group LEP, except that they run to exhaustion 24 h after the exercise and were sacrificed 0.5 h after the exhaustion.

Animals were anesthetized with trichloroacetaldehyde monohydrate (400 mg/kg intraperitoneally) and fixed in the dorsal position on an animal operation table. After abdominal cavity was quickly opened and heart was exposed, blood was drawn via inferior caval vein. Partial heart was rapidly excised, and left ventricle (LV) free wall was isolated at the level of the near apex for real-time fluorescent quantitative PCR and western blot analysis. Once the tissue was divided, the pieces were quick frozen in liquid nitrogen. The rest heart was exposed to perfusion fixation for *in situ* hybridization and immunohistochemistry.

2.3. Detection of serum cTnl and NT-proBNP

The serum levels of cTnl and NT-proBNP in the inferior caval vein blood samples were quantified. The serum cTnl levels were determined by automated immunochemiluminescence on Access 2 immunoassay system (Beckman Coulter, USA), while the serum NT-proBNP levels were determined by using rat NT-proBNP ELISA kits (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's instructions. The sensitivity thresholds for cTnl and NT-proBNP were 0.01 μg/L and 1 μg/L, respectively.

2.4. Hematoxylin basic fuchsin picric acid (HBFP) staining

Following deparaffinization and rehydration, tissue section was stained in alum hematoxylin for 5 min and differentiated in 1% acid alcohol for 4–6 s. After being stained in 0.1% basic fuchsin for 3 min, the section was rinsed with absolute acetone for 15–22 s. Then, it was differentiated in 0.1% picric acid in absolute acetone for 5–10 min. Finally, it was cleared with xylene and covered with a coverslip. The ischemic/hypoxic myocardial fibers stain a crimson red color under light microscope.

2.5. In situ hybridization

The tissue section was first deparaffinized and rinsed with distilled water and phosphate-buffered saline (PBS). After that, it was soaked in 3% hydrogen peroxide for 30 min, followed by pepsin digestion for 5–7 min. After the second washing with PBS and distilled water and prehybridization at 42 °C thermostat, the section was hybridized, blocked with serum, incubated, washed with PBS, colorated, counterstained with hematoxylin, dehydrated in graded series of ethanol and mounted. Serial adjacent sections were hybridized, and probe hybridization solution was replaced by PBS as a negative control. Cx43 *in situ* hybridization kit and DAB chromogenic reagents were purchased by Wuhan Boster Biological Engineering (Wuhan, China). The sequences of three intermediate mRNA fragment probes for rat *Cx43* gene are as follows: 5′-TCT CTC ACG TGC GCT TCT GGG TCC TTC AGA TCA TA-3′, 5′-CTC ATC CAG TGG TAC ATC TAT GGG TTC AGC TTG AG-3′, and 5′-AAC AAT TCC TCG TGC CGC AAT TAC AAC AAG CAA GC-3′.

2.6. Real-time fluorescent quantitative PCR

Total RNA was isolated from LV myocardium using a commercial kit (RNeasy, Mini Kit, Qiagen) according to the manufacturer's instructions. Strand cDNA was synthesized with random hexanucleotides from 1.5 μg of total RNA using a reverse transcription system kit (SuperScript™ III Reverse Transcriptase, Invitrogen). Real-time quantitative PCR was done using Biorad's iQ 10000× SYBR Green Supermix (Invitrogen). The primer sequences are as follows: 5′-AAA GGC GCG TTA AGG ATC GCG TG-3′ (the first primer for *Cx43* gene), 5′-GTC