Differential effects of exercise on aortic mitochondria

Christal G. Young,1 Cynthia A. Knight,2 Kasey C. Vickers,2 David Westbrook,2 Nageswara R. Madamanchi,3 Marschall S. Runge,3 Harry Ischiropoulos,4 and Scott W. Ballinger2

1Sealy Center for Vaccine Development, University of Texas Medical Branch, Galveston, Texas; 2Division of Molecular and Cellular Pathology, Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama; 3Department of Medicine, University of North Carolina, Chapel Hill, North Carolina; and 4Abramson Pediatric Research Center, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania

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Routine exercise is widely recognized as cardioprotective. Exercise induces a variety of effects within the cardiovasculature, including decreased mitochondrial damage and improved aerobic capacity. It has been generally thought that the transient increase in oxidative stress associated with exercise initiates cardioprotective processes. Somewhat paradoxically, increased oxidative stress associated with cardiovascular disease (CVD) risk factors is thought to play an important role in the promotion and development of CVD. Hence, it is possible that CVD risk factors that increase oxidative stress (e.g., hypercholesterolemia) may modulate the cardioprotective effects of exercise. In this regard, the interaction between CVD risk factors and exercise on atherosclerotic lesion development and basal oxidant load is less defined. To determine the influence of preexistent hypercholesterolemia on cardioprotective effects of exercise, atherosclerotic lesion formation, oxidant load, mitochondrial damage, protein nitration (3-nitrotyrosine levels), and mitochondrial enzyme activities were determined in aortic tissues from normocholesterolemic (C57 control) and hypercholesterolemic (apolipoprotein E-deficient [apoE−/−]) mice after 16 wk of regular exercise. In normocholesterolemic mice, regular exercise was associated with decreased mitochondrial damage and oxidant load and increased SOD2 and adenine nucleotide translocator activities. Exercise did not decrease endogenous oxidant load and mitochondrial damage in hypercholesterolemic mice and did not reduce atherosclerotic lesion development. These data are consistent with the notion that CVD risk factors associated with increased oxidative stress can alter the benefits of exercise and that mitochondrial damage appears to be correlated with the cardiovascular effects of exercise.

atherosclerosis; oxidative stress; risk factors

Routine exercise has been broadly accepted as a deterrent for cardiovascular disease (CVD) (11, 27). Because exercise has been reported to induce a variety of effects, ranging from increased levels of reactive species to improved aerobic capacity, endothelium-dependent vasoreactivity (NO production), increased antioxidant activity, induction of heat shock proteins, and energetic capacities (26, 29), it has been generally thought that the increased oxidant load induced by exercise induces a variety of antioxidant defenses and vasoregulatory systems in the cardiovasculature. Indeed, it has been shown that increases in oxidant load can be correlated with the modulation of antioxidant defenses (reviewed in Ref. 10) and that antioxidant supplementation (reducing oxidant load) can prevent the exercise-related induction of endogenous antioxidant enzymes in vivo (19). These studies are consistent with the notion that reactive species play a significant role in mediating the cardioprotective benefits of exercise.

In contrast, increased oxidative stress has also been suggested as an important mediator of atherosclerosis (4, 7). Reactive oxygen and nitrogen species have been implicated in atherogenesis, and increased oxidative stress is a shared feature among many CVD risk factors (24, 25). Studies in hypercholesterolemic animal models and in humans have revealed increased oxidant loads (22, 25); moreover, it has been shown that modulation of oxidant load can correlate with the severity of CVD development (2, 14, 22). Consequently, although the clear series of events that unequivocally lead to CVD have not been fully characterized, increased oxidative stress appears to be a consistent feature in disease development. Hence, although the paradigm of increased oxidative stress as a significant factor in the etiology of CVD gathers mounting support, exercise, another form of oxidative stress, is viewed as cardioprotective.

Parameters of mitochondrial function and damage are a contrasting feature between the pro- and antiatherogenic forms of oxidative stress. Whereas CVD risk factors that are known to increase vascular oxidative stress have been shown to increase mitochondrial damage and dysfunction (2, 14, 30), exercise appears to improve mitochondrial function and decrease mitochondrial damage (3, 10, 28, 29). One possibility is that the chronic oxidative stress associated with CVD risk factors results in mitochondrial damage and dysfunction, whereas the transient oxidative stress associated with regular exercise increases levels of antioxidants and mitochondrial gene expression, resulting in decreased mitochondrial damage and improved function. Studies have shown that regular exercise is associated with decreased lipid peroxidation products in fit individuals (27), and it has also been reported that exercise can prevent the cardiotoxic effects of doxorubicin (a reactive oxygen species generator) in the mitochondrion (12). Exercise diminishes the age-related increase in mitochondrial oxidative stress and decline in mitochondrial function and, thus, “thwarts” mitochondrial aging (3). Finally, endurance training results in a 20–30% increase in mitochondrial enzyme activities (15), and several studies have reported a significant increase in mitochondrial ATP production rates associated with
even mild forms of exercise (29). These studies are consistent with the notion that regular exercise benefits mitochondrial function and reduces damage in normal populations. However, little is known about the impact of regular exercise on these same parameters in populations with preexistent CVD risk factors that increase basal oxidant loads. It is possible that the chronic levels of oxidative stress associated with CVD risk factors, such as hypercholesterolemia, may blunt the cardio-protective effects of exercise.

Hypercholesterolemia has been associated with increased oxidative stress and mitochondrial damage and can act synergistically with other CVD risk factors to increase mitochondrial damage and atherogenesis (2, 14). In an effort to further understand the interrelations among exercise, CVD risk factors, and the mitochondrion, the impact of regular exercise on atherosclerotic lesion formation and mitochondrial function and damage was examined in vascular tissues from normocholesterolemic and hypercholesterolemic mice. It was hypothesized that the intrinsic level of oxidative stress plays an important role in influencing the vascular benefits of exercise and that these benefits would be altered in animals with increased CVD risk (e.g., hypercholesterolemia). Consistent with this hypothesis, it was found that the cardiovascular benefits of exercise are diminished if the basal oxidant load within a tissue is high, as is the case with certain CVD risk factors.

MATERIALS AND METHODS

Mice. C57 and apolipoprotein E-deficient (apoE−/−) mice (C57BL/6J backgrounds) were purchased from Jackson Laboratories. The apoE−/− mouse lacks apolipoprotein E, a high-affinity ligand for lipoprotein receptors, and, consequently, has significantly elevated levels of serum LDL cholesterol and triglycerides and develops atherosclerotic lesions in a fashion similar to humans (23). All mice were fed chow diets (PicoLab Rodent Chow 20) that contain 4.5% fat by weight (0.02% cholesterol). Diet and water were supplied ad libitum. All procedures involving live mice were approved by the University of Alabama and the University of Texas Medical Branch institutional animal care and use committees.

Exercise. Mice were divided into three exercise groups (5 days/wk, n = 15 per genotype per group): 1) sedentary, 2) 15 min of swimming per day, and 3) 60 min of swimming per day. Initially, all mice (including those in the sedentary group) underwent a 2-wk training period (commencing at 6 wk of age) to sustain 60 min of swimming. After the initial training, animals were separated into respective activity groups for 16 additional weeks. Circular (60 cm diameter), heated (30°C, 10–15 cm of water) pools were used for swimming. Sedentary mice were placed in −1 cm of water in otherwise identical conditions. At the end of each exercise period, mice were placed on 37°C heating pads for 60 min. All animals successfully completed the exercise protocol.

Animals were intraperitoneally anesthetized with ketamine (Ketaset, 40 mg/kg) and xylazine (10 mg/kg), and mitochondria isolated (31) from freshly dissected aortas were used for enzyme activity assays and for 3-nitrotyrosine immunoblotting. Whole aortas were used for oil red-O staining. Tissues were harvested within 24 h of completion of the 16 wk of exercise.

Atherosclerotic lesion assessment. Hearts with attached aortas (down to the iliac artery) were dissected free and rinsed in PBS, and atherosclerotic lesion assessment was performed as previously described (14).

Isolation of mitochondria. Mitochondria were isolated using standard differential centrifugation techniques (31). Briefly, aortic tissues were collected and homogenized in isolation buffer (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 0.5% fatty acid-free BSA, and 5 mM HEPES, pH 7.2). Fresh tissue was processed immediately for mitochondrial isolation, with all manipulations carried out at 1–4°C. Homogenates were centrifuged at 1,500 g for 15 min at 4°C. The supernatant was transferred, and the first set of spin conditions was repeated two more times. After the three low-speed spins, the supernatant was subjected to a high-speed centrifugation (8,000 g, 15 min, 4°C). The supernatant was discarded, the mitochondrial pellet was resuspended in isolation buffer, and the high-speed spin was repeated. Finally, the supernatant was discarded, and pellets were used immediately or stored at −80°C. For quantification of mitochondrial protein, an aliquot was removed, centrifuged at 8,000 g for 15 min, and washed in BSA-free isolation buffer, pelleted, and used for determination of protein concentration.

Adenine nucleotide translocator activity. Adenine nucleotide translocator (ANT) activity was determined by atracyloside-sensitive ADP uptake, as previously described (35). Counts correspond to ANT activity (normalized to total mitochondrial protein).

Quantitative PCR for evaluation of mitochondrial DNA damage. Quantitative PCR was performed as previously described (14).

Aconitase activity as a measure of superoxide. Superoxide (O2−) levels were determined by measurement of the activity of aconitase, an enzyme that is specifically inactivated by O2− (8). Briefly, aconitase activity was determined from 5 μg of mitochondrial protein by monitoring the formation of NADPH at 340 nm. Citrate is converted to isocitrate by aconitase and then to α-ketoglutarate by NADP+-dependent isocitrate dehydrogenase in 50 mM Tris-HCl (pH 7.4), 30 mM sodium citrate, 0.5 mM MnCl2, 0.2 mM NADP+, and isocitrate dehydrogenase (2 U/ml) at 25°C. Aconitase activity (pmol NADPH·min⁻¹·μg mitochondrial protein⁻¹) is expressed relative to the sedentary C57 control group.

Total mitochondrial SOD activity. Total mitochondrial SOD (SOD2) activity from 25–40 μg of mitochondrial protein was determined using the cytochrome c reduction assay, as previously described (6, 14).

Protein nitration. Immunoblots using 30 μg of total mitochondrial protein were incubated with antinitrosytrosine antibody for determination of the level of protein nitration. After incubation with the nitrosotyrosine antibody, blots were washed with Tris-buffered saline-Tween 20 and incubated with goat anti-mouse antibody for 1 h, washed in Tris-buffered saline-Tween 20, and developed using an enhanced chemiluminescence kit (Amersham/Pharmacia). For determination of the levels of SOD2 nitration, 100 μg of total mitochondrial protein were diluted to 100 μg/ml in 1× RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EGTA, 0.25% sodium deoxycholate, 1% NP-40, 1 mM sodium orthovanadate, 0.05% Triton X-100, 0.5% protease inhibitors, and centrifuged at 13,000 g for 10 min). The protein concentration in the supernatant was quantitated (Bio-Rad DC Protein Assay), and 50 μg were used for immunoprecipitation with a rabbit polyclonal antibody specific for SOD2 (Research Diagnostics). Protein A/protein G bound to Sepharose (Pierce Chemical) was added to bind and pellet the specific immune complex by centrifugation at 3,000 g for 3 min. The immunoprecipitates were washed three times in 1× RIPA buffer and resuspended in Laemmli buffer, boiled, and run on 12% SDS polyacrylamide gels, transferred, and immunoblotted with monoclonal 3-nitrotyrosine antibody as described above for Western blotting.

Cholesterol determination. Total blood plasma cholesterol levels were determined as previously described (14).

Statistical analysis. Values are means ± SE. ANOVA was used to test for the global hypothesis that all the samples were drawn from a
single population. If this test yielded a significant value \( P < 0.05 \), Student-Newman-Keuls test was used for group comparisons.

**RESULTS**

As expected, cholesterol levels were significantly higher in the apoE\(^{-/-}\) than in the C57 mice among all groups (Fig. 1). Exercise did not cause significant changes in the total cholesterol levels among the apoE\(^{-/-}\) or C57 mice compared with sedentary, genotype-matched controls: 98.00 ± 13.27 and 67.90 ± 7.62 mg/dl for control and exercised mice, respectively.

To determine whether regular exercise reduced atherosclerotic lesion formation in apoE\(^{-/-}\) mice, whole aortas were stained with oil red-O. Percent oil red-O staining area did not change with exercise in aortas from apoE\(^{-/-}\) mice: 1.70 ± 0.36, 1.80 ± 0.63, and 1.89 ± 0.39 mm\(^2\) for sedentary, 15 min of swimming/day, and 60 min of swimming/day, respectively (Fig. 2). Hence, swimming exercise did not reduce percent oil red-O staining area in aortas from apoE\(^{-/-}\) mice.

Regular exercise was associated with increased levels of SOD2 activity in aortas from normocholesterolemic C57 mice compared with sedentary controls or exercised-matched apoE\(^{-/-}\) mice (Fig. 3A). In contrast, exercise did not alter SOD2 activity in apoE\(^{-/-}\) mice (Fig. 3A). The exercise-related increase in SOD2 activity in C57 aortas was associated with increased SOD2 protein levels (Fig. 3B). Although SOD2 protein was elevated in sedentary apoE\(^{-/-}\) mice compared with their C57 counterparts, these levels did not change with exercise in the apoE\(^{-/-}\) mice (Fig. 3B). Because exercise was associated with differential SOD2 activities in normocholesterolemic (C57) and hypercholesterolemic (apoE\(^{-/-}\)) mice, we hypothesized that exercise would be similarly linked with

Fig. 1. Total cholesterol levels determined from blood plasma samples from sedentary and exercised [15 and 60 min/day (15’ and 60’ swim)] mice. Known cholesterol standards were used to extrapolate sample cholesterol values from standard curves. Values are means ± SE. *Significantly different from C57 mice. apoE\(^{-/-}\), apolipoprotein E-deficient mice.

Fig. 2. Percent oil red-O staining of whole aortas from sedentary and exercised apolipoprotein E-deficient (apoE\(^{-/-}\)) mice. Positively stained areas are expressed as percent oil red-O area relative to total aortic area. Values are means ± SE.

Fig. 3. A: total mitochondrial SOD (SOD2) activity in sedentary and exercised mice. SOD2 enzyme activity was assessed in aortic mitochondrial isolates from normocholesterolemic (C57) and hypercholesterolemic (apoE\(^{-/-}\)) mice. SOD2 activities are expressed relative to each sedentary group. B: SOD2 protein levels determined by immunoblot in sedentary and exercised mice. SOD2 protein levels are expressed relative to the C57 sedentary group. Values are means ± SE. *Significantly different from sedentary counterparts.
altered oxidant loads in these mice. Oxidant load was assessed by measuring aconitase, an enzyme that is specifically inactivated by \( \text{O}_2^- \) and ONOO\(^-\) (8). Hence, aconitase activity will inversely reflect basal levels of \( \text{O}_2^- \) and ONOO\(^-\) formation. Consistent with the observed changes in SOD2 activity, aconitase activity was significantly increased (reflecting decreased steady-state levels of \( \text{O}_2^- \)) in regularly exercised C57 mice (Fig. 4A), whereas aortic aconitase activity decreased by 43–59% in regularly exercised apoE\(^{-/-}\) mice (Fig. 4A), demonstrating that regular exercise was associated with increased basal levels of oxidative stress in apoE\(^{-/-}\) mice. Because antibody to aconitase is not commercially available, fumarase (another citric acid cycle enzyme that is not inactivated by oxidative stress) activity was determined to verify that these observed differences were not simply due to altered levels of citric acid cycle enzymes. Fumarase activities were not significantly altered between exercise groups (Fig. 4B).

Similarly, ANT activity was increased in normocholesterolemic, but not hypercholesterolemic, mice with exercise. ANT is the most abundant protein in the mitochondrial inner membrane and serves as the mitochondrial transport system that exchanges matrix ATP for extramitochondrial ADP. Aortic ANT activity was increased by 65–77% in regularly exercised C57 mice compared with sedentary C57 mice (Fig. 5A). In contrast, aortic ANT activity was unchanged in regularly exercised apoE\(^{-/-}\) mice compared with sedentary apoE\(^{-/-}\) animals. Quantification of ANT protein revealed that activity was directly related to the amount of protein present (Fig. 5B).
Nitrated protein (3-nitrotyrosine) levels from aortic mitochondrial isolates were increased in C57 mice that performed 60 min of swimming per day compared with sedentary controls (Fig. 6A). In contrast, 3-nitrotyrosine levels among the sedentary and exercised apoE<sup>−/−</sup> mice remained unchanged, although they were significantly higher than in sedentary C57 mice. In parallel experiments on sedentary apoE<sup>−/−</sup> and C57 mice, the nitration of SOD2 protein was significantly higher in the apoE<sup>−/−</sup> than in the C57 mice (Fig. 6B). Because it has been previously shown that nitration of SOD2 can result in its inactivation (17), this may potentially explain the apparent decrease in SOD2-specific activity (increased protein level and no change in enzyme activity) observed in the apoE<sup>−/−</sup> mice (Fig. 3).

To assess general levels of DNA damage in the mitochondrial genome, we used quantitative PCR. Exercise was associated with significantly decreased aortic mitochondrial DNA (mtDNA) damage in the normocholesterolemic mice (Fig. 7), whereas exercise was associated with increased aortic mtDNA damage in hypercholesterolemic mice: 15 and 60 min of swimming per day resulted in higher levels of aortic mtDNA damage (Fig. 7), consistent with the notion of increased oxidant load (as reflected by decreased aconitase activity) in the exercised apoE<sup>−/−</sup> mice.

**DISCUSSION**

Despite the preponderance of evidence supporting the concept that regular exercise is a beneficial form of oxidative stress, the mechanisms that mediate these effects have not been completely defined in terms that reconcile the concept that increased oxidative stress can also be atherogenic. It has been previously shown that apoE<sup>−/−</sup> mice have intrinsically higher endogenous levels of vascular oxidant stress, mitochondrial damage, and altered function than normocholesterolemic mice (2, 14, 22). If the cardioprotective aspects of exercise are primarily mediated by processes induced by transient increases in oxidative stress, the preexistent (basal) oxidant load within an individual should be an important factor in determining the effects of exercise. Consistent with this notion, mice with lower basal oxidant loads (normocholesterolemic, C57) appeared to benefit from regular exercise, whereas the apoE<sup>−/−</sup> animals, which have been shown to have significantly higher basal levels of oxidative stress (22), did not. Similarly, the exercise regimen used here did not significantly reduce atherosclerotic lesion formation in the apoE<sup>−/−</sup> mice. These data are consistent with other exercise studies in mice and primates, which also show that regular exercise did not reduce atherosclerotic lesion development on a background of hypercholesterolemia and absence of pharmacotherapy (20, 33). Consequently, these results support the concept that preexistent CVD risk factors that are associated with increased oxidative stress can potentially blunt the benefits of exercise. Consequently, such risk factors should be significantly reduced to receive the complete cardiovascular benefits of regular exercise.
The observation that exercise increased mitochondrial protein nitration in C57 mice suggests that exercise increased reactive nitrogen species formation in these animals. Increased NO production mediated by exercise could potentially contribute to the formation of additional nitrogen species capable of nitrating proteins. However, the increase in mitochondrial protein nitration observed in C57 mice did not appear to be associated with any overall negative effects in terms of the measured parameters in this study. 3-Nitrotyrosine levels were higher in apoE<sup>-/-</sup> than in C57 mice and remained essentially unchanged. Specific investigation of SOD2 nitration levels in parallel experiments using sedentary apoE<sup>-/-</sup> and C57 mice revealed that levels were higher in apoE<sup>-/-</sup> mice (Fig. 6). Because it has been shown that nitration of SOD2 can lead to its inactivation (17), these data potentially explain the discrepancy between SOD2 activity and protein levels in apoE<sup>-/-</sup> mice (Fig. 3). The apparent plateau in SOD2 activity between the 15- and 60-min exercise groups within the C57 mice may similarly be due to increased nitration of mitochondrial proteins (Fig. 6A), including SOD2.

In addition to its observed vascular effects, NO also acts as an important physiological regulator of mitochondrial respiration. Exogenous and endogenous sources of NO inhibit mitochondrial respiration (O<sub>2</sub> consumption), resulting in greater O<sub>2</sub><sup>-</sup> production but also allowing greater O<sub>2</sub> diffusion into tissues, thereby decreasing the "steepness" of the O<sub>2</sub> gradient from the vascular lumen (26). Increased antioxidant production, a feature observed with exercise (27), can offset increased mitochondrial O<sub>2</sub><sup>-</sup> production mediated by NO (15). However, in the absence of increased antioxidant activity (e.g., SOD2), increased mitochondrial O<sub>2</sub><sup>-</sup> production will scavenge NO (generating ONOO⁻ and ONOOO⁻), effectively lowering NO concentration and inhibiting its effects on O<sub>2</sub> diffusion. Studies have shown that rodents with compromised SOD2 activity (34) are exercise intolerant (16), as have studies using nitric oxide synthase inhibitors (18), suggesting that O<sub>2</sub><sup>-</sup> and NO levels are important in modulating the effects of exercise. Hence, preexercise CVD risk factors that increase basal levels of oxidative stress may also alter the equilibrium of O<sub>2</sub><sup>-</sup> and NO within the mitochondrion and, thus, mediate mitochondrial damage and dysfunction.

Hypercholesterolemia is a significant CVD risk factor that increases basal oxidant loads and reduces endothelium-dependent vasoreactivity (5). The combination of these effects mediated by hypercholesterolemia can likely impact the cardiovascular aspects of exercise, a notion supported within this study and others (20, 33). In a previous report, exercise significantly reduced cholesterol levels and atherosclerotic lesion formation in hypercholesterolemic LDL receptor-null (LDLR<sup>-/-</sup>) mice and increased catalase activity (19). In the present study, exercise did not decrease cholesterol levels in the apoE<sup>-/-</sup> mice nor was it associated with increased antioxidant activities. Moreover, loss of apolipoprotein E in mice has a greater impact on lipoprotein clearance than on LDL receptor loss (9), and it has been shown that apolipoprotein E expression in LDLR<sup>-/-</sup> mice inhibits atherosogenesis (32). In addition, this study differed from the LDLR<sup>-/-</sup> mouse study in terms of diet (LDLR<sup>-/-</sup> mice were fed a high-fat, high-cholesterol diet) and exercise regimen (LDLR<sup>-/-</sup> mice were subjected to 1, 6, or 12 wk of running). Finally, it has been shown that LDLR<sup>-/-</sup> and apoE<sup>-/-</sup> mice respond differently in other studies assessing atherosclerotic lesion development in response to pharmacological therapies (1, 13).

In summary, these results suggest that certain CVD risk factors can potentially modulate the effects of exercise on the vasculature. Consequently, exercise regimens, in combination with CVD risk factor reduction strategies, should be carefully considered when evaluating the long-term benefits of exercise programs.

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