Swimming reduces the severity of atherosclerosis in apolipoprotein E deficient mice by antioxidant effects

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Received 5 September 2006; received in revised form 8 February 2007; accepted 15 February 2007
Available online 21 February 2007

Time for primary review 33 days

Abstract

Objective: It was shown that aerobic exercise training may protect against the development of atherosclerosis. However, the precise mechanisms are still unknown. We assessed the hypothesis that exercise training reduced the severity of experimental atherosclerosis in apolipoprotein (apo) E-deficient mice by antioxidant effects.

Methods: Exercise training (45 min swimming, 3 times/week) was conducted on apo E-deficient mice fed a high fat diet. Over 8 and 16 weeks on alternate days, mice were treated with and without exercise, and additional exercise-treated mice were orally given 25 mg/kg/day of N\textsuperscript{G}-nitro-L-arginine methylester (L-NAME), an inhibitor of nitric oxide synthase (NOS). In addition, the effect of L-arginine against L-NAME was also tested.

Results: Fatty streak formation at 8 weeks and fibrofatty plaques at 16 weeks developed in apo E-deficient mice fed a high fat diet, and were suppressed in mice treated with swimming for 8 and 16 weeks. In contrast, atherosclerotic lesions were not ameliorated in mice treated with exercise training associated with oral L-NAME. However, in mice treated with swimming associated with L-NAME and L-arginine, the atherosclerotic lesions were reduced. Immunohistochemical analysis revealed that macrophage and CD4+ cell accumulation in the fatty streak lesions was suppressed in mice treated with exercise, but not in those treated with exercise associated with L-NAME administration. The severity of atherosclerotic lesions was inversely correlated with the endothelial NOS expression and the expression of an endogenous antioxidant protein, thioredoxin. Namely, the expression of thioredoxin in mice treated with exercise was suppressed compared with mice without exercise. Plasma thiobarbituric acid-reactive substance levels were significantly lower in groups with exercise than in those without exercise or with exercise associated with L-NAME administration, suggesting exercise-induced less lipid peroxidation. Differences in lesion area did not correlate with any significant alterations in serum lipid levels. The exercise load used in the current study did not affect energy metabolism efficacies in the hearts.

Conclusion: Exercise training, in which the load did not affect energy metabolism efficacy of the heart, suppressed atherosclerosis by antioxidant effects via the vascular NO system.

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Keywords: Atherosclerosis; Nitric oxide; Redox signaling

1. Introduction

Exercise is a deterrent of cardiovascular disease, and its antiatherogenic effects have been described in different animal models [1,2]. Exercise can also influence risk factors that are associated with cardiovascular diseases, such as hypertension, diabetes mellitus, obesity, hyperlipidemia, and endothelial dysfunction [1–3]. However, the mechanisms by
which exercise might benefit cardiovascular diseases are still unknown. Since the oxidation hypothesis of atherosclerosis was suggested [4,5], a plethora of experiments involving cell culture, animal, and human studies, has shown that oxidized lipids could exhibit numerous proatherogenic effects [6–9]. Paradoxically, exercise also induces an oxidative stress in animals and humans [10,11], and this would appear incompatible with its antiatherogenic effects. As a resolution to this paradox, some recently proposed that either the overall beneficial effects of exercise would overwhelm the deleterious effects of oxidative stress, or the exercise-induced oxidative stress might itself be beneficial by inducing arterial antioxidant enzymes [9,10,12].

Enzymes associated with antioxidant defense, such as manganese superoxide dismutase, endothelial nitric oxide (NO) synthase (eNOS), heme oxygenase, and catalase can be induced by oxidants in vitro and in vivo [13–17]. Significant evidence indicates that exercise training increases coronary endothelium-dependent relaxation [18,19], and that these effects are associated with increases in NO [18–21] production and eNOS expression [21,22]. Moreover, in vivo studies have shown that exercise as well as other oxidant stimulation could induce antioxidant enzymes in different tissues [23–25]. Taken together, these observations alerted us to the possibility that extracellular oxidative stress induced by exercise could be potentially beneficial for the protection against atherosclerosis though the induction of arterial antioxidant enzymes would not only minimize oxidative damage but also reduce in situ the generation of oxidants.

In the present study, using apolipoprotein E-deficient mice, we tested the hypothesis that exercise training may reduce the severity of experimental atherosclerosis by antioxidant effects.

2. Materials and methods

2.1. Experimental atherosclerosis

The apolipoprotein E (apo E)-deficient 129iola × C57BL/6 hybrid mice were generous gifts of Dr. Edward M. Rubin (University of California, Berkeley, CA). These mice were mated with C57BL/6 mice to produce F1 hybrids. The F1 apo E/− mice were then backcrossed to C57BL/6 mice for 10 generations. Mice homogeneous for the apo E-null allele on a C57BL/6 background were subsequently generated. Male mice were subjected to the subsequent experiments. The mice were kept in a temperature-controlled facility on a 14:10-h light-dark cycle with free access to food and water.

After being weaned at 4 weeks of age, mice were fed a normal chow diet (NCD, Oriental Yeast) until 6 weeks of age, when the animals were switched to a high-fat diet (HFD) containing 20% fat and 0.3% cholesterol as previously described [26]. In this animal model of experimental atherosclerosis, the lesions in mice fed HFD at 8 weeks or 16 weeks may be equal to those of fatty streak or fibrofatty plaque lesions, respectively [26]. We performed animal experiments in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and these were approved by the institutional ethics committee for animal experiments of Kyoto University.

2.2. Exercise protocol

At 6 weeks of age, the mice were subjected to exercise protocol (Fig. 1). They were forced to swim in a hot water bath at 37 °C for 30 min to 45 min per day 3 times on alternate days per week. The protocol was conducted for 8 weeks (for fatty streak lesions) or 16 weeks (for fibrofatty plaque lesions). Exercise time was gradually increased from 30 min up to 45 min in the first 2 weeks. These mice were divided into the following groups that were received HFD or NCD with and without 

\[ \text{N}^G\text{-nitro-L-arginine methylester (L-NAME) supplementation during an 8 week treatment (HFD alone; } n=8, \text{ HFD+Swimming; } n=11, \text{ HFD+L-NAME; } n=8, \text{ HFD+L-NAME+Swimming; } n=8, \text{ HFD+L-NAME+Swimming+L-Arginine; } n=6, \text{ or during 16 week treatment (NCD alone; } n=3, \text{ NCD+Swimming; } n=2, \text{ HFD alone; } n=4, \text{ HFD+Swimming; } n=3). \text{ L-arginine (2.25%, Sigma) and L-NAME (100 μg/ml) was administered orally via the drinking water [27]. The mice consumed L-arginine and L-NAME, and daily fluid consumption was monitored. Estimated dose of L-NAME (25 mg/kg/...} \]

![Fig. 1. Swimming mice during exercise training. Mice were forced to swim in a warm water bath at 37 °C for 45 min 3 times per week. All mice tolerated the exercise protocol well throughout the study.](https://academic.oup.com/cardiovascres/article-abstract/74/3/537/367851)
body weight per day) was based on fluid consumption and drug concentration. The oral route of administration, in the dose range studied, has been shown to produce systemic inhibition of NOS [27].

2.3. Tissue processing

Mice were killed by bleeding with puncture of the right ventricle [28]. The vasculature was perfused with sterile phosphate buffered saline (PBS) and 6.8% sucrose. The root of the aorta was dissected under a macroscope and frozen in optimal cutting temperature (OCT) embedding medium for serial cryosectioning covering 1.0 mm of the root. The first section was harvested when the first cusp became visible in the lumen of the aorta. Four sections of 6 μm thickness were harvested per slide, and thus 8 slides per mouse were prepared. All sections were immersed for 15 s in 60% isopropanol, stained for 30 min in a saturated oil-red-O solution at room temperature, counterstained with hematoxylin, and then mounted under coverslips with glycerol gelatin.

The surface areas covered by fatty streak lesions at 8 weeks and fibrofatty plaque lesions at 16 weeks were quantified in oil red-O-stained samples, and specimens from exercise-treated mice with and without L-NAME supplementation were compared with those from exercise-untreated controls. In addition, the effect of L-arginine against L-NAME in the exercise-treated mice was evaluated.

2.4. Immunohistochemistry

Aortic root cryosections were also processed for immunohistochemistry as described previously with minor modifications [28–30]. In brief, anti-macrophage (anti-Mϕ, M3/84, 1:400, PharMingen), anti-CD4 (GK1.5, 1:50, PharMingen), anti-CD8 (53-6.7, 1:50, PharMingen), anti-I-Ab (anti-major histocompatibility class II, 25-9-17, 1:25, PharMingen), anti-intercellular adhesion molecule (ICAM)1 (M-19, 1:100, Santa Cruz Biotechnology), and anti-eNO (1: 200, Affinity BioReagents Inc) antibodies were applied to acetone-fixed cryosections. After being washed, the sections were then exposed to a second antibody (horseradish peroxidase-conjugated), and the antibody binding was visualized with diaminobenzidine. Sections were counterstained with 1% methyl green. The percentage of positively stained cells per infiltrating cells in the lesions was calculated for each antibody as previously described [29–31]. Data were obtained by dividing the number of positively stained cells by all methyl green-stained cells inside the internal elastic lamina. Three to five random microscopic fields were analyzed at ×200.

For the analysis of the expression of thioredoxin (TRX), an endogenous ubiquitous antioxidant protein [32–37], the indirect immunoperoxidase staining method was used as previously described [32,33]. The reactivity of TRX was semiquantitatively determined according to the previously reported methods [33,34]. The most frequently scored reactivity was considered to be representative for the lesions.

2.5. Lipid measurement

Serum was separated by centrifugation and stored at −80 °C. Serum total cholesterol (TC) and triglyceride (TG) levels were measured with assay kits (Wako) according to the manufacturer’s instructions.

2.6. Thiobarbituric acid-reactive substance (TBARS) assay

Plasma TBARS levels were determined for the evaluation of lipid peroxidation using a biochemical assay kit as previously described [38,39].

2.7. Cardiac myosin isoform analysis

To confirm the effects of swimming exercise upon heart, cardiac myosin isoform was analyzed as previously described [40]. In brief, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used for the analysis of isoforms. The hearts were rinsed in cold PBS and then

<table>
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<th>Table 1</th>
<th>Physiological and TBARS parameters</th>
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<tr>
<td>(n)</td>
<td>BW (g)</td>
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<tr>
<td>8 weeks (Fatty streak stage)</td>
<td></td>
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<tr>
<td>HFD alone</td>
<td>8</td>
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<tr>
<td>HFD + Swimming</td>
<td>11</td>
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<tr>
<td>HFD + L-NAME</td>
<td>8</td>
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<tr>
<td>HFD + L-NAME + Swimming</td>
<td>8</td>
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<tr>
<td>HFD + L-NAME + Swimming + L-Arginine</td>
<td>6</td>
</tr>
<tr>
<td>16 weeks (Fibrofatty plaque stage)</td>
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<tr>
<td>HFD alone</td>
<td>4</td>
</tr>
<tr>
<td>HFD + Swimming</td>
<td>3</td>
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<tr>
<td>NCD alone</td>
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*p<0.05 vs HFD alone. (Mean±SD).

HFD = high fat diet, L-NAME = N(G)-nitro-L-arginine methylster, NCD = normal chow diet, TBARS = thiobarbituric acid-reactive substance, BW = body weight, HW = heart weight, HW/BW = heart weight to body weight ratio.
Fig. 2. Representative oil-red-O and thioredoxin stained (insets) atherosclerotic lesions. The root of the aorta was dissected under a microscope and frozen in OCT embedding medium for serial cryosectioning covering 1.0 mm of the root. The first section was harvested when the first cusp became visible in the lumen of the aorta. Four sections of 6 μm thickness were harvested per slide, and thus 8 slides per mouse were prepared. Controls developed extensive plaque formation (a, f). In the mice treated with swimming, the lesion was markedly reduced (b, g). In the mice treated with swimming associated with l-NAME, the lesion was not reduced (d) compared with the mice treated with HFD with l-NAME (c). In the mice treated with swimming associated with l-NAME and arginine, the lesion was reduced (e). Thioredoxin (TRX)-positivity (brown area, arrows) was found less predominantly in the plaques in the mice fed HFD with swimming (b, inset) HFD with l-NAME (c, inset), and HFD with l-NAME and swimming (d, inset) compared with the mice fed HFD alone (a, inset) and HFD with l-NAME (c, inset).

(A) 8W (Fatty Streak Stage)

a) HFD alone

b) HFD + Swimming

c) HFD + L-NAME

d) HFD + L-NAME + Swimming

e) HFD + L-NAME + Swimming + L-Arginine

(B) 16W (Fibrofatty Plaque Stage)

f) HFD alone

g) HFD + Swimming
homogenized in 1.5 ml of Guba-Straub solution. The homogenate was left on ice for 60 min and then centrifuged at 10,000 × g for 20 min. The supernatant was then dialyzed overnight against 300–400 volumes of a solution containing 10 mM KCl, 0.1 mM dithiothreitol, and 1 mM tris (hydroxymethyl) aminomethane (Tris)–HCl (pH 7.2). Protein concentration was determined. Equivalent amounts of the samples (μg) were mixed with equal volumes of 2× gel sample buffer [0.125 M Tris–HCl (pH6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.004% bromphenol blue], boiled for 5 min, and then electrophoresed on a 4% polyacrylamide slab gel and stained with 0.1% Coomassie brilliant blue or silver stain. Fetal hearts were also prepared.

2.8. Statistical analysis

Values were expressed as means±SD. Statistical analysis of the data was determined by t-test or by one-way analysis of variance (ANOVA), followed by the Fisher protected least-significant-difference test. A value of p<0.05 was considered statistically significant.

3. Results

3.1. Effects of exercise on organ weights

There were no significant changes of heart weight (HW), body weight (BW) and heart weight to body weight ratio (HW/BW) at 8 weeks or 16 weeks (Table 1).

3.2. Effects of exercise on fatty streak at 8 weeks and fibrofatty plaque formation at 16 weeks

Apo E-deficient mice were forced to swim or not, and were kept on a cholesterol-rich diet for 8 weeks to induce fatty streak formation and for 16 weeks to form fibrofatty plaques [26,28,31]. Controls developed extensive lesions in the root of the aorta in fatty streak (8 weeks) and fibrofatty streak formation (16 weeks) stages (Fig. 2). In the mice treated with swimming, the fraction area of lesions was markedly reduced as shown in Table 2. In contrast, in the mice treated with swimming associated with oral L-NAME treatment, the fraction area of lesions was not reduced. However, in the mice treated with swimming associated with both oral L-NAME and L-arginine, the lesions were reduced (Table 2).

3.3. Immunohistochemistry

As previously reported [28], a significant amount of macrophages and CD4+ T cells appeared in the fatty streak lesions of mice fed HFD over 8 weeks. However, the percentages of macrophage and CD4+ cell were significantly reduced in the swimming-trained group than in the untrained group (Table 3). This may be due to the antiinflammatory effects of exercise [3].

Immunohistochemical staining showed that eNOS-positivity was found more predominantly in the fatty streak lesions at 8 weeks in the exercise-treated mice compared with the mice without exercise and the mice with exercise and oral L-NAME treatment (Fig. 3). Also, in the fibrofatty plaque lesions at 16 weeks, eNOS -positivity was more predominant in the exercise-treated mice than in the mice without exercise (data not shown).

The degree of the expression of TRX in the plaque lesions reflected the severity of atherosclerotic lesions (Fig. 2); i.e., the expression of TRX in the lesion of the mice with exercise was suppressed compared with the mice without exercise.

3.4. Effects of exercise on lipid profiles

Swimming training did not significantly modify the serum lipids profiles (Table 1).

3.5. Effects of exercise on TBARS

Swimming-trained groups showed lower plasma TBARS levels compared with untrained mice. Concurrent
administration of L-NAME prevented the decrease in the plasma TBARS levels in the trained groups compared with the untrained groups (Table 1).

3.6. Cardiac myosin isoform patterns

SDS-PAGE showed that the \( \alpha \)-isoform alone was presented both in the heart of mice fed with HFD alone and in the heart of exercise-trained mice fed with HFD (Fig. 4). Both \( \alpha \)- and \( \beta \)-isoforms were noted in a fetal heart (Fig. 4). Isoform specificity was confirmed by immunoblotting with murine isoform-specific antibodies (data not shown). The reproducibility of the results was confirmed. Thus, the current exercise protocol did not affect the changes of myosin isoform patterns, suggesting that the energy metabolism of the heart was not affected.

4. Discussion

It was shown that swimming suppressed the development of experimental atherosclerosis in apo E-deficient mice by antioxidant effects and that concomitant L-NAME treatment with swimming and L-arginine improved the severity of the lesions, suggesting the effect of exercise may be via NO system.

4.1. Oxidative stress in atherosclerosis

Many studies report the induction of antioxidant enzymes by exercise in different species, including humans [23–25]. In the present study, we hypothesized that exercise-induced...
aortic antioxidant response could eventually be beneficial against atherosclerosis. We chose an endogenous antioxidant, thioredoxin (TRX), as a representative antioxidant enzyme because of previous findings that TRX is stress-inducible which also protects cells from various types of stresses [32–37]. We had already reported that the expression of TRX is related to the severity of myocardial lesions in animal models of myocarditis [32,33], which might be supposed to reflect the excess oxidative stress in myocarditis caused by reactive oxygen species associated with inflammation. Okuda et al. also showed by human pathological study that TRX expression in stenotic coronary arteries are associated with the severity of atherosclerotic lesions [37]. Compatible with this report, the present study showed that the less severe expression of TRX in the mice treated with swimming were noted, reflecting the less severe oxidative stress overload in the mice.

4.2. NO and atherosclerosis

In this study, it was clearly demonstrated that exercise induced eNOS expression in the mice fed with HFD, but did not in those with L-NAME treatment (Fig. 3). The induction of eNOS by exercise has been previously reported in the coronary vessels of dogs [21] and in apo E-deficient mice [22]. In this study, exercise training associated with oral L-NAME supplementation, a blocker of NOS, did not induce the reduction of atherosclerotic lesions. However, it was shown that, in mice treated with swimming associated with oral L-NAME and L-arginine, the lesions were reduced. It was also demonstrated that exercise-treated mice showed the less intense TRX expression in the lesions compared with exercise-untreated mice, and that concomitant L-NAME administration with exercise restored the decreased TRX expression. Less lipid peroxidation in exercise-treated groups was additively confirmed by the data of plasma levels of TBARS.

The role of NO and the importance of nitrosative–oxidative stress in atherosclerosis were already reported in apo E-deficient mice [38,39]. That is, exercise may enhance endothelium-dependent vasodilation with increases in eNOS expression by shear stress [38]. On the contrary, peroxynitrate, resulted from the interaction between NO and free radicals, sustains oxidative injury to the endothelium and reduces NO availability [39]. TRX can protect against nitrosative stress by favoring the reaction between TRX or reduced glutathione sulfhydryl (GSH) and peroxynitrite to form s-nitrosothiols [35,40]. Accordingly, it may be that exercise therapy may suppress the development of atherosclerosis by antioxidant effects via endothelial NO system.

4.3. Exercise load

Intact ventricular myosin can be electrophoretically separated into three distinct components, V1 to V3, in order of decreasing Ca²⁺-activated ATPase activity [41]. The change from V1 (α, α) to V3 (β, β) results in improved energy metabolism efficacies [41]. In the present study, SDS-PAGE analysis demonstrated distinctly the α-isofrom alone in the hearts of mice fed HFD with and without swimming exercise treatment. Thus, the current exercise load did not affect cardiac energy metabolism efficacies in mice [1]. From the data reported previously [22], this work load is considered to be as moderate. To clarify the effect of exercise load on the atherosclerotic lesions of apo E-deficient mice with the analysis of oxidative stress, experimental protocols of more severe exercise load appear warranted.

4.4. Exercise in atherosclerosis

The benefits of exercise on atherosclerosis cannot exclusively be attributed to an induction of aortic antioxidant defenses alone because several risk factors for coronary heart disease are favorably modified by physical activity [3]. We showed that exercise decreased plaque size in atherogenic diet-fed mice. High fat diet-fed animals might induce a high concentration of plasma lipids, as mentioned before, which could be taken up and oxidized by surrounding tissues. NOS is more likely to be important in the context of atherosclerosis and endothelial function. In this study, there were no significant changes in the lipid profiles in all groups.

Most recently, Kinugawa et al. demonstrated a significant contribution of exercise capacity in endogenous antioxidant system using superoxide dismutase (SOD) knockout mice [42]. They showed that superoxide anion is increased by moderate exercise, that exercise capacity is reduced in SOD2 knockout mice, and that antioxidants may be useful for the treatment of reduced exercise capacity in subjects with heart failure, where reactive oxygen species play a crucial role in the pathogenesis. Linker et al. demonstrated antioxidative effects of exercise training in patients with chronic heart failure, by the analysis of expression of antioxidative enzyme activity in their biopsied skeletal muscles [43]. Accordingly, exercise training exerts beneficial effects in the patients with chronic heart failure, due not only to an augmentation in activity of radical scavenger enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase [42], but to the suppression of inflammatory cytokines [3]. Taken altogether with our data, it may be that exercise performance is related to the interaction between an endogenous antioxidant system and antiinflammation [44,45].

4.5. Inflammation and atherosclerosis

It is suggested that chronic inflammation is thought to be of central importance in atherosclerosis [46]. It was also shown that regular and chronic exercise could suppress overt and subclinical inflammation [3,47], based on the fact that atherosclerosis can be considered as generalized manifestations of an inflammatory disease [46]. Therefore, we and other
investigators had already reported that experimental atherosclerosis in apo E-deficient mice was markedly suppressed by Fc portion of immunoglobulin administration, possibly by an antiinflammatory action via inhibitory Fcγ receptor IIb [28,31]. However, in this study, there were no data on C-reactive protein or inflammatory cytokines in mice with and without swimming exercise treatment.

4.6. Conclusions

We have provided evidence for the lowering effects of atherosclerotic lesions by exercise-induced antioxidant effects. More importantly, L-NAME supplementation, an inhibitor of NOS, to exercise-trained mice diminished the effects of exercise on atherosclerotic lesions. In addition, L-arginine administration canceled the effects of L-NAME. In conclusion, exercise training conducted in the present study protects against experimental atherosclerosis in apo E-deficient mice by antioxidant effects via NO system.

Acknowledgment


References


