Exercise Training Reduces Severity of Atherosclerosis in Apolipoprotein E Knockout Mice via Nitric Oxide

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Background  Exercise training may protect against the development of atherosclerosis, although the precise mechanisms are still unknown. The present study assessed the hypothesis that exercise training would reduce the severity of experimental atherosclerosis in apolipoprotein-E (apoE)-deficient mice via nitric oxide (NO).

Methods and Results  ApoE-deficient mice fed a high-fat diet underwent exercise training (30 min swimming) 3 times per week for 8 weeks. The exercise group were also given oral NO-nitro-L-arginine methylester (L-NAME; 25 mg·kg⁻¹·day⁻¹), an inhibitor of NO synthase. Fatty streak plaque lesions developed in ApoE-deficient mice fed the high-fat diet, and were suppressed in the mice that underwent swimming training. In contrast, atherosclerotic lesions were not ameliorated in mice that had exercise training plus oral L-NAME treatment. Immunohistochemical analysis revealed that the expression of endothelial NO increased in mice undergoing exercise compared with the mice that did not exercise, and that the expression was suppressed in the mice having exercise plus oral L-NAME treatment. Differences in lesion area did not correlate with any significant alterations in serum lipid levels.

Conclusion  Exercise training suppressed atherosclerosis via the NO system.  (Circ J 2007; 71: 1147–1151)

Key Words:  Atherosclerosis; Exercise; Nitric oxide

Exercise is deters the development of cardiovascular disease. Its antiatherogenic effects have been described in different animal models,1,2 and exercise can also influence risk factors that are associated with cardiovascular diseases (ie, hypertension, diabetes mellitus, obesity, hyperlipidemia, and endothelial dysfunction).1–3 However, the mechanisms by which exercise is beneficial with regard to cardiovascular diseases are still unknown. Enzymes associated with antioxidant defense, such as manganese superoxide dismutase, endothelial nitric oxide synthase (eNOS), heme oxygenase, and catalase4–8 can be induced by oxidants in cell culture studies. There is significant evidence to indicate that exercise training increases coronary endothelium-dependent relaxation9,10 and that these effects are associated with increased NO production9–12 and eNOS gene expression12,13.

In the present study, using apolipoprotein-E (apoE)-deficient mice, we looked for evidence of induction of fatty and fibrofatty streaks in the arterial wall and the lessening of atherosclerotic lesions by exercise. We also investigated the effect of NO-nitro-L-arginine methylester (L-NAME) supplementation, an inhibitor of NOS, on atherosclerotic lesions in exercise-trained mice.

Methods

Experimental Atherosclerosis  The apoE-deficient 129ola×C57BL/6 hybrid mice were the generous gift of Dr Edward M. Rubin (University of California, Berkeley, CA, USA). They were mated with C57BL/6 mice to produce F1 hybrids. The F1 apoE+/– mice were then backcrossed to C57BL/6 mice for 10 generations. Male mice were used in the present 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, the mice were fed a normal chow diet (Oriental Yeast) until 6 weeks of age, when they were switched to a high-fat diet (HFD) containing 20% fat and 0.3% cholesterol as previously described.14 We performed the animal experiments in accordance with the Declaration of Helsinki, and they were approved by the institutional ethics committee for animal experiments of Kyoto University.

Exercise Protocol  At 6 weeks of age, the mice underwent an exercise protocol of swimming in a hot bath (37°C) for 30 min per day 3 times per week for 8 weeks. Exercise time was gradually increased up to 45 min in the first 2 weeks. These mice were divided into the following 4 groups, which on day 0 received HFD with and without L-NAME supplementation: HFD alone; n=9, HFD + Swimming; n=9, HFD + L-NAME; n=8, HFD + Swimming + L-NAME; n=9. L-NAME (100 μg/ml) was administered orally via the drinking water15 and daily fluid consumption was monitored. The estimated dose of L-NAME (25 mg·kg⁻¹·body weight⁻¹·day⁻¹) 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. Blood pressure and heart rate were periodically determined by the tail-cuff method using a photoelectric tail-cuff detection system (model BP-98A, Softron, Tokyo, Japan).

Tissue Processing
Mice were killed by bleeding after puncture of the ventricle. The vasculature was perfused with sterile phosphate-buffered saline and 6.8% sucrose. The root of the aorta was dissected under a macroscope 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. 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.

Immunohistochemistry
Aortic root cryosections were also processed for immunohistochemistry, as described previously with minor modifications. In brief, an anti-endothelial NO antibody (1:200, Affinity BioReagents Inc) was applied to acetone-fixed cryosections. After being washed, the sections were then exposed to a second antibody (horseradish peroxidase-conjugated antibodies), and the antibody binding was visualized with diaminobenzidine. Sections were counterstained with 1% methyl green. Three to 5 random microscopic fields were analyzed at ×200.

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

Statistical Analysis
Values are expressed as means ± SD. Statistical analysis of the data was determined by 1-way ANOVA, followed by
Results

Effects of Exercise on Organ Weights and Hemodynamics

There were no significant changes in heart weight, body weight or the heart weight to body weight ratio (Table 1). Also, there were no significant changes in blood pressure or heart rate among the 4 groups (data not shown).

Effects of Exercise on Fatty Streaks

The ApoE-deficient mice either exercised or not, and were kept on a cholesterol-rich diet for 8 weeks to induce formation of fatty streaks.14,16,19 The surface area covered by the fatty streak lesions was quantified in oil red O-stained samples, and specimens from exercise-treated mice with and without L-NAME supplementation were compared with those of unexercised controls. Controls developed extensive lesions in the root of the aorta (Fig 1). In the exercising mice, the fractional area of lesions was reduced compared with the controls (Fig 3). In contrast, in mice that exercised and were given oral L-NAME treatment, the fractional area of the lesions was not reduced.

Immunohistochemistry

Immunohistochemical staining showed that eNOS-positivity was found more predominantly in the fatty streak plaques of the exercising mice than in the mice not exercising and the mice with exercise and oral L-NAME treatment (Fig 2).

Discussion

The exercise training performed in the current experiment suppressed the development of experimental atherosclerosis in apoE-deficient mice, and L-NAME treatment concomitant with exercise did not improve the severity of the lesions, suggesting the effect of exercise on the cardiovascular system may be via the NO system.

No is thought to be a key regulator in the development of atherosclerosis.20,21 Classical cardiovascular risk factors, such as hypertension, diabetes mellitus, hypercholesterolemia, and smoking, all impair NO function. NO is formed from L-arginine by NOS, and 3 distinct isoenzymes of NOS are known to exist: constitutive-type isoforms, such as neural NOS (nNOS) and eNOS, and the inducible type of the enzyme (iNOS). The latter is widely distributed in a variety of cell types, including vascular smooth muscle cells and leukocytes, responds to a variety of stimuli, and can produce a very high output of NO.22 NO is a pleiotropic signaling molecule involved in numerous processes, includ-
ing liquid oxidation, molecular cell infiltration, and vascular smooth muscle homeostasis. Notably, eNOS is essential for the survival, migration, and angiogenic response of mature endothelial cells.

Using apoE-deficient mice on a HFD, we have shown that exercise can reduce the size of atherosclerotic lesions. Oil-red-O stain analysis showed that plaque size in the exercise group was smaller than that of the control group. The induction of eNOS by exercise, reported here, has been previously reported. Exercise training associated with oral L-NAME supplementation, a blocker of iNOS, did not reduce the atherosclerotic lesions in this study. In addition, iNOS-positivity was found more predominantly in the plaque from the exercising mice compared with the unexercised mice and the mice given exercise and oral L-NAME treatment. Taking together the results from previous reports it appears that exercise training may suppress the development of atherosclerosis via the NO system.

**Study Limitations**

This study could not identify the actual data of the blocking effects of L-NAME against the expression of eNOS in vivo. As mentioned previously, the method and dose of L-NAME used in the current study have been already reported and established. Furthermore, other authorized reports support the feasibility of the administered activity of L-NAME. Accordingly, L-NAME might counterbalance the effects of shear stress-induced endothelium-dependent vasodilation, which is related to the NO system, as mentioned earlier, resulting in suppression of eNOS in the exercising mice.

Exercise could increase the plasma level of oxidized fatty acids in mice fed a HFD and further upregulate eNOS. The benefits of exercise on atherosclerosis cannot exclusively be attributed to induction of the aortic NO system alone because several risk factors for coronary heart disease are favorably modified by physical activity. We showed here that exercise decreased plaque size in mice fed an atherogenic diet. A HFD might induce a high concentration of plasma lipids, 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. The HFD itself seemed to stimulate eNOS expression, which could be attributed to the presence of plasma lipid peroxides, as has been described in in-vivo models. In this study, there were no significant changes in the lipids profile of any group.

It has been suggested that chronic inflammation is of central importance in atherosclerosis, and it has been shown that regular and chronic exercise can suppress overt and subclinical inflammation, based on the fact that atherosclerosis can be considered as a generalized manifestation of an inflammatory disease. Therefore, we and other investigators have already reported that experimental atherosclerosis in apoE-deficient mice is markedly suppressed by administration of the Fc compartment of immunoglobulin, possibly by an anti-inflammatory action via the inhibitory Fc receptor IIb/IIIa. However, in this study, we did not obtain data on C-reactive protein or inflammatory cytokines.

**Conclusion**

Exercise training conducted in the present study protected against experimental atherosclerosis in apoE-deficient mice via the NO system.

**References**

Exercise in ApoE-Deficient Mice


