Original Article

Voluntary Exercise Ameliorates the Progression of Atherosclerotic Lesion Formation via Anti-Inflammatory Effects in Apolipoprotein E-Deficient Mice

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Aim: A sedentary lifestyle with insufficient exercise is associated with cardiovascular disease. Previous studies have demonstrated that endurance exercise benefits atherosclerosis and cardiovascular disorders; however, the mechanisms by which physical activity, such as voluntary exercise (Ex), produces these effects are not fully understood.

Methods and Results: Eight-week-old male apolipoprotein (ApoE)-deficient mice were fed a standard diet (STD) or high fat diet (HFD) for 10 weeks. The HFD + Ex group mice performed Ex on a running wheel for 10 weeks. No significant differences in lipid profiles were observed between the HFD and HFD + Ex groups. Although changes in body and brown adipose tissue weights were comparable between the HFD and HFD + Ex groups, white adipose tissue weight was significantly lower in the HFD + Ex group than in the HFD group. The areas of atherosclerotic lesions in the aortic sinus and thoracoabdominal aorta were significantly reduced in the HFD + Ex group than in the HFD group ($p < 0.001$). There was a strong negative correlation between atherosclerotic areas and the mean running distance per day in the HFD + Ex group ($r = -0.90$, $p = 0.01$). Endothelial function was significantly preserved in the HFD + Ex group ($p < 0.05$). Serum interleukin-6 and macrophage chemoattractant protein-1 levels were significantly lower and those of adiponectin were significantly higher in the HFD + Ex group than in the HFD group ($p < 0.05$).

Conclusions: These results suggest that Ex ameliorates the progression of endothelial dysfunction and atherosclerotic lesion formation through anti-inflammatory effects, despite continued consumption of HFD.

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Key words; Voluntary exercise, Atherosclerosis, Endothelial function, Apolipoprotein E-knockout mice, Adiponectin

Introduction

Regular physical activity reduces the morbidity

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and mortality associated with cardiovascular diseases¹⁻³. Physical exercise not only improves traditional risk factors, such as obesity, hypertension, dyslipidemia and glucose intolerance, but also the inflammatory state, which is closely linked to cardiovascular disorders^{4, 5}. However, despite the wealth of evidence derived from epidemiological observations, interventional trials, and experimental animal studies, the mechanisms by which physical activity benefits ath-

erogenesis are not fully understood.

Exercise assessment models in mice use either voluntary or forced activity. Forced activities, such as swimming and treadmill exercise, have the advantage that mice can be made to exercise at reproducible duration and distances; however, these methods are non-physiological and stressful^{6, 7}. Both swimming and treadmill exercises are unpleasant for mice because of the shock bar to avoid discontinuity of running or the fear of drowning^{6, 7}; therefore, such unpleasant exercises may lead to adverse stress^{8, 9}. Furthermore, these exercises are mostly performed during the day, in contrast to the nocturnal activity of mice. These factors are confounding for assessing the underlying mechanisms by which innate exercise, including the maintenance of physical activity, has beneficial effects on cardiovascular diseases. In contrast, voluntary running exercise can be performed in a non-stressful environment without disruption of the normal diurnal rhythm.

The present study aimed to assess the effects of voluntary exercise (Ex) on the initiation and progression of atherosclerotic lesions in apolipoprotein E (ApoE)-deficient mice. In addition, we examined the endothelial function of thoracic aorta, circulating pro- and anti-inflammatory parameters, and changes in white and brown adipose tissue weights, which play important roles in systemic inflammation. We demonstrated a marked reduction in atherosclerotic lesions, with a strong negative correlation between atherosclerotic areas and the mean running distance per day in mice fed a high-fat diet (HFD) supplemented with Ex. Moreover, voluntary running exercise ameliorated the progression of endothelial dysfunction and atherosclerotic lesion formation via anti-inflammatory effects, including an increase in adiponectin concentration followed by a decrease in the weight of white adipose tissue without a decrease in that of brown adipose tissue, despite continued consumption of HFD.

Materials and Methods

Experimental Animals

The Animal Care and Use Committee of Juntendo University School of Medicine reviewed and approved all protocols described in this study. ApoE-deficient mice were obtained from Jackson Laboratories (West Grove, PA). Male mice were provided with the diet and water and were maintained on a 12-h light/dark cycle in the specific pathogen-free facility of Juntendo University. The investigation conformed to 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).

Study Protocol

Experimental mice were weaned at 4 weeks of age and provided a normal chow diet (0.09% cholesterol and 5.6% fat; Oriental Yeast Co., Tokyo, Japan) until 8 weeks of age, and then randomly assigned to 3 groups as follows: a standard diet (STD), HFD, and HFD and Ex (HFD+Ex) groups. The STD group was provided a normal chow diet for 10 weeks. The HFD and HFD+Ex groups were provided a HFD (normal chow diet containing an additional 0.15% cholesterol and 15% fat; Oriental Yeast Co., Tokyo, Japan) for 10 weeks. The HFD+Ex group mice were kept in individual cages supplied with a running wheel (14 cm in diameter) and equipped with a counter to record the daily running rotation for 10 weeks. Mice were euthanized and sacrificed at 18 weeks of age and tissue samples were harvested immediately.

Histological Analysis of Atherosclerotic Lesions

Atherosclerotic lesions at the aortic sinus were analyzed by histological staining as follows. The upper portion of the heart and proximal aorta were obtained at sacrifice, embedded in OCT compound, and stored at -80°C . Serial 5- μm thick aortic cryosections beginning at the aortic root were collected from 200- μm segments of the aortic sinus, stained with saturated Oil Red-O solution at room temperature for 30 min, and counterstained with hematoxylin. For each mice, lipid lesions at the aortic sinus assessed as positive by Oil Red O-staining were quantified for the comparison of atherosclerotic lesions¹⁰. The ascending and descending aortas were dissected, cleaned of peripheral fat under a dissecting microscope to expose the endothelial surface, and stained with Sudan IV solution (0.5% wt/vol.; Sigma Chemical, St Louis, MO, USA). Aortic images were digitized using a microscope (Olympus AX80; Olympus Optical, Tokyo) equipped with a high-resolution camera (Nikon D2X; Nikon, Tokyo). The proportion of aortic intima surface area occupied by red staining of atherosclerotic lesions was quantified by normalizing the area stained with Sudan IV to that of the entire aorta using an imaging system (KS400; Carl Zeiss Imaging Solutions GmbH, Germany).

Endothelium-Dependent Relaxation to Acetylcholine

After the mice were sacrificed under anesthesia, their descending thoracic aortas were excised, cleaned of connective tissue, and cut into transverse vascular rings (approximately 2 mm in length). The protocol was conducted in temperature-controlled (37°C) and

an oxygenated Krebs bicarbonate buffer (pH 7.4) containing the following: 118 mM NaCl, 4.7 mM KCl, 1.5 mM CaCl₂, 25 mM NaHCO₃, 1.1 mM MgSO₄, 1.2 mM KH₂PO₄, and 5.6 mM glucose, as we described previously¹⁰. Optimal passive tension of 0.5 g was applied to the vascular rings throughout the experiment. The vascular rings were initially exposed to a high-K⁺ Krebs bicarbonate buffer containing 30 mM KCl instead of NaCl to produce maximal force generation and to enhance the reproducibility of subsequent contractions. After washing and observing a steady-state level of contraction, the vessels were submaximally contracted with 1.0 μ M phenylephrine. The vasodilator responses to the endothelium-dependent agonist acetylcholine, the endothelium-independent agonist, and NO donor sodium nitroprusside were studied to investigate endothelial function. Relaxation was expressed as the percent change in the steady-state level of contraction. R_{max} was determined from the maximum relaxation of the established tone and ED₅₀ was calculated from log transformation of the relaxation data.

Quantification of Lipids

Serum was separated by centrifugation and stored at -80°C. Serum total cholesterol and triglyceride levels and as well as lipoprotein concentrations were analyzed by an online dual enzymatic method for simultaneously quantifying cholesterol and triglycerides by high-performance liquid chromatography at Skylight Biotech Inc. (Akita, Japan), as we described previously¹⁰.

Real-Time PCR

Total RNA from mouse thoracoabdominal aortas was extracted using a RNeasy Mini Kit (Qiagen) and treated with RNase-free DNase according to the manufacturer's instructions. Then, 1 μ g RNA was reverse transcribed to cDNA with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR (7500 Real-time PCR System, Applied Biosystems) was used to determine mRNA levels of CD68 (macrophage marker), CD4 (T-helper cell marker), and CD11c (dendritic cell marker). TaqMan[®] Gene Expression Assays for CD68 (Cat # Mm00839636_g1), CD4 (Cat # Mm00442754_m1), CD11c (Cat # Mm00498690_g1), and β -actin (Cat # Mm01205647_g1) were purchased from Applied Biosystems.

Measurement of Serum Parameters

Serum interleukin (IL)-6 and tumor necrosis factor (TNF)- α levels were measured using the Cytomet-

ric Bead Array Mouse Soluble Protein Master Buffer Kit (BD Biosciences). Serum monocyte chemoattractant protein (MCP)-1 and adiponectin levels were measured using commercial mice ELISA kits (Otsuka Pharmaceutical, Tokyo, Japan; R&D Systems, Minneapolis, MN, USA, respectively).

Statistical Analysis

Continuous variables are presented as the mean $\pm$ SD with the exception of analysis of vascular ring relaxation as the mean $\pm$ SEM. Differences between groups were analyzed by unpaired *t*-tests. Differences among 3 groups were analyzed by one-way ANOVA followed by appropriate post hoc analysis with a Bonferroni correction for multiple comparisons. Differences with *p* < 0.05 were considered significant.

Results

Body Composition and Lipid Profiles

Table 1 shows the body weights and lipid profiles in the STD, HFD, and HFD + Ex groups. Body weight in the HFD + Ex group was slightly but significantly lower than in the HFD group at 18 weeks of age (HFD + Ex: 28.6 $\pm$ 1.0 g, HFD: 31.1 $\pm$ 0.9 g, *p* < 0.05); however, there were no significant differences in the percent change in body weight from 8 to 18 weeks of age among the 3 groups. The mean running distance per day in the HFD + Ex group was 15,145 $\pm$ 3,619 m/24 h. There were no significant differences in food consumption, heart rate, or blood pressure among the 3 groups (data not shown). There were no significant associations between body weight changes and the mean running distance per day or body weight changes and food consumption. HFD induced a marked increase in total cholesterol in the HFD and HFD + Ex groups (HFD: 1,023 $\pm$ 170 mg/dL, HFD + Ex: 974 $\pm$ 330 mg/dL, STD: 521 $\pm$ 84 mg/dL, HFD vs. STD, *p* < 0.05, HFD + Ex vs. STD, *p* < 0.05); however, there was no significant difference between the HFD and HFD + Ex groups. The very low-density lipoprotein cholesterol levels were significantly higher in the HFD and HFD + Ex groups than in the STD group (HFD: 653 $\pm$ 146 mg/dL, HFD + Ex: 586 $\pm$ 297 mg/dL, STD: 295 $\pm$ 74 mg/dL, HFD vs. STD, *p* < 0.05, HFD + Ex vs. STD, *p* < 0.05). The high-density lipoprotein cholesterol or triglyceride levels were not significantly different among the 3 groups.

Table 2 presents a comparison of epididymal, perirenal, omental, and brown adipose tissues from fat pads among the STD, HFD, and HFD + Ex groups. The values of each fat/body weight of epididymal (10.1 $\pm$ 1.2 g/g $\times$ 1,000 vs. 7.8 $\pm$ 1.2 g/g $\times$ 1,000, *p* < 0.05),

Table 1. Body weights and lipid profiles

	STD (<i>n</i> =8)	HFD (<i>n</i> =8)	HFD + Ex (<i>n</i> =8)
Initial BW, g	24.1 ± 1.0	24.5 ± 2.8	23.1 ± 0.9
Final BW, g	29.9 ± 1.4	31.1 ± 0.9	28.6 ± 1.0 [#]
BW changes, %	23.9 ± 9.8	28.5 ± 15.6	24.2 ± 7.3
Total cholesterol, mg/dL	521 ± 84	1023 ± 170 [*]	974 ± 330 [*]
VLDL-cholesterol, mg/dL	295 ± 74	653 ± 146 [*]	586 ± 297 [*]
LDL-cholesterol, mg/dL	191 ± 51	285 ± 75 [*]	251 ± 74
HDL-cholesterol, mg/dL	29 ± 14	36 ± 9	32 ± 21
Triglyceride, mg/dL	33 ± 22	32 ± 17	30 ± 6

Data are expressed as the mean ± SD.

STD: standard diet, HFD: high fat diet, Ex: voluntary exercise, BW: body weight, VLDL: very low-density lipoprotein, LDL: low-density lipoprotein, HDL: high-density lipoprotein.

^{*}*p* < 0.05 vs. STD, [#]*p* < 0.05 vs. HFD.

Table 2. Comparison of epididymal, perirenal, omental, and brown adipose tissues from fat pads among the STD, HFD, and HFD + Ex groups

	STD (<i>n</i> =6)	HFD (<i>n</i> =6)	HFD + Ex (<i>n</i> =6)
Epididymal fat/BW, g/g × 1,000	7.8 ± 1.2	10.1 ± 1.2 [*]	7.4 ± 1.6 [#]
Perirenal fat/BW, g/g × 1,000	1.8 ± 0.3	2.4 ± 0.4 [*]	2.1 ± 0.3
Omental fat/BW, g/g × 1,000	3.0 ± 0.6	3.1 ± 0.9	2.4 ± 0.4
BAT/BW, g/g × 1,000	2.2 ± 0.5	1.9 ± 0.4	1.8 ± 0.3
WAT/BW, g/g × 1,000	12.5 ± 1.9	15.5 ± 2.4 [*]	11.9 ± 2.2 [#]

Data are expressed as the mean ± SD.

STD: standard diet, HFD: high fat diet, Ex: voluntary exercise, BW: body weight, BAT: brown adipose tissue, WAT: white adipose tissue (epididymal fat + perirenal fat + omental fat).

^{*}*p* < 0.05 vs. STD, [#]*p* < 0.05 vs. HFD.

perirenal (2.4 ± 0.4 g/g × 1,000 vs. 1.8 ± 0.3 g/g × 1,000, *p* < 0.05), and total white adipose tissues (15.5 ± 2.4 g/g × 1,000 vs. 12.5 ± 1.9 g/g × 1,000, *p* < 0.05) were significantly higher in the HFD group than in the STD group. The values of epididymal (7.4 ± 1.6 g/g × 1,000 vs. 10.1 ± 1.2 g/g × 1,000, *p* < 0.05) and total white adipose tissues (11.9 ± 2.2 g/g × 1,000 vs. 15.5 ± 2.4 g/g × 1,000, *p* < 0.05) were significantly lower in the HFD + Ex group than in the HFD group. There was no significant difference in the values pertaining to brown adipose tissue among the 3 groups.

Voluntary Running Exercise Reduces Aortic Atherosclerotic Lesions in ApoE-Deficient Mice

Fig. 1 and **2** show atherosclerotic lesion formation in the aortic sinus and thoracoabdominal aorta, respectively, in the STD, HFD, and HFD + Ex groups. HFD for 10 weeks significantly induced atherosclerotic lesions in the aortic sinus (**Fig. 1B**, HFD: 19.9 ± 7.0%, STD: 8.2 ± 5.9%, *p* < 0.005) and thoracoabdominal aorta (**Fig. 2B**, HFD: 31.6 ± 6.1%, STD:

22.6 ± 4.7%, *p* < 0.005) in the HFD group. Histomorphometric analysis revealed that voluntary running exercise for 10 weeks significantly decreased atherosclerotic lesion formation in the HFD + Ex group than in the HFD group (**Fig. 1B**, HFD: 19.9 ± 7.0%, HFD + Ex: 7.2 ± 4.9%, *p* < 0.0005, **Fig. 2B**, HFD + Ex: 18.6 ± 4.4%, HFD: 31.6 ± 6.1%, *p* < 0.0001). Interestingly, there was a strong negative correlation between atherosclerotic areas in the thoracoabdominal aorta and mean running distance per day in the HFD + Ex group (*r* = -0.90, *p* = 0.01) (**Fig. 3**).

Voluntary Running Exercise Preserves HFD-Induced Endothelial Dysfunction in ApoE-Deficient Mice

Fig. 4 shows cumulative concentration curves for acetylcholine and sodium nitroprusside in the phenylephrine-contracted rings of thoracic aortas from STD, HFD, and HFD + Ex mice. In this experiment, we added a STD with Ex (STD + Ex) group to assess whether exercise itself could improve acetylcholine- and sodium nitroprusside-induced relaxation. Endo-

Fig. 1. Comparison of atherosclerotic lesion formation in the aortic sinus among the STD, HFD, and HFD + Ex groups.

Atherosclerotic lesion formation in the aortic sinus was determined by Oil red-O staining in the STD, HFD, and HFD + Ex groups. Representative examples (A) and histomorphometric analysis (B: mean $\pm$ SD; $n=6$ per group). The Y-axis is the percent area of atherosclerotic lesion formation in the aortic sinus.

Fig. 2. Comparison of atherosclerotic lesion formation in the ascending and descending aortas among the STD, HFD, and HFD + Ex groups.

Ascending and descending aortas of STD, HFD, and HFD + Ex group mice were dissected to expose the endothelial surface, and developed for Sudan IV staining. Representative examples (A) and histomorphometric analysis (B: mean $\pm$ SD; $n=6$ per group). The Y-axis is the percent area of atherosclerotic lesion formation in the thoracoabdominal aorta.

thelium-dependent relaxation in response to acetylcholine at 1×10^{-8} ($23.8 \pm 3.1\%$ vs. $2.6 \pm 1.4\%$, $p < 0.05$), 3×10^{-8} ($50.5 \pm 3.3\%$ vs. $12.6 \pm 3.0\%$, $p < 0.05$), 1×10^{-7} ($77.9 \pm 1.9\%$ vs. $40.8 \pm 4.4\%$, $p < 0.05$), 3×10^{-7} ($92.4 \pm 0.9\%$ vs. $66.5 \pm 3.4\%$, $p < 0.05$), and 1×10^{-6} M ($96.6 \pm 0.5\%$ vs. $75.2 \pm 3.3\%$, $p < 0.05$) was significantly higher in the HFD+Ex group than in the HFD group. The endothelium-dependent relaxation with acetylcholine at 1×10^{-8} , 3×10^{-8} , 1×10^{-7} , 3×10^{-7} , and 1×10^{-6} M was also significantly higher in the HFD+Ex group than in the STD group (all $p < 0.05$). Endothelium-dependent relaxation with acetylcholine at 1×10^{-8} ($17.5 \pm 2.8\%$ vs. $2.6 \pm 1.4\%$, $p < 0.05$), 3×10^{-8} ($44.9 \pm 5.7\%$ vs. $12.6 \pm 3.0\%$, $p < 0.05$), 1×10^{-7} ($75.7 \pm 4.0\%$ vs. $40.8 \pm 4.4\%$, $p < 0.05$), 3×10^{-7} ($90.6 \pm 1.2\%$ vs. $66.5 \pm 3.4\%$, $p < 0.05$) and 1×10^{-6} M ($96.0 \pm 0.7\%$ vs. $75.2 \pm 3.3\%$, $p < 0.05$) was significantly higher in the STD+Ex group than in the HFD group. Endothelium-dependent relaxation in response to acetylcholine at 1×10^{-8} , 1×10^{-7} , 3×10^{-7} , and 1×10^{-6} M was also significantly higher in the STD+Ex group than in the STD group (all $p < 0.05$). In addition, endothelial-dependent relaxation with acetylcholine at 1×10^{-8} , 3×10^{-8} , 1×10^{-7} , 3×10^{-7} , and 1×10^{-6} M was significantly higher in

Fig. 3. Correlation between the percent area of atherosclerotic lesions in the thoracoabdominal aorta and the mean running distance per day.

Ascending and descending aortas from HFD + Ex group mice were dissected to expose the endothelial surface, and developed for Sudan IV staining ($n=6$).

Fig. 4. Comparison of relaxation of vascular aortic rings with acetylcholine and sodium nitroprusside among the STD, HFD, HFD+Ex, and STD+Ex groups.

(A) Endothelium-dependent relaxation was elicited by the cumulative concentration of acetylcholine (0.1 nM – $1 \mu\text{M}$) in the STD ($n=12$), HFD ($n=12$), HFD+Ex ($n=11$), and STD+Ex ($n=12$) groups. (B) An endothelium-independent response was elicited by the cumulative concentration of sodium nitroprusside (0.1 nM – $1 \mu\text{M}$) in the STD ($n=12$), HFD ($n=12$), HFD+Ex ($n=11$), and STD+Ex ($n=12$) groups. The relaxation results are expressed as the percent change in the steady-state levels of $0.3 \mu\text{M}$ phenylephrine concentration. Error bars represent SEM. * $p < 0.05$ when compared between the HFD and HFD+Ex groups. # $p < 0.05$ when compared between the STD and HFD+Ex groups. † $p < 0.05$ when compared between the STD and STD+Ex groups. †† $p < 0.05$ when compared between the HFD and STD+Ex groups.

Fig. 5. Comparison of aortic mRNA expression for markers of inflammatory cells, i.e., macrophages, CD4 T cells, and dendritic cells among the STD, HFD, and HFD + Ex groups.

Levels of aortic mRNA expression of CD68, CD4, and CD11c were measured using real-time PCR in the STD ($n=5$), HFD ($n=5$), and HFD + Ex ($n=5$) groups at 18 weeks of age. Error bars represent SD.

the STD + Ex group than in the HFD group (all $p < 0.05$). No significant differences were observed in endothelial-dependent relaxation between the STD + EX and HFD + Ex groups. Endothelium-independent relaxation in response to sodium nitroprusside was not different among the four groups.

Voluntary Running Exercise Decreases Aortic mRNA Expression for Markers of Inflammatory Cells

Fig. 5 shows the levels of aortic mRNA expression for markers of inflammatory cells, i.e., macrophages (CD68), CD4 T cells (CD4), and dendritic cells (CD11c). HFD significantly increased mRNA expression levels of CD68 (1.96 ± 0.78 vs. 1.00 ± 0.40 , $p < 0.05$) and CD11c (2.45 ± 1.11 vs. 1.00 ± 0.48 , $p < 0.05$) in the aortas of ApoE-deficient mice. The expression levels of CD4 (0.66 ± 0.32 vs. 1.60 ± 0.73 , $p < 0.05$) and CD11c (0.97 ± 0.72 vs. 2.45 ± 1.11 , $p < 0.05$) were significantly lower, and those of CD68 (1.06 ± 0.60 vs. 1.96 ± 0.78 , $p < 0.08$) tended to be lower in the HFD + Ex group than in the HFD group.

Voluntary Running Exercise Decreases Circulating Proinflammatory Cytokine Levels and Increases those of the Anti-Inflammatory Cytokine Adiponectin

Fig. 6 shows the levels of circulating proinflammatory cytokines and the anti-inflammatory cytokine adiponectin. HFD increased circulating IL-6 (14.6 ± 11.2 pg/mL vs. 4.3 ± 3.3 pg/mL, $p < 0.05$), TNF- α (4.6 ± 3.2 pg/mL vs. 1.7 ± 0.8 pg/mL, $p < 0.05$), and MCP-1 levels (177.7 ± 75.9 pg/mL vs. 99.4 ± 29.5 pg/

mL, $p < 0.05$), in ApoE-deficient mice. The serum IL-6 (2.5 ± 1.2 pg/mL vs. 14.6 ± 11.2 pg/mL, $p < 0.05$) and MCP-1 levels (68.4 ± 34.3 pg/mL vs. 177.7 ± 75.9 pg/mL, $p < 0.01$) were significantly lower, and those of TNF- α tended to be lower (1.9 ± 1.0 pg/mL vs. 4.6 ± 3.2 pg/mL, $p = 0.07$) in the HFD + Ex group than in the HFD group. Adiponectin levels increased significantly in the HFD + Ex group (17.2 ± 2.2 μ g/mL) compared to the HFD (13.9 ± 2.2 μ g/mL) and STD (14.0 ± 2.2 μ g/mL) groups (both $p < 0.05$).

Discussion

This is the first study demonstrating that voluntary running exercise preserved endothelial function and ameliorated the progression of atherosclerotic lesions in the aortic sinus and thoracoabdominal aorta with a strong negative correlation between atherosclerotic areas and the running distance per day in ApoE-deficient mice. Moreover, Ex decreased the white adipose tissue weight, including that of epididymal fat, without loss of brown adipose tissue, whereas adiponectin levels increased and those of pro-inflammatory mediators, MCP-1, IL-6, and TNF- α , decreased.

Previous studies have shown that exercise training, such as on a treadmill and swimming, reduced atherosclerotic lesion formation and neointimal growth after carotid injury in mice models¹¹⁻¹⁸; however, such unpleasant exercise may be forced under stressful conditions with disruption of the normal diurnal rhythm⁶⁻⁹. In contrast, voluntary running exercise can be performed to assess the underlying mechanisms by which

Fig. 6. Comparison of serum interleukin (IL)-6, tumor necrosis factor (TNF)- α , monocyte chemoattractant protein (MCP)-1, and adiponectin levels among the STD, HFD, and HFD + Ex groups.

Serum IL-6 and TNF- α levels were measured using the Cytometric Bead Array Mouse Soluble Protein Master Buffer Kit in the STD ($n=8$), HFD ($n=6$), and HFD + Ex ($n=6$) groups at 18 weeks of age. Serum MCP-1 and adiponectin levels were measured using commercial mouse ELISA kits in the STD ($n=6$), HFD ($n=6$), and HFD + Ex ($n=6$) groups at 16 weeks of age. Error bars represent SD.

physical activity benefits cardiovascular diseases in a non-stressful environment without disruption of the normal diurnal rhythm. Recent studies have reported that Ex increases the number of circulating endothelial progenitor cells in wild-type¹⁹⁾ and ApoE-deficient mice²⁰⁾ as well as promoting anti-oxidative and anti-inflammatory effects^{20, 21)}, and preserves endothelial function in ApoE-KO mice²¹⁾. Our results provide new evidence that Ex reduces atherosclerotic lesion formation via an increase in adiponectin levels following a reduction in adiposity, especially white adipose tissue.

Adiponectin, which is derived only from adipose tissue and is abundantly present in circulating blood, has been identified as an "adipocytokine" with protective actions against the initiation and progression of

atherosclerosis through anti-inflammatory and anti-atherogenic effects^{22, 23)}. The present study is the first to demonstrate that Ex may reduce atherogenesis with consequent increased circulating adiponectin levels in atherogenic ApoE-deficient mice fed HFD. Although body weight changes did not differ in the HFD + Ex group compared with those in the HFD group, the fat mass of white adipose tissues decreased significantly. A recent clinical interventional study demonstrated that the increase in adiponectin concentration in response to physical exercise was related to a reduction in fat mass and inflammatory factor levels²⁴⁾. Adiponectin mRNA levels in epididymal fat tissue increased in rats after treadmill exercise²⁵⁾. Furthermore, 3T3-L1 adipocytes treated with TNF- α showed decreased adiponectin mRNA expression²⁶⁾, suggesting that TNF- α

may be responsible for regulating adiponectin expression and the production of adiponectin^{23, 27}. In the present study, TNF- α , IL-6 and MCP-1 levels decreased. These cytokines and chemokines play important roles in the pathogenesis of atherosclerosis and an increase in their levels leads to endothelial dysfunction. Therefore, we speculate that Ex may ameliorate endothelial dysfunction and atherosclerotic lesion formation via anti-inflammatory effects as a consequence of increased adiponectin by a reduction in fat mass. Indeed, a significant and negative correlation between adiponectin levels and atherosclerotic areas in the thoracoabdominal aorta was observed (data not shown). In addition, we assessed aortic mRNA expression of adiponectin receptor 1 (*AdipoR1*) and adiponectin receptor 2 (*AdipoR2*) in the three groups. HFD significantly decreased aortic mRNA expression of *AdipoR1* and *AdipoR2* in ApoE-deficient mice. Expression of *AdipoR2* was higher in the HFD + Ex group than in the HFD group (data not shown). To date, the changes in the arterial expression of adiponectin, *AdipoR1*, and *AdipoR2* before and after exercise training remain unclear. Adiponectin has vascular actions that directly stimulate the production of nitric oxide (NO) in endothelial cells using phosphatidylinositol 3-kinase-dependent pathways involving the phosphorylation of eNOS at Ser1179 by AMPK²⁸. Thus, the increase in adiponectin may contribute to improved vasodilatory action in the HFD + Ex group. Two studies have investigated the association between Ex and adiponectin levels in mouse models. Kimura *et al.* reported that Ex does not affect circulating adiponectin levels in Otsuka Long Evans Tokushima Fatty rats²⁹. Bradley *et al.* also showed no significant changes in plasma adiponectin concentration after Ex in wild-type mice made obese by feeding a high fat and sucrose diet³⁰. An explanation for the different results between the present and previously reported studies may be derived from the difference in mouse models.

Kishimoto *et al.* reported that NO and antioxidants may play an important role in the effects of exercise^{13, 18}. In addition, the vascular response to acetylcholine is related to an increase in NO bioavailability as a result of the upregulation of the antioxidant defense system³¹. In the present study, markedly enhanced endothelium-dependent vasorelaxation in mice with Ex was observed compared with mice fed a standard diet. This result suggests that the NO-related system may also contribute to the preserved endothelial function.

Ajjola *et al.* recently reported that voluntary wheel-running exercise for 8 weeks reduces atherosclerotic plaque formation in 20-week-old ApoE-KO mice

fed a normal chow diet, but not in those fed HFD, suggesting that Ex may reduce atherosclerosis progression in mice with early lesions but not in those with advanced complex lesions²⁰. In the present study, Ex for 10 weeks reduced atherosclerotic lesion formation in 8-week-old ApoE-KO mice even on HFD. Laufts *et al.* reported that endothelium-dependent but not endothelium-independent vasorelaxation improved after voluntary wheel running exercise for 6 weeks in ApoE-deficient mice fed HFD²¹. Significant differences in endothelium-dependent vasorelaxation induced by acetylcholine between the HFD + Ex and HFD groups as well as between the HFD + Ex and STD groups were observed in the present study. These results suggest that a long period (at least 10 weeks) of voluntary running exercise may reduce the progression of atherosclerosis even in mice fed HFD that have advanced atherosclerotic lesions. In addition, endothelium-dependent vasorelaxation in the STD + Ex as well as HFD + Ex groups was significantly higher than in the STD group. These results may suggest that exercise itself could improve acetylcholine-induced relaxation regardless of the diet composition in ApoE-deficient mice. No significant differences were observed in lipid profiles between the HFD and HFD + Ex groups in the present study; however, the levels of total cholesterol and low-density lipoprotein cholesterol were significantly lower in the STD + Ex group than in the HFD and HFD + Ex groups. Furthermore, low-density lipoprotein cholesterol levels were also significantly lower in the STD + Ex group than in the STD group (data not shown); therefore, we could not reject the fact that the difference in lipid profiles between the STD + Ex and STD groups could affect endothelium-dependent vasorelaxation. We would like to clarify the precise mechanism underlying the anti-inflammatory effect of voluntary exercise in the four groups (STD/STD + Ex/HFD/HFD + Ex) in the next step.

A strong and significant correlation between the amount of exercise and the area of atherosclerotic lesion in the thoracoabdominal aorta was observed in the present study. We also confirmed the correlation between rotations (rpm/day) and atherosclerotic lesions in the aortic sinus. A moderate and negative correlation between rotations and atherosclerotic lesions in the aortic sinus was also observed, but it was not statistically significant (data not shown). There are several possible explanations, including the small number of observations in this experiment and differences in atherosclerotic process in the aortic sinus and thoracoabdominal aorta. This point will be clarified in future studies.

Conclusion

In conclusion, Ex ameliorated the progression of endothelial dysfunction and atherosclerotic lesion formation with a strong negative correlation between atherosclerotic areas and the mean running distance per day in ApoE-deficient mice, despite continued consumption of HFD. These protective roles of Ex against the progression of atherosclerosis might be derived from its anti-inflammatory effects, including an increase in adiponectin concentration followed by a decrease in the weight of white adipose tissue without a decrease in that of brown adipose tissue.

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