NOS inhibition accelerates atherogenesis: reversal by exercise

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—In this study, we assessed the effects of chronic exercise training (12 wk) on atherosclerotic lesion formation in hypercholesterolemic apolipoprotein E-deficient mice (n = 31). At the age of 9 wk, mice were assigned to the following groups: sedentary (Sed; n = 9); exercise (Ex; n = 12); sedentary and oral Nω-nitro-L-arginine (l-NNA, Sed-NA; n = 4), or exercise and oral l-NNA (Ex-NA; n = 6). Chronic exercise training was performed on a treadmill for 12 wk (6 times/wk and twice for 1 h/day) at a final speed of 22 m/min, and an 8° grade. l-NNA was discontinued 5 days before final treadmill testing. The farthest distance run to exhaustion was observed in Ex-NA mice (Sed: 306 ± 32 m; Ex: 640 ± 87; Sed-NA: 451 ± 109 m; Ex-NA: 820 ± 49 m; all P < 0.05). Lesion formation was assessed in the proximal ascending aorta by dissection microscopy after oil red O staining. The aortas of Sed-NA mice manifested a threelfold increase in lesion formation compared with the other groups. This l-NNA-induced lesion formation was reduced by chronic exercise training (Sed, 786 ± 144; Ex, 780 ± 206; Sed-NA, 2,147 ± 522; Ex-NA, 851 ± 253; Sed-NA vs. all other groups: P < 0.001). In conclusion, treatment with oral l-NNA (an nitric oxide synthase antagonist) leads to accelerated atherogenesis in genetically determined hypercholesterolemic mice. This adverse effect can be overcome by chronic exercise training.

apilipoprotein E deficiency; atherosclerotic lesions; Nω-nitro-L-arginine; nitric oxide; treadmill

ENDOTHELIUM- DERIVED nitric oxide (NO) is an endogenous antiatherogenic molecule. NO is known to inhibit key processes involved in atherogenesis including monocyte adherence, platelet aggregation, vascular smooth muscle proliferation, and oxidative enzyme activity (2, 13, 21, 30, 33, 44, 51, 52). NO also suppresses the activation of oxidant-responsive genes such as monocyte chemoattractant protein-1 and vascular adhesion molecule-1 (14, 27, 43, 45). In hypercholesterolemic animals, there is reduced bioactivity of NO due to increased degradation of NO by superoxide anion and an inadequate increase in NO synthesis, the latter being secondary to elevated levels of the endogenous NO synthase (NOS) inhibitor asymmetric dimethylarginine (6, 7, 10, 32). Administration of NOS antagonists accelerates lesion formation (9, 22, 28).

Another approach to manipulate the NOS pathway is through exercise interventions. Chronic exercise is known to enhance endothelium-dependent vasodilation, in association with increases in endothelial NOS (eNOS) expression (41, 49). This effect of exercise may be mediated by the known effect of shear stress to upregulate eNOS expression (41, 43, 46). Shear stress also has other effects on the endothelium, which may affect NOS bioactivity and/or influence atherogenesis, such as increasing the expression of superoxide dismutase (21) or increasing prostacyclin release (12, 23). These effects of shear stress on the endothelium may explain the observation that endurance runners as well as patients with coronary artery disease who participated in a high-intensity exercise program have greater vasodilatory capacity of the epicardial coronary arteries (17, 19). The exercise-induced enhancement of endothelial function may account in part for the known effects of exercise to attenuate atherogenesis and reduce cardiovascular morbidity and mortality (18, 31, 34). Accordingly, the current study was designed to determine whether the acceleration of atherogenesis induced by NOS inhibition could be reversed by chronic exercise.

METHODS

Animals. Female apolipoprotein E (apoE)-deficient C57BL/6J mice were purchased at the age of 8 wk (n = 31) (Jackson Laboratories; Bar Harbor, ME) and entered into the experimental protocol after 1 wk of acclimation in the housing facilities of the Stanford Department of Comparative Medicine (DCM). These genetically determined hypercholesterolemic, apoE-deficient mice have been shown to develop the entire spectrum of atherosclerotic lesions similar to those seen in humans (38). All mice were inspected before the study by the DCM veterinarian and monitored daily by DCM technicians and investigators. All experimental protocols were approved by the Administrative Panel on Laboratory Animal Care of Stanford University and were performed in accor-

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dance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care. All mice were housed three to four per cage under standard conditions in conventional cages. They were maintained on a 12:12-h light-dark cycle and given unlimited access to food and water for the duration of the study. All mice were handled daily and taught to run on a treadmill with a shock-plate incentive (Exer-4 Treadmill, Columbus Instruments; Columbus, OH) but were otherwise confined to cages for the duration of the study.

ApoE mice were generated from targeted disruption of the apoE gene in the 129 embryonic stem cell line. Germ line chimeras were mated and back-crossed for 10 generations with C57BL/6J wild-type mice (37).

Experimental protocol. ApoE mice were randomized at 9 wk of age to the following four groups: sedentary (Sed, n = 9); exercise (Ex, n = 12); sedentary and receiving oral \( N^\circ\)-nitro-L-arginine (t-NNa) (Sed-NA, n = 4); and exercise and receiving oral t-NNa (Ex-NA, n = 6) (see Fig. 1). All apoE mice received the same regular mouse chow (32, 35). t-NNa was discontinued 5 days before final exercise testing and death.

After 12 wk of dietary and/or exercise intervention, all mice underwent treadmill testing. Subsequently, mice were euthanized in random order at 21 wk of age by an overdose of pentobarbital sodium (Methoxyflurane, Pitman-Moore; Mundelein, IL). Blood was collected from the right atrium for measurement of serum cholesterol levels. The heart was removed by transection of the major vessels at the base. After the fat was removed, the heart was blotted dry and weighted. Gastrocnemius and vastus medialis muscles were removed, frozen in liquid nitrogen, and then stored at \(-80^\circ\)C until assayed. Maximal citrate synthase activity was assayed on muscle homogenates by the method of Srere (42). Values were expressed as an average of both muscles (in \( \mu\)mol/min \( \cdot \)g \(-1\) of muscle).

Western blot analysis. To determine the relative amount of eNOS protein, samples of hindlimb muscle were homogenized in buffer containing protease inhibitors to isolate protein content. The protein content was determined by the Lowry method and 50 \( \mu\)g of protein from each sample were subsequently separated on a 6.0% SDS-polyacrylamide minigel. Eluted proteins were electroblotted onto nitrocellulose membranes (HyBond, Amersham). The blots were incubated for 1 h in 5% nonfat dry milk-0.05% Tween in Tris-buffered saline to block nonspecific binding of the antibody. Blots were then incubated for 3 h with primary monoclonal antibodies against human eNOS diluted 1:500 in Tris-buffered saline-Tween. The blots were then incubated with peroxidase-labeled goat anti-mouse IgG in the same buffer for 1 h. Peroxidase-labeled protein was visualized with an enhanced chemiluminescence detection system (Amersham) on X-ray film.

Histochemistry. At death, the heart was excised and placed in PSS, pH 7.2, for 5 min. It was then embedded in optimum cutting temperature compound (Fischer; Santa Clara, CA), snap frozen on dry ice, and kept at \(-80^\circ\)C until being cryosectioned. Sectioning and lesion evaluation was performed by following the protocol of Paigen et al. (25, 36). The basal portion of the heart and the proximal ascending aorta were sectioned transversely into 10-\( \mu\)m-thick slices. Serial sections were collected on polylysine-coated slides and stored at \(-80^\circ\)C. Lipid deposits were identified by use of oil red O with hematoxylin and light green counterstain (25, 36). The basal content of the lesion was determined by an experienced observer blinded to the treatment group, and the mean lesion area per section per animal was calculated for each individual and group of animals. The intraobserver variability was <5%; the results of a representative set of sections were further verified by a second blinded individual with an interobserver variability of <10%.

Statistical analyses. For all statistical tests, differences were considered statistically significant if the two-sided probability of the observed result under the null hypothesis was \( p \leq 0.05 \). Results are expressed as means \( \pm \) SE. All calculations were performed using SPSS software (SPSS; Chicago, IL). For statistical evaluation nonparametric tests (Wilcoxon signed-rank test for intragroup comparisons within groups, and the Mann-Whitney U-test for interindividual
changes between groups). ANOVA was performed to identify a significant difference among the mean values of a variable measured in more than two groups. When ANOVA was significant, comparisons of the mean values were made by paired Student's t-test with Fisher's exact test correction. Correlation coefficients were calculated by Pearson product-moment correlations.

RESULTS

Body weight and serum cholesterol. At 9 wk of age, the average baseline body weight for all apoE-deficient mice was 19.8 ± 0.6 g. After 12 wk of study, body weight increased significantly in all groups (P < 0.001). Although body weight was lowest in the exercise groups, there was no significant difference observed between groups (P = not significant). Total serum cholesterol levels were measured after death in all mice. Values were not different between groups (see Table 1).

Maximal distance run. L-NNA was discontinued 5 days before maximal exercise testing (see Fig. 2). The farthest distances run until exhaustion were found in Ex and Ex-NA mice, which ran twice as far as their respective sedentary controls (both P < 0.001). Distance run to exhaustion correlated significantly with citrate synthase levels (r = 0.809, P < 0.001).

Effect of exercise on citrate synthase and eNOS expression in hindlimb. Citrate synthase levels were measured in hindleg muscles after death at the end of the study (Fig. 3). Levels reached in the exercise groups were significantly higher than in their respective sedentary control groups (both P < 0.05). Values of mice in Sed and Sed-NA were not different from each other. Western blot analysis revealed that eNOS expression was increased in the hindlimb of exercised mice (Fig. 3).

Lesion formation. Lesion formation was assessed in the proximal ascending aorta by dissection microscopy after oil red O staining (see Figs. 4, 5, and 6). Sed-NA mice showed an almost threefold increase in lesion formation compared with the other groups. This L-NNA-induced lesion formation was reduced by chronic exercise training (P < 0.001). Among mice treated with L-NNA (Sed-NA, Ex-NA), there was a significant inverse correlation between distance run and atherosclerotic lesion formation (r = −0.936, P < 0.001).

DISCUSSION

The main findings of this study are the following: 1) L-NNA accelerates atherogenesis in genetically determined hypercholesterolemic apoE-deficient mice; and 2) the acceleration of atherosclerosis by L-NNA is reversed by chronic exercise training.

In this study, atherogenesis was accelerated by L-NNA (a NOS antagonist). Inhibition of NOS has previously been shown to impair endothelial function, enhance monocyte binding, and promote atherosclerotic lesion formation in hypercholesterolemic animals (9, 11, 22, 28, 44). Similarly, in this study, administration of L-NNA increased lesion formation threefold. Exercise reversed the effects of NOS inhibition so that the extent of lesion formation was not different from that observed in untreated sedentary animals. Indeed, running distance was inversely correlated with lesion formation in these animals.

Table 1. Biochemical assays after 12 wk of study

<table>
<thead>
<tr>
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<th>Ex</th>
<th>Sed-NA</th>
<th>Ex-NA</th>
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<td>28.3</td>
<td>27.4</td>
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<td>Cholesterol, mg/dl</td>
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<td>857</td>
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<td>849</td>
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<td>Citrate synthase, mg - min^-1 - g muscle^-1</td>
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<td>681</td>
<td>608</td>
<td>862</td>
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</tbody>
</table>

Values are means ± SE; n, no. of mice. Sed, sedentary; Ex, exercise; Sed-NA, Sed + N^O2-nitro-L-arginine; Ex-NA, exercise and oral N^O2-nitro-L-arginine. *P < 0.001 for all groups; †P < 0.05 vs. Sed-NA.

Fig. 2. Distance run to exhaustion after 12 wk. N^O2-nitro-L-arginine was discontinued 5 days before maximal exercise testing. *P < 0.001, the farthest distance run to exhaustion was observed in Ex-NA mice.

Fig. 3. Effect of endothelial nitric oxide synthase (eNOS) expression in the hindlimb. Western blot analysis revealed that exercise increased eNOS expression in the mouse hindlimb (n = 3). One representative blot is shown. L-NNA, N^O2-nitro-L-arginine.
An exercise-induced increase in blood flow may exert its beneficial effects on vascular reactivity and structure through an increase in the elaboration of several endothelium-derived substances such as NO, prostacyclin, and superoxide dismutase (12, 21, 23, 41, 43, 46, 49). Chronic exercise training reversed the effects of L-NNA on lesion formation, possibly through an increased expression of NOS, which may have reduced the sensitivity to NOS antagonism.

Alternatively, the effect of exercise observed in this study may be due to exercise-induced release of prostacyclin (12, 23). Prostacyclin may exert its antiatherogenic effects by inhibiting the uptake of cholesterol esters into macrophages or into smooth muscle cells (16, 50), although compared with NO it may contribute less to the inhibition of monocyte adhesion (15, 23, 24). Furthermore, it inhibits platelet aggregation at much lower concentrations than those needed to inhibit adhesion (20), thereby allowing platelets to participate in the repair of the vessel wall while at the same time preventing or limiting thrombus formation.

Increases in flow may also trigger the release of endothelium-derived hyperpolarizing factor (EDHF), another endothelium-dependent vasodilator (3). The release of EDHF is increased when NO elaboration is inhibited (3). Therefore, inhibition of NOS would not be expected to obliterate flow-mediated vasodilation in most vessels, where prostacyclin and EDHF mechanisms are operative. Whether EDHF has antiatherogenic properties (like prostacyclin and NO) is not known.

Flow also modulates the expression of numerous paracrine substances, including endothelial growth factors, matrix modulators, chemokines, and regulators of blood fluidity, all of which may participate in the beneficial effects of exercise-induced vascular remodeling and reactivity (10, 39, 40).

Finally, exercise-induced changes in flow might be expected to have other anti-atherogenic effects. Endothelial cells exposed to shear stress elaborate less superoxide anion (43); this may in part be due to increased transcription of superoxide dismutase (21, 43). There are also shear-stress-responsive elements in the promoter region of several adhesion molecules (e.g., intercellular adhesion molecule) that may reduce their gene expression (27, 39). However, none of these mechanisms provide a satisfactory explanation for the lack of effect of exercise on the extent of internal lesions that occurs in the absence of L-NNA.

Several reasons may account for this observation: the mice in this study were still very young compared with adolescence or young adulthood in humans.
Thus lesion formation was rather modest and further improvement unlikely to be detected. Also, 12 wk of study may not have been long enough or the exercise intensity not high enough to induce a sufficient release of vasoprotective endothelial substances which could counterbalance the deleterious atherogenic effects of severe hypercholesterolemia (~800 mg/dl). This is in keeping with other human studies that report beneficial effects of exercise on coronary stenoses only in the presence of satisfactory low-density lipoprotein levels induced by lipid-lowering therapy (18, 31, 34). Although one of these studies (31) revealed an independent role of chronic exercise training, this study was performed in patients with only moderately elevated serum cholesterol levels.

We have recently shown that running distance and aerobic capacity are diminished in the hypercholesterolemic state and that this dysfunction correlates with a reduction in endothelium-dependent relaxation, endothelial NO production, and postexercise urinary nitrite excretion (26, 32). This dysfunction can be mimicked by inhibition of NOS activity by L-NNA (26) and can be reversed by chronic exercise training as reported in this study, indicating a critical role for NO in the distribution of blood flow to exercising skeletal muscle.

In this study, chronically exercised mice that were treated with L-NNA had the NO inhibitor discontinued 5 days before maximal exercise testing. Under these conditions, the treated mice ran the greatest distances until exhaustion. It is possible that chronic NOS antagonism led to a further upregulation of vascular NOS in these animals or an upregulation of other vasodilation mechanisms such that withdrawal of the NOS inhibitor 5 days before testing led to a “rebound” enhancement of vasodilator capacity (and limb blood flow) during exercise.

We (29) hypothesized that exercise-induced shear stress and subsequent increase in NO synthesis may contribute to the beneficial cardiovascular effects of exercise on vascular function and structure. During physical exercise intracoronary blood flow increases, which results in an endothelium-dependent vasodilatation of the epicardial coronary arteries (4, 17, 41, 53). Chronic exercise in dogs has been shown to increase mRNA expression of NOS, which augments NO activity, and subsequently leads to an improvement in vascular reactivity in coronary arteries (41). NO inhibits multiple processes involved in atherogenesis and restenosis, including generation of superoxide anion, adherence of monocytes, aggregation of platelets, and proliferation of vascular smooth muscle (2, 13, 21, 30, 33, 44, 51, 52). Enhancement of vascular NO activity inhibits atherogenesis (5, 6, 47, 48) and may even induce regression of preexisting intimal lesions (8, 48).

In summary, antagonism of NOS accelerates atherogenesis in genetically determined hypercholesterolemic mice. This effect of NOS inhibition could be reversed by exercise training. The antiatherogenic effect of exercise may be mediated in part by its enhancement of NO biosynthesis.

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DISCLOSURES

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