Exercise preconditioning attenuates pressure overload-induced pathological cardiac hypertrophy

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Abstract: Pathological cardiac hypertrophy, a common response of the heart to a variety of cardiovascular diseases, is typically associated with myocytes remodeling and fibrotic replacement, cardiac dysfunction. Exercise preconditioning (EP) increases the myocardial mechanical load and enhances tolerance of cardiac ischemia-reperfusion injury (IRI), however, is less reported in pathological cardiac hypertrophy. To determine the effect of EP in pathological cardiac hypertrophy, Male 10-wk-old Sprague-Dawley rats (n=30) were subjected to 4 weeks of EP followed by 4-8 weeks of pressure overload (transverse aortic constriction, TAC) to induce pathological remodeling. TAC in untrained controls (n=30) led to pathological cardiac hypertrophy, depressed systolic function. We observed that left ventricular wall thickness in end diastole, heart size, heart weight-to-body weight ratio, heart weight-to-tibia length ratio, cross-sectional area of cardiomyocytes and the reactivation of fetal genes (atrial natriuretic peptide and brain natriuretic peptide) were markedly increased, meanwhile left ventricular internal dimension at end-diastole, systolic function were significantly decreased by TAC at 4 wks after operation (P < 0.01), all of which were effectively inhibited by EP treatment (P < 0.05), but the differences of these parameters were decreased at 8 wks after operation. Furthermore, EP treatment inhibited degradation of IκBα, and decreased NF-κB p65 subunit levels in the nuclear fraction, and then reduced IL-2 levels in the myocardium of rats subject to TAC. EP can effectively attenuate pathological cardiac hypertrophic responses induced by TAC possibly through inhibition of degradation of IκBα and blockade of the NF-κB signaling pathway in the early stage of pathological cardiac hypertrophy.

Keywords: Exercise preconditioning, pathological cardiac hypertrophy, pressure-overload, IκB, NF-κB

Introduction

Pathological cardiac hypertrophy, accompanying with hypertension, aortic stenosis and valve defects, is typically associated with myocytes remodeling and fibrotic replacement, cardiac dysfunction, and increases risk of heart failure and sudden death [1-3]. A pathological stimulus causing pressure overload (e.g. aortic stenosis, hypertension) produces an increase in systolic wall stress which results in concentric hypertrophy. If the chronic stimulus is not relieved, the hypertrophied heart will dilate, contractile function will fall and then heart failure will develop. At present there is no good way to cure heart failure, and long term survival following heart failure remains poor, with one third of patients dying within a year of diagnosis [4-7]. Thus, recent studies have focused on how to prevent or reverse cardiac hypertrophy and transition to heart failure, and identifying the molecular mechanisms and finding new therapeutic targets. It is well known that physiological stimuli (e.g. regular aerobic exercise) enhance cardiac function. Exercise preconditioning (EP), a physiologic favorable adaptation in many organs, including the heart, increases the myocardial mechanical load and enhances tolerance of cardiac ischemia-reperfusion injury (IRI), however, is less reported in pathological cardiac hypertrophy, accompanied by a positive alteration in stroke volume and sympathetic nervous system activity, therefore, reduces the incidence of arrhythmias [8, 9] and myocardial apoptosis [10], and improves heart function [11].

Nuclear transcription factor(NF)-κB is a ubiquitous transcription factor which regulates relative genes involved cardiac remodeling,
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It can play an important role in the pathophysiology of myocardial ischemia/reperfusion injury, atherosclerosis and heart failure [14-19]. The NF-κB family has five subunits, including p65, RelB, c-Rel, p50, and p52, which form homo- or hetero-dimers [12, 20]. Under sedentary conditions, inactive NF-κB dimers are bound to inhibitor of κB (IκB) in the cytoplasm, whereas on stimulation, IκB kinase (IκK)-mediated IκB phosphorylation results in IκB ubiquitination and nuclear translocation of NF-κB. The activation of the NF-κB pathway plays a key role in the development of cardiac hypertrophy, and the inhibition of NF-κB pathway could promote the regression of cardiac hypertrophy induced by pressure overload [21, 22].

The transverse aortic constriction (TAC) model is performed by banding the aorta between the innominate artery and the left carotid artery and is a convincing method for producing pressure overload-induced pathological hypertrophy and heart failure [23]. Similar to aortic stenosis and hypertension, TAC initially leads to compensated hypertrophy of the heart, which often is associated with a temporary enhancement of cardiac contractility. Over time, however, the response to the chronic hemodynamic overload becomes maladaptive, resulting in cardiac dilatation and heart failure [24]. Hence, in light of this and the fact that NF-κB is a potential critical mediator of cardiac hypertrophy, we tested in the present study the hypothesis that EP could attenuate pathological cardiac hypertrophy induced by pressure overload through the inhibition of the NF-κB pathway. Our data presented below unveils EP can effectively suppress the activation of the NF-κB pathway in myocardium, as well as TAC-induced cardiac hypertrophic responses in rats.

Materials and methods

Experimental animals

The experimental protocols were approved by the Committee on Animal Research at the Second Military Medical University laboratory animals center. All rats were maintained on a 12 h:12 h light: dark cycle and received food and water ad libitum.

Experimental protocol

All animals were randomly divided into three groups (30 rats per group): sham group, TAC group and EP-treated TAC group (EP+TAC group). According to the Bedford standard [25], rats of EP group were subjected to moderate-intensity exercise (about 60% of maximal aerobic velocity) [26] on a motor-driven treadmill (Hangzhou, China) for 4 wks. In the first 5 days, these rats were trained for 0% grade, 40 min/day at 15 m/min, and duration and intensity increased daily until the animals were trained for 60 min at 18 m/min, 0% grade. Thereafter, exercise intensity was kept constant at moderate level at each training period (5 days per week). All post training experiments in trained rats were performed 48 h after the last exercise training bout to avoid acute effects of exercise. The other two groups rats remained at sedentary for the training period. After EP, sixty rats of EP+TAC and TAC group were performed by tying a 3-0 silk suture over an 8 gauge needle to produce pressure overload-induced pathological cardiac hypertrophy as previously described [19, 27-29]. The sham group procedure was performed identically but without the aortic ligation. No further exercise training was undertaken after TAC. Four wks and 8 wks after surgery, sham, TAC and EP+TAC rats were examined with echocardiography, and were weighed and decapitated under anesthesia, followed by collection of cardiac tissues and tibia length. Heart weight-to-body weight (HW/BW) ratios and the heart weight-to-tibia length ratio (HW/TL) were recorded at the time of tissue collection.

Echocardiography

At 4 wks and 8 wks after operation, Rats (n=10, per group) were anesthetized by isoflurane and transthoracic echocardiographic measurement was carried out by an animal specific instrument (Visual Sonics Vevo770, Visual Sonics Inc, Toronto, Canada) [30, 31]. LV wall thicknesses, LV chamber dimensions, LV volumes, and fractional shortening were determined from M-mode tracings. All measurements were performed by two experienced persons and taken in a double-blind manner.
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Morphology and histological analyses

Excised hearts (n=5, per group) were weighed, perfused with PBS and fixed with 4% polyformaldehyde for global morphometry and then with 10% formalin for further histological analysis. Paraffin embedded hearts were sectioned at 4-6 μm thickness and stained with hematoxylin and eosin (H-E). Cardiomyocyte morphology and histology of LV free wall was visualized under a high magnification to assess cross-sectional area (CSA) using a video camera (Leica Qwin 3) attached to a micrometer. Images were analyzed using an Image J (NIH, USA). CSA of cardiomyocyte measured from 5 sections for one heart and 5 hearts (4 wks after TAC) examined, and five randomly chosen fields were evaluated from each cross section of the LV free wall.

Quantitative real-time polymerase chain reaction analysis

Total RNA was isolated from the LV free wall of animal models (n=6, per group) using TRIzol reagent (Invitrogen). SYBR Green qRT-PCR (Takara Bio Inc, Otsu, Japan) was carried out with reverse primer and forward primers designed specifically for each of the mRNAs (Table 1). qRT-PCR primers were compounded and purchased from Sangon Biological (Shanghai, China). Gene expression was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for mRNA. The expression of target genes was quantified by qRT-PCR by using One Step SYBR Prime Script RT-PCR Kit II (Takara) on an ABI StepOne Real-Time PCR System (Life Technologies) with conventional protocols. The 2^△△Ct method was used to calculate the expression of target genes relative to the reference gene GAPDH.

Western blot analysis

Total protein lysates were prepared from left ventricles (n=6, per group) as previously described [32]. Isolation of nuclear protein was performed according to the manufacturer’s instructions using Nuclear Extraction Kit from Merck. Individual protein lysates (10 mg/lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (7.5%, 15%, or gradient 4~20%; Bio-Rad and Pierce) and transferred to nitrocellulose membranes (GE Healthcare Amersham Biosciences, Oslo, Norway). After incubation in SuperBlock T20 PBS Blocking Buffer (Thermo Fisher Scientific Inc, Waltham, Mass), the membranes were incubated with individual antibodies according to manufacturer instructions. Antibodies for protein detection were as follows: atrial natriuretic peptide (ANP) (Santa Cruz, CA, USA, dilution 1:200), brain natriuretic peptide (BNP) (Abcam, UK, dilution 1:1000), IкBα (Santa Cruz, CA, USA, dilution 1:200), NF-κB p65 (Santa Cruz, CA, USA, dilution 1:200), limin B2 (Abcam, UK, dilution 1:1000) and interleukin 2 (IL2) (Santa Cruz, CA, USA, dilution 1:200). Blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (EarthOx LLC, CA, USA; dilution 1:5000) and developed with ECL (GE Healthcare Biosciences). Images were acquired and analyzed using a BioDocIt Imaging System (UVP, USA). Relative amounts of proteins were expressed as the percent increase over sham values.

Statistics

Data were expressed as the means ± standard deviation (means ± SD). All computations were carried out using the SPSS version 18.0 software for Windows (SPSS Inc, IL, USA). The statistical significance of differences among experimental groups was evaluated by two-way analysis of variance (ANOVA). When the ANOVA revealed significant between-group differences, post hoc analysis was performed using Bonferroni’s method of multiple comparisons. Single between-group comparisons were made by Student’s t-tests. A two-tailed P value of 0.05 was considered statistically significant.

Results

Elimination and mortality rate of the experimental animals

At EP stage, 2 rats (6.7%) were eliminated because they could not adapt to the treadmill exercise training. Four weeks after operation, the mortality rate of TAC and EP+TAC group were 13.3% (2/15) and 7.1% (1/14), respec-

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>5’- GCCATCACTGCACTCACGA-3’</td>
<td>5’- GCCATGTCAGATCCACAACG-3’</td>
</tr>
<tr>
<td>ANP</td>
<td>5’- AGAGAGTGAAGGCCAGAG-3’</td>
<td>5’- TGGACACCGCCTGATACG-3’</td>
</tr>
<tr>
<td>BNP</td>
<td>5’- CCAGTCCAGAGAGACTCTC-3’</td>
<td>5’- TCTCGAGCCAGGTCTTC-3’</td>
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Table 1. Primers for quantitative real-time polymerase chain reaction
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tively, and the mortality rate went up separately to 33.3% (5/15) and 21.4% (3/14) at 8 wks after TAC. There was not death in sham group.

**EP attenuated pathological cardiac hypertrophy induced by pressure overload**

At 4 wks after TAC, there were no significant differences in heart rate and body weight (BW) between TAC and EP+TAC rats (Figure 1A). The parameters for assessing cardiac hypertrophy, including LV wall thickness in end diastole (LVWd), heart rise, HW/BW, HW/TL (Figure 1B) and cross-sectional area of cardiomyocytes (CSA) (Figure 1C) were increased, meanwhile LV internal dimension at end-diastole (LVIDd), systolic function (Figure 1A) were decreased in TAC rats, however, this increases were signifi-
Figure 2. Examination of ANP and BNP expression levels in the LV myocardium. The expressions of ANP and BNP were confirmed by qPCR and Western blot. 

A: The mRNA expression of ANP was quantified as the ratio of ANP to GAPDH and expressed as 100% of sham. 

B: Representative photographs of the protein expression.
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of ANP; β-actin in whole cell lysate used as the loading control. C: The mRNA expression of BNP was quantified as the ratio of BNP to GAPDH and expressed as 100% of sham. D: Representative photographs of the protein expression of BNP; β-actin in whole cell lysate used as the loading control. Data were shown as mean ± SE from three individual experiments *P < 0.05, **P < 0.01.

Figure 3. The effect of EP on myocardial NF-κB signaling pathway in rats subject to TAC. A: Detection of cell lysate IκBα expression by Western blot; representative photographs of the protein expression of IκBα; expression of IκBα was quantified as the ratio of IκBα to β-actin and expressed as 100% of sham. B: Detection of cytoplasm NF-κB p65 subunit expression by Western blot; cytoplasm NF-κB p65 subunit levels; expression of NF-κB p65 subunit was quantified as the ratio of NF-κB p65 to β-actin and expressed as 100% of sham. C: Detection of nuclear NF-κB p65 subunit expression by Western blot; nuclear NF-κB p65 subunit levels; expression of NF-κB p65 subunit was quantified as the ratio of NF-κB p65 to lamin B2 and expressed as 100% of sham. D: Detection of cell lysate IL2 expression by Western blot; representative photographs of the protein expression of IL2; expression of IL2 was quantified as the ratio of IL2 to β-actin and expressed as 100% of sham. Data were shown as mean ± SE from six individual experiments *P < 0.05, **P < 0.01.
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significantly attenuated by EP treatment. At 8 wks after TAC, compared with sham group, the above parameters for assessing cardiac hypertrophy of two surgical groups were increased ($P < 0.05$), while, between TAC and EP+TAC group, these parameters revealed no significant differences.

**Effects of EP on the ventricular myocardial expression of fetal genes in the hypertrophic hearts**

Many genes such as ANP and BNP, which are expressed in fetal heart, are no longer expressed in the adult heart. The expression of these so-called fetal genes is reactivated when the heart undergoes pathological insults such as pathological cardiac hypertrophy. The reactivation of the fetal gene program has been extensively used as an important marker at the gene expression level for cardiac pathological responses. Hence, we evaluated the level of ANP and BNP expression by quantitative RT-PCR and Western blotting. QRT-PCR and Western blotting were thus performed to assess the effects of EP on the relative expression levels of ANP and BNP mRNA and protein in the cardiac tissue. Compared with sham group, TAC induced increases in the mRNA and protein levels of both ANP (Figure 2A, 2B) and BNP (Figure 2C, 2D) and the increases were considerably less in EP+TAC at 4 wks after TAC ($P < 0.01$). At 8 wks after TAC, compared with the sham group, the mRNA and protein levels of both ANP and BNP of two surgical groups were increased ($P < 0.01$), however, the differences between TAC and EP+TAC group were decreased than those at 4 wks after TAC. These data suggest that EP can effectively suppress the reactivation of the fetal gene program induced by pressure overload, but the protective effect of EP to on pressure overload-induced pathological cardiac hypertrophy is not sustained over time.

**Effects of EP on the NF-κB pathway in the heart during pathological cardiac hypertrophy**

NF-κB was well known to act, in general, worsening cardiac remodeling or dysfunction by activation of proinflammatory pathway, previous studies showed that the activation of NF-κB is essential for the development of cardiac hypertrophy induced by pressure overload in vivo [33]. Our data show that compared with the sham group, myocardial IκBα protein levels were significantly decreased in the TAC group but were markedly increased in the EP+TAC group, (Figure 3A) at 4 weeks after TAC, but the differences between TAC and EP+TAC group were significantly decreased at 8 wks after TAC than those of at 4 wks after TAC.

To further investigate the effects of EP on the nuclear translocation of NF-κB in hypertrophic cardiac tissue, which is an important step in the activation of NF-κB pathway, NF-κB p65 subunit levels in the cytoplasm(Figure 3B) and nucleus(Figure 3C) were explored by western blotting analysis. At 4 wks after TAC, compared with sham rats, nuclear NF-κB p65 subunit levels were increased in the TAC, which were significantly inhibited by EP treatment. IL2 protein levels were significantly lower in the EP+TAC group compared with those in the sham or the TAC groups (Figure 3D).

**Discussion**

Pathological cardiac hypertrophy is an independent risk factor for cardiovascular morbidity and mortality in humans [34], hence, it is of high clinical significance to attenuate pathological cardiac hypertrophy. In this study, we demonstrated that EP can effectively reduce cardiac hypertrophic responses induced via TAC possibly through inhibition of degradation of IκB and blockade of the NF-κB pathway.

EP, as a physiologic favorable adaptation, increases the myocardial mechanical load, accompanied by a positive alteration in stroke volume and sympathetic nervous system activity. Moreover, exercise modulates important features of maladaptive cardiac remodeling induced by chronic pressure overload, resulting in an improved cardiac phenotype [35-38]. Here we show that LVWD, systolic function, LVIDd, HW/BW and HW/TL were remarkably increased during cardiac hypertrophy induced by TAC-triggered pressure overload at 4 wks after TAC, however, this increases were significantly attenuated by EP treatment. We also found that the differences of these parameters between TAC and EP+TAC group were decreased at 8 wks after operation. These data suggest that the effect of EP on pressure overload-induced pathological cardiac hypertrophy is significant in the early stage of pathological cardiac hypertrophy, but at 8 wks after operation, the protective effect was lost.
In the process of cardiac hypertrophy, several fetal genes, including ANP and BNP, are reacti-
vated. Some evidence suggests that ANP and BNP as cardiac failure marker has been increas-
ingly used in the diagnosis of cardiac failure, differencial diagnosis, risk stratification, prog-
osis and treatment guideline [39-43]. One proposed mechanism for increased BNP gene
expression in cardiac hypertrophy in vivo is the activation of the NF-κB pathway [44]. Our data
also showed that the relative expression levels of ANP and BNP mRNA and protein in the hyper-
trophic cardiac tissue were inhibited by EP treatment, especially in the early stages.
Furthermore, according to the above research, because of timeliness of EP, we find the effects
of EP on the TAC model were not sustained over time. However, confirmation of critical molecu-
lar mechanisms relative EP is essential for the development of novel and viable therapeutic
intervention strategies for patients with cardio-
vascular disease who are either unable or unwilling to undertake regular physical activity
intervention programs.

Numerous stimuli and signaling pathways can be involved in the process of cardiac hypertro-
phy, and the NF-κB pathway has been shown to mediate cardiac hypertrophy and maladaptive
remodeling [45]. It has been reported that persistent myocyte NF-κB p65 subunit activation in
cardiac failure exacerbates cardiac remodeling by imparting pro-inflammatory, pro-fibrotic,
and pro-apoptotic effects [21, 46]. In addition, NF-κB p65 subunit is a mainly classical signal
pathway, and plays an important role in the development of inflammation [14]. To better
understand the mechanism of EP on cardiac hypertrophy, we investigated the NF-κB path-
way in this research. TAC stimuli, activates IkK, the upstream kinase of IkB. Upon activation by
inflammatory factors, IkK phosphorylates IkB, causing rapid IkB polyubiquitination and degra-
dation by proteosomes, however, this abnor-
mality was reversed by exercise training [47].
Degradation of IkB allows the cytoplasmic
NF-κB to translocate into the nucleus where NF-κB activates its target genes such as IL2;
therefore, inhibition of degradation of IkB may block the NF-κB signaling pathway [48, 49]. We
discovered that myocardial protein levels of IkBo, a pivotal negative regulator of NF-κB acti-
vation, were decreased in rats subject to TAC but the decrease was effectively reduced by EP
treatment. Since EP suppressed degradation of
IkB in the pressure-overload early stage, we then verified changes in NF-κB signaling. An on-
off experiment suggested that among the two groups in the TAC cohort, the EP treated groups
showed a lower nuclear NF-κB p65 subunit protein level and a lower protein level of myocardial
IL2, an inflammatory cytokine that is stimulated by NF-κB activation [50]. Taken together,
these findings provide strong evidence that EP treatment inhibits the NF-κB signaling, including
the degradation of IkB, NF-κB p65 subunit activation and express of myocardial IL2 in rat
hearts undergoing cardiac hypertrophy induced by TAC, which may contribute to the antihyper-
trophy effects of EP, specifically in early patho-
logical cardiac hypertrophy.

In the present study, we demonstrates that EP
can effectively attenuate cardiac hypertrophic
responses induced by TAC through echocardi-
ographic, morphological and histological param-
eters for assessing cardiac hypertrophy and
the level of ANP and BNP expression, especially
in the early stage of pathological cardiac hyper-
trophy. Furthermore, we found the possibly
mechanism is that EP treatment inhibits the
NF-κB signaling, including the degradation of
IkB, NF-κB p65 subunit activation and expres-
son of myocardial IL2 in rat hearts undergoing cardiac hypertrophy induced by TAC. The beneficial
effects of regular physical activity on cardiovas-
cular health are well established. However, the
key molecular mechanisms underlying the cardio-
protective effects of exercise are not well
defined. Therefore, although the effects of EP
on NF-κB signaling in the TAC model were not
sustained over time, identification of critical
pathways that are activated by exercise may
lead to novel and viable therapeutic interven-
tion strategies.

This study has some limitations. First, a miss-
ing element is preoperative echocardiography
on these experimental animals. Second, there
is yet no scientific consensus in the critical
point of intensity and duration of EP protective
effect.

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**Disclosure of conflict of interest**

None.
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EP attenuates pathological cardiac hypertrophy


