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Original article

Chronic exercise downregulates myocardial myoglobin and attenuates nitrite reductase capacity during ischemia–reperfusion

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ABSTRACT

The infarct sparing effects of exercise are evident following both long-term and short-term training regimens. Here we compared the infarct-lowering effects of nitrite therapy, voluntary exercise, and the combination of both following myocardial ischemia–reperfusion (MI/R) injury. We also compared the degree to which each strategy increased cardiac nitrite levels, as well as the effects of each strategy on the nitrite reductase activity of the heart. Mice subjected to voluntary wheel running (VE) for 4 weeks displayed an 18% reduction in infarct size when compared to sedentary mice, whereas mice administered nitrite therapy (25 mg/L in drinking water) showed a 53% decrease. However, the combination of VE and nitrite exhibited no further protection than VE alone. Although the VE and nitrite therapy mice showed similar nitrite levels in the heart, cardiac nitrite reductase activity was significantly reduced in the VE mice. Additionally, the cardiac protein expression of myoglobin, a known nitrite reductase, was also reduced after VE. Further studies revealed that cardiac NFAT activity was lower after VE due to a decrease in calcineurin activity and an increase in GSK3β activity. These data suggest that VE downregulates cardiac myoglobin levels by inhibiting calcineurin/NFAT signaling. Additionally, these results suggest that the modest infarct sparing effects of VE are the result of a decrease in the hearts ability to reduce nitrite to nitric oxide during MI/R.

1. Introduction

Despite the well documented beneficial cardiovascular effects of exercise [1,2], the signaling mechanisms, particularly in the heart, that mediate these effects have not been fully elucidated [3]. However, growing evidence from animal studies suggest that exercise leads to the activation and/or upregulation of numerous proteins that are classically associated with cytoprotective signaling cascades [4]. For instance, exercise increases components of the endogenous antioxidant defenses, increases the expression of heat-shock proteins, activates AMP-activated protein kinase, increases the expression and activity of endothelial nitric oxide synthase (eNOS) resulting in an increase in nitric oxide (NO) levels, and activates ATP-sensitive potassium channels [5]. Additionally, we recently discovered a novel role for β_3-adrenergic receptors (β_3-ARs) in exercise-mediated cardioprotection, as we found that β_3-ARs play a critical role in regulating the phosphorylation of eNOS and the generation of NO in response to exercise [6]. Based on the existing evidence in the literature that the upregulation and/or activation of any of these proteins results in robust cardioprotection, it was rather surprising that our recent study found that 4 weeks of voluntary wheel running reduced infarct size by only 23% [6].

Previously, we reported that exercise increased the cardiac levels of the NO metabolites, nitrite and nitrosothiols, and that the generation and storage of these metabolites play an important role in the infarct sparing effects of exercise [6]. Nitrite is a storage reservoir of NO in blood and tissue that is reduced to NO during ischemia [7]. Nitrite reductase activity in mammalian tissue has been linked to several proteins including deoxyhemoglobin [8] and xanthine oxidase [9]. However, emerging evidence suggest that myoglobin is the major reductase in the heart [10,11]. Nitrosothiols, which are formed by the ubiquitous redox-related modification of cysteine thiols in a process known as nitrosylation, have emerged as one of the most important mechanisms by which NO imparts its cellular effects [12]. Previously we have reported that oral nitrite therapy provides significant cardioprotection (48% reduction in infarct size) against acute myocardial ischemia–reperfusion (I/R) injury by increasing the steady-state levels of both nitrite and nitrosothiols in the heart [13]. Based on this evidence, one can speculate that exercise training does not increase the cardiac levels of nitrite to the same extent as oral nitrite therapy and/or alters the ability of the heart to reduce nitrite to NO.

To address these issues, we investigated if supplementing mice with nitrite in their drinking water during voluntary exercise (VE) training could provide additional infarct sparing effects against acute myocardial

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I/R injury. Furthermore, we also compared the degree to which each strategy increased cardiac nitrite levels, as well as the effects of each strategy on the nitrite reductase activity of the heart. Here, we report that the nitrite reductase activity of the heart is diminished after VE due to a decrease in myoglobin levels. These results uncover a previously unrecognized mechanism to account for the modest infarct lowering effects of VE.

2. Materials and methods

2.1. Animals

Six strains of mice were utilized in this study: (1) Male C57BL6/J mice (Jackson Labs, Bar Harbor, ME; 8–10 weeks of age); (2) Male NFAT-luciferase reporter mice (10–12 weeks of age); (3) Male Calcineurin Aβ deficient, (4) Male NFATc2 deficient; (5) Male NFATc4 deficient; and (6) Male NFATc2/NFATc4 deficient mice. The generation of NFAT-luciferase reporter mice [14] has been described previously. The generation of Calcineurin Aβ [15], NFATc2 [16], NFATc4 [17], and NFATc2/NFATc4 [18] global non-conditional deficient mice have been described previously. All experimental procedures were approved by the Institute for Animal Care and Use Committee at Emory University School of Medicine and conformed to the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (NIH Publication No. 86-23, Revised 1996) and with federal and state regulations.

2.2. Materials

DETA/NO, 2,2′-(Hydroxynitrosohydrazino)bis-ethanamine (DETANO) was purchased from EMD Millipore. DETANO was dissolved in saline and administered using a 32-gauge needle at a dose of 0.5 mg/kg (final volume of 50 μL) as an injection into the LV lumen at the time of reperfusion. Saline was administered in the same manner for the vehicle groups.

2.3. Voluntary exercise protocol

Mice were placed in custom designed cages fitted with running wheels (Mini Mitter, Bend, OR) for a period up to 4 weeks. Running distances were monitored daily. After the exercise-training period, the running wheel was removed from the cage and the mice were allowed to rest for a 24-hour period before further experimentation was conducted.

2.4. Myocardial ischemia–reperfusion protocol and myocardial injury assessment

Surgical ligation of the left coronary artery (LCA), myocardial infarct size determination, and Troponin-I measurements were performed similar to methods described previously [19].

2.5. NO metabolite measurements

Nitrite concentrations were quantified by ion chromatography (ENO20 Analyzer, Eicom). Tissue nitrosothiol compounds were quantified using group specific reductive denitrosation by iodine–iodide with subsequent detection of the NO liberated by gas-phase chemiluminescence. Nitrosyl-heme (NO-heme) levels were determined by parallel injection of replicate aliquots of tissue homogenates into a solution of 0.05 M ferricyanide in PBS at pH 7.5 and 37 °C. All NO analysis procedures have been previously described in detail [13].

2.6. Nitrite reductase activity measurements

Tissue was homogenized in PBS. An aliquot of the homogenate was then placed in a reaction vessel connected to a NO chemiluminescence detector and purged with helium. NO generation was then measured in the presence and absence of nitrite (200 μM) as described previously [20]. In separate experiments, samples were incubated with myoglobin (25 μM) prior to placement in the reaction vessel. NO generation was then measured after the addition of nitrite.

2.7. Subcellular fractionation and western blot analysis

Subcellular Fractionation and Western blot analysis was performed as described previously [19].

2.8. Isolation of mRNA and Taqman qPCR

RNA was isolated using the RiboPure kit according to manufacturer's instructions (Ambion). Reverse transcription was performed in a standard fashion with QuantiTect Reverse Transcription Kit (QIAGEN) supplemented with DNase treatment. Taqman qPCR was carried out according to the manufacturer's instructions using probe sets for myoglobin and 18S. Analysis was carried out using the ΔΔ-CT method with 18S correction and reported as relative fold change versus sham.

2.9. NFAT luciferase activity

Luciferase enzymatic activity in heart extracts was measured with a commercially available kit (luciferase assay system, Promega Corp.).

2.10. Calcineurin activity assay

Calcineurin activity was measured in heart samples with a commercially available kit (Calcineurin Cellular Activity Assay Kit, Calbiochem) according to the manufacturer's instructions as previously described [21]. Calcineurin activity was measured as the dephosphorylation rate of a synthetic phosphopeptide substrate (RII peptide).

2.11. GSK-3β activity

The activity of GSK-3β was measured in nuclear fractions prepared from heart tissue. The samples were first immunoprecipitated with a specific anti-GSK-3β antibody (Cell Signaling). An aliquot of the immunoprecipitated samples was incubated in a reaction buffer containing 12.5 mM Tris–HCl (pH 7.5), 2.5 mM β-glycerophosphate, 1 mM dithiothreitol, 0.05 mM Na_3VO_4, 5 mM MgCl2, 0.0625 mM ATP, and 10 μg of GSM (GSK-3β synthetic substrate peptide). The rate of ADP formed from the incorporation of ATP in the synthetic peptide was then measured with the ADP-Glo Kinase Assay kit (Promega) according to the manufacturer's instructions. Activity was expressed as ADP generated (in picomoles) per minute per milligram of protein.

2.12. Xanthine oxidase activity

The activity of xanthine oxidase was measured in whole cell homogenates with a commercially available kit (Xanthine Oxidase Fluorometric Assay Kit, Cayman Chemical) according to the manufacturer's instructions.

2.13. Echocardiography

Echocardiography was performed with a Vevo 2100 (Visualsonics) as previously described [22].