Exercise Protects Against Myocardial Ischemia–Reperfusion Injury via Stimulation of $\beta_3$-Adrenergic Receptors and Increased Nitric Oxide Signaling: Role of Nitrite and Nitrosothiols


Rationale: Exercise training confers sustainable protection against ischemia–reperfusion injury in animal models and has been associated with improved survival following a heart attack in humans. It is still unclear how exercise protects the heart, but it is apparent that endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) play a role.

Objective: To determine the role of $\beta_3$-adrenergic receptors ($\beta_3$-ARs), eNOS activation, and NO metabolites (nitrite and nitrosothiols) in the sustained cardioprotective effects of exercise.

Methods and Results: Here we show that voluntary exercise reduces myocardial injury in mice following a 4-week training period and that these protective effects can be sustained for at least 1 week following the cessation of the training. The sustained cardioprotective effects of exercise are mediated by alterations in the phosphorylation status of eNOS (increase in serine 1177 and decrease in threonine 495), leading to an increase in NO generation and storage of NO metabolites (nitrite and nitrosothiols) in the heart. Further evidence revealed that the alterations in eNOS phosphorylation status and NO generation were mediated by $\beta_3$-AR stimulation and that in response to exercise a deficiency of $\beta_3$-ARs leads to an exacerbation of myocardial infarction following ischemia–reperfusion injury.

Conclusions: Our findings clearly demonstrate that exercise protects the heart against myocardial ischemia–reperfusion injury by stimulation of $\beta_3$-ARs and increased cardiac storage of nitric oxide metabolites (ie, nitrite and nitrosothiols). (Circ Res. 2011;108:1448-1458.)

Key Words: $\beta_3$-adrenergic receptor ■ nitric oxide ■ cardioprotection ■ exercise ■ nitrite ■ nitrosothiol

Exercise training reduces many risk factors related to cardiovascular disease. Exercise also consistently provides sustainable protection against myocardial infarction in animal models and is associated with improved survival following ischemic insults in humans. The acute cardioprotective effects of exercise have been attributed to an increase in a number of classical preconditioning molecules, such as catalase, heat shock proteins (HSPs), and adenosine-5'-triphosphate (ATP)-sensitive potassium channels. Interestingly, the cardioprotective effects of exercise are not confined to the period of exercise, as it has been reported that protection is sustained against experimental myocardial ischemia–reperfusion (MI/R) injury for as long as 9 days after the cessation of the training. The mechanism or mechanisms responsible for this sustained protection are currently not known, but it is known that HSPs and catalase do not play a role since their upregulation wanes by 7 to 9 days, suggesting that some other cardioprotective molecule or molecules are responsible for this sustained protection.

Previous studies have suggested a role for endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) in exercise-mediated cardioprotection, as it has been reported that plasma levels of the NO metabolites, nitrite and nitrosothiols, increase during exercise in both rodents and humans. Nitrite represents a critically important storage reservoir of NO in blood and tissues that can readily be reduced to NO and nitrosothiols during ischemia or hypoxia. The circulating levels of both metabolites directly regulate their tissue stor-
Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>AAR</td>
<td>area at risk</td>
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<td>β-AR</td>
<td>β-adrenergic receptor</td>
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<td>β1-AR</td>
<td>β1-adrenergic receptor</td>
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<td>β2-AR</td>
<td>β2-adrenergic receptor</td>
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<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<tr>
<td>eNOS-PSer1177</td>
<td>eNOS phosphorylated at serine residue 1177</td>
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<tr>
<td>eNOS-PThr495</td>
<td>eNOS phosphorylated at threonine residue 495</td>
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<td>HSPs</td>
<td>heat shock proteins</td>
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<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
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<td>INF</td>
<td>infarct size</td>
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<td>LCA</td>
<td>left coronary artery</td>
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<td>MI/R</td>
<td>myocardial ischemia–reperfusion</td>
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<td>NO</td>
<td>nitric oxide</td>
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<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
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<td>RSNO</td>
<td>s-nitrosothiol</td>
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<tr>
<td>RXNO</td>
<td>nitrosothiol</td>
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<tr>
<td>SED</td>
<td>sedentary</td>
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<td>SOD</td>
<td>superoxide dismutase</td>
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<td>VE</td>
<td>voluntary exercise</td>
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age,7 and increasing the cardiac levels of both nitrite and nitrosothiols is an effective cardioprotective strategy.8,9 On the basis of this evidence, one can speculate that increasing the levels of NO metabolites during exercise may contribute to its cardioprotective effects. However, it is currently not known whether exercise can augment the cardiac levels of NO metabolites.

An additional question that remains to be fully answered relates to the molecular mechanisms that lead to the activation of eNOS during exercise. Recently, the β1-adrenergic receptor (β1-AR) has emerged as a potential target for the treatment of cardiovascular diseases including hypertension, acute MI, and heart failure.10 This is partially related to the evidence indicating that its stimulation increases eNOS activity and NO bioavailability.11 During exercise, there is an increase in β1-AR stimulus (ie, catecholamines),12 making the β1-AR a possible source for eNOS activation. However, the role that β1-ARs play in mediating the cardioprotective effects of exercise is currently unknown.

To address these issues, we examined the protective effects of voluntary exercise (VE) training in an established in vivo mouse model of MI/R injury. Specifically, we investigated whether NO metabolites formed during exercise were stored in the heart and whether they contributed to the sustained cardioprotective effects of exercise. Additionally, we investigated the role that β2-ARs play in mediating the cardioprotective effects of exercise.

Methods

Animals

Male C57BL/6J mice (Jackson Labs, Bar Harbor, ME; 8 to 10 weeks of age) were utilized. The eNOS deficient mice (eNOS−/−; 8 to 10 weeks of age) on a C57BL/6J background13 and β2-AR-deficient receptor (β2-AR−/−; 8 to 10 weeks of age) mice,14 as well as littermate controls, were also utilized. The β-AR−/− mice were developed on a FVB background and backcrossed 9 generations to a C57BL/6J background. 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.

Subjects and Study Procedures

All procedures with human subjects were approved by the Institutional Review Board (Human Research Committee) of the University of Colorado at Boulder. The nature, benefits, and risks of the study were explained to the volunteers, and their written informed consent was obtained before participation. A total of 23 healthy young (ages 18 to 31 years) men were studied: 16 non-exercise–trained individuals and 7 endurance athletes. The nonexercise-trained subjects had performed no regular exercise for >2 years, whereas the trained endurance athletes performed >3 sessions per week of vigorous aerobic-endurance exercise for >2 years.

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, 1-week, or 4-week period before further experimentation was conducted.

Myocardial Ischemia–Reperfusion (I/R) 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.15

Western Blot Analysis

Western blot analysis was performed as described previously.15

Analysis of Nitrite, Nitrate, and Nitrosothiols

Nitrite and nitrate concentrations were quantified by ion chromatography (ENO20 Analyzer, Eicom, Dayton, OH). Tissue nitrosothiol compounds were quantified using group-specific reductive denitrosation by iodine-iodide with subsequent detection of the NO liberated by gas-phase chemiluminescence. All NO analysis procedures have been previously described in detail.8

Analysis of Catecholamine Levels

Catecholamines were measured in blood samples taken from mice using the Bi-CAT Elisa (ALPCO, Salem, NH) according to the manufacturer’s instructions.

Statistical Analysis

All the data in this study are expressed as mean±standard error (SEM). Differences in data between the groups were compared using Prism 4 (GraphPad Software, Inc., La Jolla, CA) with Student paired 2-tailed t test, 1-way analysis of variance (ANOVA), or 2-way ANOVA (comparison of results from experiments using eNOS−/− and β2-AR−/− mice). For the 1-way and 2-way ANOVAs, if a significant variance was found, the Tukey or Bonferroni test was used as the post hoc analysis. A probability value less than 0.05 was considered statistically significant.

Results

VE Training Reduces Infarct Size Following MI/R Injury

To determine whether VE training attenuated myocardial injury following myocardial I/R, we housed groups of mice in
cages fitted with running wheels and allowed them to exercise voluntarily for 4 weeks (VE 4 weeks). The intensity of the exercise training remained constant throughout the 4-week training period with an average of 7.4±0.2 km/d (Online Figure I). Control mice (sedentary, SED) were housed in cages without running wheels for the same durations as were the VE mice. At the end of the 4-week training period, the mice were subjected to 45 minutes of left coronary artery occlusion, followed by 24 hours of reperfusion, at which time myocardial injury was assessed by determining infarct size (INF) and measuring circulating levels of troponin-I. The VE mice displayed a 23% reduction (P<0.05 versus SED) in INF in relation to the area at risk (AAR) (58.6±3.6% for SED versus 45.2±3.3% for VE 4 weeks) and a 39% (39.6±3.7 ng/mL for SED versus 24.0±2.7 ng/mL for VE 4 weeks) reduction in circulating troponin-I levels (Figure 1B and 1C).

We next evaluated whether the cardioprotective effects of VE training could be maintained after the mice stopped exercising. For these experiments, mice were allowed to exercise for 4 weeks (average running distance of 7.3±0.2 km/d, Online Figure I). At the end of the training period, the mice were removed from the cages for a period of 1 week (VE 4 weeks+1 week SED) and then subjected to 45 minutes of left coronary artery occlusion followed by 24 hours of reperfusion. The VE 4 weeks+1 week SED mice displayed a 30% reduction (P<0.01 versus SED; Figure 1B) in INF in relation to the AAR and a 37% reduction (P<0.05 versus SED; Figure 1C) in circulating troponin-I levels. This demonstrates that VE training provides significant cardioprotection against I/R injury for at least 1 week following the cessation of the training.

VE Training Increases CuZnSOD Expression and Alters the Phosphorylation Status of AMPK

We next investigated whether VE training altered the expression of several cardioprotective signaling molecules purported to play a role in mediating exercise-induced cardioprotection. Western blot analysis (Figure 2) revealed that the expression of HSP70 remained unchanged in the hearts of mice following VE training when compared with SED mice. However, a significant increase in CuZnSOD was observed in the hearts of the VE 4-week group (P<0.05 versus SED), but not in the hearts of the VE 4 weeks+1 week SED group. AMP-activated protein kinase (AMPK) is another cardioprotective signaling molecule that is activated by exercise. We next studied the effects of VE on eNOS expression and Metabolite Levels

VE Training Alters the Phosphorylation Status of eNOS and Increases Circulating and Cardiac NO
Exercise has been associated with increasing plasma nitrite levels, but it is not known whether exercise increases nitrite levels in the heart. Because we have previously demonstrated that increasing nitrite levels in the heart results in cardioprotection, we evaluated the effects of exercise training on the levels of nitrite, nitrate, and nitrosothiols (RXNO and RSNO) in the plasma and heart. As shown in Figure 4A through 4F, a significant increase in the levels of nitrite and nitrosothiols were observed in the plasma and hearts of the VE 4-week group when compared with the SED group. Importantly, these elevations were still present 1 week after the end of the training.
Interestingly, plasma and heart nitrate levels were only significantly increased 1 week after the end of the training period (\( P < 0.05 \) versus SED). These results suggest that NO metabolites may be responsible for the sustained cardioprotective effects of VE training, given that nitrite, nitrate, and nitrosothiols have all been shown to provide cardioprotection during myocardial ischemia by increasing NO levels and signaling.\(^8,9,18\)

We next investigated whether eNOS was critical for the cardioprotection afforded by VE training. Mice deficient in eNOS (eNOS\(^{-/-}\)) were subjected to 4 weeks of VE training followed by MI/R (Figure 4G). Analysis revealed that VE training did not abrogate myocardial infarct size in eNOS\(^{-/-}\) mice (\( P = \text{N.S.} \) [not significant] versus SED), suggesting that eNOS plays an important role in the cardioprotective actions of VE. Additionally, the eNOS\(^{-/-}\) mice did not exercise to the same extent as the C57BL/6j wild-type mice exercised (Online Figure I, 4.0\( \pm 0.9 \) versus 7.4\( \pm 0.2 \) km/d, \( P < 0.001 \)), suggesting that eNOS is important for exercise training.

**VE Increases the Expression of eNOS and Increases the Levels of NO Metabolites in the Skeletal Muscle**

Because VE involves changes in blood flow in active muscles, it is conceivable that the circulating levels of NO metabolites are derived from additional sources other than the heart. Therefore, we evaluated whether VE could alter the expression of eNOS in another working muscle, the gastrocnemius. As is shown in Online Figure II, the expression of eNOS was found to be significantly increased at the end of the 4-week training period (VE 4 weeks; \( P < 0.01 \) versus SED), but had returned to control levels 1 week after the cessation of exercise (VE 4 weeks +1 week SED). We also found that there was no difference in the expression of eNOS-P\(^{\text{Ser177}}\) in either group of exercised mice when the expression was compared with the expression of total eNOS. However, there was a significant increase in eNOS-P\(^{\text{Ser177}}\) in the VE 4-week group when the expression was compared with the expression of \( \alpha \)-tubulin (\( P < 0.05 \) versus SED), suggesting that there was more eNOS-P\(^{\text{Ser177}}\) after VE.
Additionally, a significant decrease in the expression of eNOS-PThr495 in the gastrocnemius of the VE 4-week group (P < 0.05 versus SED), but not the VE 4 weeks +1 week SED group was observed. VE also significantly increased the levels of nitrite and nitrosothiols in the skeletal muscle of the VE 4-week group (Online Figure III; P < 0.01 versus SED). However, unlike the observation in the heart, the levels of nitrite and nitrosothiols in the skeletal muscle declined back to near baseline levels at 1 week after the end of the VE period. In contrast, nitrate levels were significantly higher 1 week after the end of VE training (P < 0.05 versus SED).

Exercise Increases Circulating Levels of Nitrosothiols in Trained Endurance Athletes

Additional studies were also performed to determine whether VE training increased the steady-state levels of NO metabolites in human athletes. Blood samples were taken from individuals who exercised for at least 45 minutes a day, >3 times a week, for at least 2 years (trained endurance athletes) and from nontrained individuals. Subject characteristics are shown in Online Table I. Analysis revealed that trained endurance athletes (age 24 ± 2, n = 7) and nontrained individuals (age 27 ± 1, n = 16) had similar plasma levels of nitrite and nitrate (Figure 4H). In contrast, trained endurance athletes were found to have significantly higher (P = 0.014) plasma nitrosothiol levels (15 ± 4 versus 7 ± 1 nmol/L versus nontrained individuals, Figure 4I).

VE Increases the Levels of Circulating Catecholamines and Increases the Expression of the β3-AR

Recent studies have indicated an upregulation in the density of β3-ARs19 and an increase in β3-AR stimulants (ie, catecholamines)12 in response to exercise training. As shown in Figure 5A, we found that VE significantly (P < 0.05 versus SED) increased the circulating levels of epinephrine and norepinephrine at the end of the training period. However, both had returned to normal levels 1 week after the end of the training. Furthermore, Western blot analysis revealed (Figure 5B and 5C) a significant increase in the protein expression of cardiac β3-AR in the hearts of the VE 4-week group (P < 0.05 versus SED), but not in the hearts of the VE 4 weeks +1 week SED group. No changes in the expression of β1-AR and β2-AR were noted in response to VE training. Given that β3-AR stimulation results in the production of NO from eNOS,20 we next examined the role that β3-ARs play in mediating the generation of NO metabolites during VE, as well as the role that they play in mediating the cardioprotective effects of VE. First, we examined the effects of VE on eNOS expression and phosphorylation status in mice deficient in β3-AR (β3-AR−/−). VE training promoted a significant decrease (Figure 6A and 6B) in the expression of eNOS-PSer1177 (P < 0.05 versus SED), as well as a significant decrease in the expression of nNOS (P < 0.001 versus SED). No changes in the expression of eNOS, eNOS-PThr495, or iNOS were noted. As a result of these alterations, VE failed...
to increase the plasma or heart levels of nitrite, nitrate, and nitrosothiols in the β3-AR−/- mice (Figure 6C through 6E).

We then evaluated how these alterations would affect VE-mediated cardioprotection. Beta3-AR−/- mice were subjected to 4 weeks of VE training followed by MI/R (Figure 6F). We found that the deficiency of β3-AR resulted in a significant increase in myocardial injury in response to VE training, as seen by a 40% increase in INF/AAR (39.2 ± 4.2 versus 54.8 ± 3.7, P < 0.05) and a 58% increase in INF/LV (18.9 ± 2.2 versus 29.9 ± 2.8, P < 0.05). Additionally, the β3-AR−/−
mice did not exercise to the same extent as the C57BL/6J wild-type mice exercised (Online Figure I, P<0.001), suggesting that a deficiency in β_{2}-ARs dampens the ability of mice to exercise.

Additional experiments were performed to further demonstrate a relationship between catecholamines and eNOS/NO. For these experiments, we studied the effects of a single injection of epinephrine on eNOS expression and phosphorylation status, as well as the levels of plasma and cardiac NO. Epinephrine dose dependently increased the heart rate and cardiac contractility of mice at concentrations ranging from 0.050 μg/kg to 10 μg/kg (Online Figure IVA and IVB). Using the higher dose (10 μg/kg), we found that epinephrine increased the expression of eNOS-P-Ser1177 in a time-dependent manner (Online Figure VA and VB). These data suggest that β-AR stimulation rapidly induces changes in eNOS phosphorylation, resulting in an increase in NO bioavailability.

The Sustained Cardioprotective Effects of VE Are Lost When NO Metabolites Return to Normal Levels

Additional experiments were performed to determine whether the sustained cardioprotective effects of VE were present at a time when NO metabolites had returned to normal levels. For these experiments, mice were allowed to exercise for 1 week and then removed from the cages for a period of either 1 week (VE 1 week +1 week SED) or 4 weeks (VE 1 week +4 weeks SED). Mice in the VE 1 week +1 week SED group displayed a significant increase in the levels of nitrite, nitrate, and nitrosothiols in both the plasma and heart (P<0.05 versus SED; Figure 7A through 7C). In contrast, mice in the VE 1 week +4 weeks SED group displayed no changes in plasma or heart NO metabolite levels when compared with SED mice, suggesting that the VE-induced alterations in eNOS/NO had returned to baseline levels 4 weeks after the end of the training period. Additionally, following MI/R, the VE 1 week +1 week SED mice displayed a 31% reduction (P<0.05 versus SED; Figure 7D) in INF in relation to the AAR when compared with the SED control mice. In contrast, the VE 1 week +4 weeks SED mice were not protected against MI/R injury, suggesting that the sustained cardioprotective effects of exercise are lost when NO metabolite levels are not elevated.

Discussion

The endothelium plays a critical role in mediating the protective effects associated with exercise. Specifically, increased vascular wall shear stress induced by exercise increases the expression and activity of vascular eNOS, which subsequently increases the production and bioavailability of NO throughout the body.21,22 In the current study, we not only confirmed this finding but also found that exercise altered the expression and phosphorylation status of eNOS in a very tissue-specific manner. In the heart, we found that exercise increased the expression of eNOS-P-Thr495 and decreased the expression of eNOS-P-Thr495 without altering the expression of total eNOS, whereas in the skeletal muscle, exercise increased the expression of total eNOS and decreased the expression of eNOS-P-Thr495. Furthermore, the degree to which exercise mediated these changes was also found to be tissue specific, because the alterations observed in the heart persisted for at least 1 week after the end of the training period, whereas those changes observed in the skeletal muscle returned to baseline levels at this time point. This suggests that the mechanism or mechanisms responsible for the alterations in eNOS may also be tissue specific.

Importantly, the observed alterations in eNOS resulted in an increase in NO bioavailability, as evidenced by an increase in the plasma, skeletal muscle, and cardiac levels of nitrite and nitrosothiols. Circulating nitrite and nitrosothiol levels have traditionally been considered an acute marker of NO production and surrogate for endothelial function and cardiovascular health status.23 As such, their role in mediating the cardioprotective effects of exercise has not been investigated. Given the recent paradigm shift in NO biology, the role of these NO metabolites in exercise should be reconsidered. In the current study, we for the first time provide strong evidence that the generation and storage of nitrite and nitrosothiols plays a role in mediating the acute and sustained cardioprotective effects of exercise. First, we report that 4 weeks of VE training increased the levels of nitrite and nitrosothiols in the plasma, skeletal muscle, and heart in mice. Given that nitrite is reduced to NO during myocardial I/R8 and that nitrosothiols act as a redox-sensitive NO donor,23 it can be suggested that some of the acute cardioprotective effects of VE can be attributed to both nitrite and nitrosothiols. Second, we found that nitrite, nitrate, and nitrosothiols were stored in the heart for 1 week after the cessation of VE. Because nitrate can also be reduced to nitrite, which can then subsequently be reduced to NO,24 it is probable that all 3 of these NO metabolites play a role in mediating the sustained cardioprotective effects of VE. This is further supported by the observation that the cardiac expression of CuZnSOD and AMPK were unchanged when compared with the SED control mice at this same time point. Third, we found that the cardioprotective effects of exercise did not extend to 4 weeks after the cessation of the training period when the levels of nitrite and nitrosothiols in both the plasma and heart had returned to baseline levels. Fourth, we found that nitrosothiol levels were increased in the plasma of trained endurance athletes. Although these data do not offer any insights into storage levels of NO metabolites in the heart, they do provide evidence that VE increases the circulating levels of nitrosothiols in humans and provide a clinical basis to support the experimental findings of the current study.

Accumulating evidence indicates that nitrosothiols (S-nitrosothiols in particular) play an important role both in normal physiological processes and in a broad spectrum of human diseases. Nitrosothiols, which are formed by the ubiquitous redox-related modification of cysteine thiols in a process known as nitrosylation, have emerged as the most important mechanisms by which NO imparts its cellular effects.24 With regard to cardiovascular physiology, protein S-nitrosylation can influence cardiac contractility through the
regulation of β-AR signaling and calcium cycling.25–27 Additionally, increasing the S-nitrosylation of proteins in the heart can reduce I/R injury by preventing the irreversible oxidation of proteins during early reperfusion,9 by inhibiting apoptosis28 and inflammation,29 by influencing blood flow and oxygen delivery,30 and by modulating angiogenesis.31 On the basis of this evidence, the findings of the current study suggest that nitrosothiols may be a more significant indicator of NO-mediated protection during exercise when compared with the other NO metabolites, given that both NO and nitrite can covalently modify a reactive cysteine to form a nitrosothiol.7,32

Another major finding of the current study relates to the role of β3-ARs in exercise. In the heart, 3 populations of β-ARs potentially modulate cardiac function (β1-AR, β2-AR, and β3-AR).20 These different subtypes belong to the G protein-coupled receptor superfamily and modulate cardiac function after stimulation by catecholamines.33 The effects of β1-AR and β2-AR are well established both in human and other mammals, because their stimulation produces positive chronotropic and inotropic effects. Although the precise physiological and pathophysiological roles of β3-ARs remain uncertain, recent observations suggests that β3-AR stimulation produces a negative inotropic effect via the production of NO from eNOS.20 The activation of eNOS during exercise can be caused by shear stress, inducing a signaling cascade involving Akt (protein kinase B), protein kinase A (PKA), or AMPK.34,35 A recent study reported that inhibition of Akt signaling with wortmannin blunts the increase in the vascular expression of eNOS-PSer1177 in mice subjected to treadmill running without altering the expression of phosphorylated CREB (PKA signaling) or AMPK.34 Because wortmannin did not completely attenuate the increase in the expression of eNOS-PSer1177, these data simply suggest that Akt plays a major role in regulating this phosphorylation. This also suggests that other signaling molecules regulate the expression of eNOS-PSer1177 during exercise.

Here, we provide strong evidence that β3-ARs are involved in this process. First, in agreement with other studies,12 we found that 4 weeks of VE significantly increased the circulating levels of epinephrine and norepinephrine, as well as the protein expression of cardiac β3-ARs. Previously in cultured cells, it has been reported that epinephrine can rapidly increase the expression of eNOS-PSer1177 in a β3-AR-dependent manner.36 We also found that acute injections of epinephrine rapidly increased the expression of eNOS-PSer1177 and the levels of NO metabolites in both the plasma and heart. Although these studies do not provide direct evidence that β3-AR stimulation can increase the expression of eNOS-PSer1177, it does suggest that epinephrine can modulate eNOS/NO levels in vivo. However, a direct relationship was demonstrated with our studies using β3-AR-deficient mice. On the basis of the existing literature, we predicted that a deficiency in β3-ARs would dampen the increase in the expression of eNOS-PSer1177 in response to exercise in a similar manner as that reported for wortmannin. Interestingly, we found that in response to exercise, the increase in the expression of eNOS-PSer1177 was not simply blunted in the hearts of β3-AR-deficient mice, but rather its expression was significantly decreased when compared with sedentary control levels. Additionally, exercise induced a significant decline in the expression of nNOS in the hearts of β3-AR deficient mice. This was also a rather surprising finding, given that nNOS levels did not change in the hearts of wild-type mice that exercised but supportive of the recent evidence indicating that β3-AR stimulation can also regulate nNOS.11 As a result of this downregulation, exercise failed to increase the plasma or heart levels of nitrate, nitrite, and nitrosothiols in the β3-AR−/− mice. Importantly, in response to exercise, a deficiency of β3-ARs leads to an exacerbation of myocardial infarction following ischemia–reperfusion injury. Taken together, these results suggest that β3-ARs not only play a major role in regulating the phosphorylation of eNOS at serine 1177, as well as maintaining the basal expression of myocardial nNOS during exercise, but also are necessary for regulating the production of NO during exercise and for mediating the cardioprotective effects of exercise.

Although it is not fully known how β3-ARs regulate the expression of eNOS, it is known that interfering with this regulation can lead to detrimental effects. Previously, it has been reported that lack of β3-AR signaling exacerbates cardiac pressure-overload–induced remodeling by enhancing eNOS uncoupling and increasing superoxide production.37 It is currently unknown whether exercise causes eNOS to become uncoupled in the hearts of β3-AR−/−. However, the uncoupling of eNOS would certainly provide an explanation as to why the β3-AR−/− mice in the current study displayed exacerbated myocardial injury following exercise training. This would also, in part, explain the different response to MI/R injury between the eNOS−/− and β3-AR−/− mice, given that the eNOS−/− could not experience eNOS uncoupling and subsequent superoxide production.

In summary, the current study demonstrates that 4 weeks of VE training provides acute and sustained cardioprotection against MI/R injury. Additionally, the current study provides evidence that NO metabolites play a major role in the observed sustained cardioprotective effects and provides novel evidence suggesting a role for β3-ARs in exercise-mediated cardioprotection. On the basis of these results, we propose that during exercise sympathetic stimulation via the β3-AR leads to the activation of eNOS, resulting in an increase in the production of NO. The NO generated would then have 2 fates. It can either be used to induce vasodilatation38 to match blood flow to metabolic demands39 or be metabolized into nitrite and nitrosothiols, which can then be stored in the heart and circulation. This can continue with each passing exercise period until the steady-state levels of the metabolites in the heart are elevated above normal baseline levels. This would be analogous to the effects of oral nitrite supplementation on the heart that was observed in a previous study.8 Increasing these stores in the heart prior to myocardial ischemia is important because the bioavailability of NO is decreased during ischemia. The cause of this decrease is still not completely understood, but it has been suggested that NO levels are reduced during myocardial ischemia due to a decrease in production from eNOS because of low oxygen and diminished substrate delivery or an increase in ROS production.6,40 In any event, the stored nitrite
can be reduced to NO during myocardial ischemia by any of the identified nitrite reductases found in the heart, thereby providing an increase in the bioavailability of NO. The nitrosothiols can act as a reversible protective shield to prevent the irreversible oxidation of proteins during the early oxidative burst of reperfusion and act as a redox-sensitive NO donor. The increase in NO can then serve as a signaling molecule to protect the heart against MI/R injury.

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Disclosures
None.

References


**Novelty and Significance**

**What Is Known?**

- Exercise training reduces many risk factors associated with cardiovascular disease. It confers sustainable protection against myocardial infarction in animal models and improves survival following myocardial ischemia in humans.
- The cardioprotective effects of exercise are not confined to the period of exercise.
- The mechanism or mechanisms by which exercise protects the heart against myocardial ischemia–reperfusion injury are not completely understood, but it is apparent that endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) play a role.

**What New Information Does This Article Contribute?**

- Mice that engaged in 4 weeks of voluntary exercise training were protected against myocardial ischemia–reperfusion injury at the end of the training period and displayed sustained protection for 1 week following the cessation of the training period.
- Voluntary exercise increased the circulating levels of epinephrine and norepinephrine, as well as the abundance of cardiac beta3- adrenergic receptors (beta3-ARs).
- The beta3-AR-deficient mice were not protected against MI/R injury following exercise.

- The cardioprotective effects of exercise were in part mediated by alterations in the phosphorylation status of eNOS leading to an increase in cardiac NO metabolite (nitrite and nitrosothiols) levels.
- The alterations in eNOS phosphorylation status and NO generation were mediated in part by beta3-AR stimulation.
- The cardioprotective effects of exercise did not extend to 4 weeks after the cessation of the training period when the levels of nitrite and nitrosothiols in the heart had returned to baseline levels.

The NO metabolites nitrite and nitrosothiols are potent signaling molecules and potential therapies for ischemic disease. The present investigation clearly demonstrates that when mice exercise, there is an activation of cardiac eNOS and NO generation resulting in increased cardiac nitrite and nitrosothiol levels. Additionally, we report a novel role for cardiac beta3-ARs in exercise-mediated cardioprotection. Specifically, we found that beta3-ARs play a critical role in regulating the phosphorylation of eNOS and the generation of NO in response to exercise. Collectively, our results provide novel insights into the cardioprotective effects of exercise and may aid in the design of treatment modalities to treat patients who suffer from ischemic heart disease.
Exercise Protects Against Myocardial Ischemia–Reperfusion Injury via Stimulation of β3-Adrenergic Receptors and Increased Nitric Oxide Signaling: Role of Nitrite and Nitrosothiols

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SUPPLEMENTAL MATERIAL

Exercise Protects the Heart Against Ischemia-Reperfusion injury via stimulation of $\beta_3$-Adrenergic Receptors and Increased Cardiac Storage of Nitrite and Nitrosothiols

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Detailed Methods

Animals. Male C57BL6/J mice (Jackson Labs, Bar Harbor, ME), 8-10 weeks of age were utilized. eNOS deficient mice (eNOS<sup>−/−</sup>; C57BL6/J background)<sup>1</sup> and β<sub>3</sub> adrenergic receptor deficient (β<sub>3</sub>-AR<sup>−/−</sup>; 8-10 weeks of age) mice<sup>2</sup> as well as littermate controls were also utilized. The β<sub>3</sub>-AR<sup>−/−</sup> mice were developed on a FVB background and backcrossed 9 generations to a C57BL6/J background. All experimental mouse 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. The number of animals used for each experiment is depicted on each figure.

Subjects and blood collection. All procedures were approved by the Institutional Review Board (Human Research Committee) of the University of Colorado at Boulder. The nature, benefits, and risks of the study were explained to the volunteers, and their written informed consent was obtained before participation. A total of 23 healthy young (aged 18-31 years) men were studied: 16 non-exercise trained individuals and 7 endurance athletes. The non-exercise trained subjects had performed no regular exercise for ≥2 years, whereas the trained endurance athletes performed >3 sessions/week of vigorous aerobic-endurance exercise (at least 45 minutes) for >2 years. All of the men had plasma LDL-cholesterol <160mg/dl, resting blood pressure <140/90 mm Hg, were non-smokers, non-diabetic (fasting blood glucose <126 mg/dl), non-obese (body mass index<30kg/m<sup>2</sup>) and free of clinical diseases as assessed by medical history, physical examination, blood chemistry and resting ECG. Subjects were not taking medications and had not taken antioxidants (e.g., vitamins C and E) within 6 week of the study. Venous blood was collected into a 10mL EDTA Tube at the University of Colorado at Boulder Clinical Translational Research Center after a 12-hour fast and a 24-hr abstention from alcohol and physical activity. Blood was centrifuged at 400g for 20 minutes at 23°C. Plasma was aliquoted into 0.65 mL eppendorf tubes and stored in -80°C until analysis.

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, 1-week, or 4-week period before further experimentation was conducted.

Myocardial Ischemia-Reperfusion (I/R) Protocol. Prior to any surgical procedure, mice were anesthetized with intraperitoneal injections of ketamine (60 mg/kg) and sodium pentobarbital (20 mg/kg). Mice also received 200 Units/kg of sodium heparin via intraperitoneal injection before surgery to prevent clot formation and allow for consistent and complete reperfusion postligation. The mice were then attached to a surgical board with their ventral side up and orally intubated with polyethylene-60 (PE-60) tubing connected via loose junction to a rodent ventilator (MiniVent Type 845, Hugo-Sachs Elektronik) set at a tidal volume 240 µL of and a rate of 110 breaths per minute and supplemented with 100% oxygen (0.1-0.2 liters/minute flow rate) via a side port on the ventilator. Effective ventilation was visually confirmed by vapor condensation in the endotracheal tube and rhythmic rising of the chest. Mice were maintained at a constant temperature of 37°C with a heat pad warmed by a circulating water bath. Temperature was monitored via a rectal probe connected to a Digisense K-Type digital thermometer. Hair remover (i.e., Nair®) was placed on the chest with a cotton swab and then removed along with the chest hair. The exposed regions were wiped with alcohol and betadine solution. A midline incision was then made along the sternum exposing the ribcage. Next, a median sternotomy
was performed and the wound edges were cauterized with an electrocautery device. The proximal left coronary artery (LCA) was visually identified with the aid of an Olympus stereomicroscope with a fiber optic light source. The LCA was ligated with a 7-0 silk suture passed with a tapered BV-1 needle. A short segment of PE-10 tubing was placed between the LCA and the 7-0 silk suture to minimized damage to the coronary artery and allow for complete reperfusion following the ischemic period. During the ischemic period the incision was covered with parafilm creating an effective barrier against desiccation and dehydration. Following 45 minutes of LCA occlusion, the ligature was removed, and reperfusion was visually confirmed. The chest wall and skin incision was carefully closed in layers with a 4-0 BIOSYN suture (CV-23 tapered needle). Animal recovery was supplemented by 100% oxygen and butorphanol (0.15 mg/kg) analgesia as well as a single dose of the antibiotic Cefazolin (80 mg/kg) to prevent infection. In the surgical recovery area, a heat pad warmed by a circulating water bath was utilized to maintain the appropriate body temperature of the mice. In addition, food and water were made available immediately and for the remainder of the first 24 hours of recovery.

**Myocardial Infarct Size Determination.** At 24 hours of reperfusion, the mice were fully anesthetized as before, intubated, and connected to a rodent ventilator. A catheter (PE-10 tubing) was placed in the common carotid artery to allow for Evans Blue dye injection. A median sternotomy was performed and the LCA was re-ligated in the same location as before. Evans Blue dye (1.25 mL of a 7.0% solution, Sigma) was injected into the carotid artery catheter into the heart to delineate the ischemic zone from the non-ischemic zone. The heart was rapidly excised and serially sectioned along the long axis in five, 1 mm thick sections that were then incubated in 1.0% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) for 4 minutes at 37°C to demarcate the viable and nonviable myocardium within the risk zone. Each of the five, 1 mm thick myocardial slices were weighed and the areas of infarction, risk, and non-ischemic left ventricle were assessed by a blinded observer using computer-assisted planimetry (NIH Image 1.57). All of the procedures for the left ventricular area-at-risk and infarct size determination have been previously described.³

**Troponin I.** A blood sample (500 µL) was collected from mice prior to the Evans blue dye injection. Serum was obtained and the levels of the cardiac-specific isoform of Troponin-I (ng/mL) were assessed using an ELISA kit from Life Diagnostics (West Chester, PA).

**Protein Extraction.** Whole cell fractions were prepared as described previously.⁴,⁵ Briefly, frozen LV samples were powdered under liquid nitrogen with mortar and pestle prior to homogenization in 1 ml of ice-cold RIPA lysis buffer (Cell Signaling). Homogenates were then centrifuged at 1,300 g to remove any cellular debris. The pellet was discarded, and the supernatant was again centrifuged at 16,000 g for 30 min at 4°C. The resultant supernatant was collected. Protein concentrations of all cellular fractions were measured with the DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

**Western blot analysis.** Western blot analysis was performed as described previously.³ Equal amounts of protein were loaded into lanes of polyacrylamide-SDS gels. The gels were electrophoresed, followed by transfer of the protein to a PVDF membrane. The membrane was then blocked and probed with primary antibodies overnight at 4°C. The following primary antibodies were used: anti-rabbit CuZnSOD, Cell Signaling; anti-rabbit AMPK-PThr172, Cell Signaling; anti-rabbit AMPK, Cell Signaling; anti-rabbit HSP70, Cell Signaling; anti-rabbit eNOS, BD Transduction Laboratories; anti-rabbit eNOS Sér177, Cell Signaling; anti-rabbit eNOS Thr495, Cell Signaling; anti-mouse nNOS, BD Transduction Laboratories; anti-rabbit β1-adrenergic receptor, Abcam; anti-rabbit β2-adrenergic receptor, Abcam; anti-chicken β3-adrenergic receptor, Abcam. Immunoblots were
next processed with secondary antibodies (anti-rabbit, anti-chicken, or anti-mouse, Cell Signaling) for 1 hr at room temperature. Immunoblots were then probed with an ECL+Plus chemiluminescence reagent kit (GE Healthcare) to visualize signal, followed by exposure to X-ray film (Denville Scientific). The film was scanned to make a digital copy and densitometric analysis was performed to calculate relative intensity with ImageJ software from the National Institutes of Health (version 1.40g) using the Rodbard function. The membranes were incubated with the phospho-specific antibody first. Membranes were then stripped and incubated with the total-specific antibody. Results are presented as the ratio of the expression of phosphorylated protein to total protein. All experiments were performed in triplicate. For each membrane the relative intensity of each band was normalized to the value of the weakest band (smallest intensity). The values for each individual sample were averaged to obtain one value for each sample. The values for each group were then averaged and subsequently normalized to the mean of the control group (SED) or vehicle group as previously described.³

**Analysis of Nitrite, Nitrate, Nitrosothiols, and NO-Heme.** Nitrite and nitrate concentrations were quantified by ion chromatography (ENO20 Analyzer, Eicom). Tissue nitrosothiol compounds (RXNO) were quantified using group specific reductive denitrosation by iodine-iodide with subsequent detection of the NO liberated by gas-phase chemiluminescence. S-nitrosothiol levels were detected by preincubation with 2% mercuric chloride followed by acidified sulfanilamide. The addition of mercuric chloride allows for the differentiation between mercury sensitive (RNNO) and mercury insensitive nitrosothiols (RSNO). All NO analysis procedures have been previously described in detail.⁶⁻⁷

**Analysis of catecholamine levels.** Catecholamines were measured in blood samples taken from mice using the Bi-CAT Elisa (ALPCO, Salem, NH) according the manufacturer’s instructions.

**Statistical Analysis.** All the data in this study are expressed as mean ± standard error (SEM). Differences in data between the groups were compared using Prism 4 (GraphPad Software, Inc) with Student’s paired 2-tailed t-test, one-way analysis of variance (ANOVA), or two-way ANOVA where appropriate. For the ANOVA, if a significant variance was found, the Tukey or Bonferroni test was used as the post hoc analysis. A p value less than 0.05 was considered significant.
References


### Online Table I. Subject Characteristics

<table>
<thead>
<tr>
<th>Subject Characteristics</th>
<th>Non-trained individuals</th>
<th>Trained endurance athletes</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>16</td>
<td>7</td>
</tr>
<tr>
<td>Age (years)</td>
<td>27±1</td>
<td>24±2</td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>81±2</td>
<td>75±3</td>
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<tr>
<td>BMI (kg/m$^2$)</td>
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<td>23±1</td>
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<tr>
<td>Systolic BP (mmHg)</td>
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<td>Diastolic BP (mmHg)</td>
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<td>Total Cholesterol (mg/dL)</td>
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<td>LDL-C (mg/dL)</td>
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<td>HDL-C (mg/dL)</td>
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<td>Triglycerides (mg/dL)</td>
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<td>Glucose (mg/dL)</td>
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<tr>
<td>VO$_2$ max (ml/kg/min)</td>
<td>47±2</td>
<td>59±2*</td>
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</table>

Data are mean ± SE. BMI indicates body mass index; BP, blood pressure; LDL-C, low-density-lipoprotein cholesterol; HDL, high-density-lipoprotein cholesterol; VO$_2$ max, maximal oxygen consumption. *p<0.05 vs non-trained individuals
Online Figure I. Average daily running distances for C57BL/6J mice subjected to 4 weeks of voluntary exercise (VE) training, 4 wks of VE training followed by 1 week of rest, eNOS deficient mice (eNOS\(^{-/-}\)) subjected to 4 weeks of VE training, and \(\beta_3\)-Adrenergic Receptor deficient mice (\(\beta_3\)-AR\(^{-/-}\)) subjected to 4 weeks of VE training. Values are means ± S.E.M. **p<0.01 and ***p<0.001 vs. VE 4 wks.
Online Figure II. VE training increased the expression and altered the phosphorylation status of skeletal muscle eNOS. (A) Representative immunoblots and densitometric analysis of (B) total eNOS, (C-D) phosphorylated eNOS at serine residue 1177 (eNOS-P\textsuperscript{Ser1177}), (E-F) phosphorylated eNOS at threonine residue 495 (eNOS-P\textsuperscript{Thr495}). The expression of phosphorylated eNOS was normalized to the expression of total eNOS and α-tubulin since the total levels of eNOS increased after VE training. Values are means ± S.E.M. Numbers inside the bars are the number of animals investigated. *p<0.05 vs. SED.
Online Figure III. VE training increased the levels of NO metabolites in skeletal muscle. Levels of (A) nitrite, (B) nitrate, and (C) nitrosothiols (RXNO) were measured in the skeletal muscle following 4 weeks of VE training. Values are means ± S.E.M. *p<0.05, and **p<0.01 vs. SED.
Online Figure IV. Single injection of epinephrine altered the phosphorylation status of cardiac eNOS and increased the bioavailability of NO. A single intravenous injection of epinephrine dose dependently increased the (A) heart rate and (B) cardiac contractility of mice at concentrations ranging from 0.050 µg/kg to 10 µg/kg. Values are means ± S.E.M. *p<0.05 and ***p<0.001 vs. Vehicle.
**Online Figure V.** Single injection of epinephrine altered the phosphorylation status of cardiac eNOS. (A) Representative immunoblots and (B-D) densitometric analysis of the expression of eNOS, eNOS-P<sup>Ser1177</sup>, iNOS and nNOS in heart samples taken from 15 minutes to 2 hours following a single injection of epinephrine (10 µg/kg). Values are means ± S.E.M. *p<0.05 and ***p<0.001 vs. Vehicle.
Online Figure VI. Single injection of epinephrine increased plasma and heart NO metabolite levels. Levels of nitrite and nitrosothiols were also measured in the (A-B) plasma and (C-D) heart from 15 minutes to 2 hours following a single injection of epinephrine (10 µg/kg). Values are means ± S.E.M. *p<0.05 and **p<0.01 vs. Vehicle.