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Exercise attenuates matrix metalloproteinase activity in preexisting atherosclerotic plaque

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ABSTRACT

Objective: Few studies have investigated if exercise by itself has anti-atherosclerotic effects, without combining interventions with a low-fat diet. We studied the effects of exercise as a stand-alone intervention on preexisting atheromata by measuring not only plaque size but also the levels of plaque-destabilizing matrix-metalloproteinase (MMP) activity *in vivo*.
Methods and results: We used near-infrared fluorescent (NIRF) molecular imaging with an MMP-2/9 activatable NIRF probe to visualize the inflammatory protease activity within preexisting atheromata of 17-week-old ApoE$^{-/-}$ mice on: (a) normal chow diet (NCD), (b) Western diet (WD), and (c) WD with treadmill exercise for 10 weeks. We also measured tissue levels of aortic lipid peroxidation (LPO) and plasma levels of glucose/lipid/cytokine profiles. Exercise did not attenuate growth of preexisting atheromatous plaques. However, exercise strongly decreased proteolytic activity in plaques for animals on WD, with levels decreasing almost to NCD levels. Exercise was associated with decreased aortic LPO levels and increased blood adiponectin/leptin levels; however, exercise did not affect WD-consumption/weight-gain or improve blood glucose/lipid profiles.
Conclusions: Exercise training reduced aortic MMP activity in mice with preexisting atheromata, even though they remained on a high fat diet and plaque-growth was not attenuated.

1. Introduction

Exercise and physical activity have anti-atherosclerotic effects in humans and animal models [1,2]. In subjects with elevated cardiovascular risk, endurance training significantly decreased circulating inflammatory markers including interleukin (IL)-8, monocyte chemoattractant protein-1 (MCP-1), and matrix metalloproteinase-9 (MMP-9) [3]. Exercise could directly affect the homeostasis of the vessel wall [4] as well as modify vascular risk factors, for instance by lowering blood pressure, improving body composition, glucose tolerance, insulin sensitivity, lipid profile, and thrombotic function [5].

One key mechanism of plaque destabilization by macrophages is the generation and secretion of MMPs [6,7]. MMPs degrade the collagen and elastin components of the matrix and contribute to atherosclerotic plaque rupture [6,7], causing thromboembolic complications such as stroke or myocardial infarction. Recently, we showed that near-infrared fluorescent (NIRF) molecular imaging closely reports on plaque inflammation by sensing the activity of MMP or cathepsin-B proteolytic enzymes in or around the carotid bifurcation area and ulcero-hemorrhagic lesions of human carotid plaques [8]. NIRF imaging has also been used to follow statin-mediated therapeutic effects on plaque inflammation [9].

Despite the well known modulation of risk factors by exercise, and its frequent recommendation as a life style change to patients

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with atherosclerosis, there have been few studies that investigated the anti-atherosclerotic effects of exercise [10] as a single factor in preexisting plaques. It is unknown if exercise by itself, without change to a healthier low-fat diet, could attenuate growth of preexisting plaques. This general gap in our knowledge is addressed in this study, where we attempt to separate out the beneficial effects of exercise and a low-fat diet in an animal model of atherosclerosis. We specifically investigate the effects of exercise on the phenotype of inflamed, vulnerable-type plaques.

We hypothesized that exercise decreases lesion size and attenuates inflammation in preexisting plaques. We used NIRF molecular imaging based on an MMP-activatable NIRF probe [8] to visualize the inflammatory protease activity within preexisting atheromata of Apolipoprotein-E (ApoE) knock-out ($ApoE^{-/-}$) mice on a Western diet (WD). Interventions tested were a daily 30 min treadmill work out. While antioxidant therapy was reported to inhibit activation and secretion of the precursor MMP-9 [11], there have been conflicting results about oxidative stress and exercise [12]. Thus, we also studied if exercise training has anti-oxidative effect on the atheromata. Moreover, exercise may change blood levels of cytokines [3] that could affect plaque inflammation or oxidative stress, which led us to perform cytokine analysis.

2. Materials and methods

All experiments were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

2.1. Animals and experimental procedures

Eight-week-old $ApoE^{-/-}$ mice (20–25 g, $n = 68$) were purchased (Japan-SLC, Shizuoka, Japan) and maintained in a controlled environment of 20 °C and 40–50% humidity, with 12 h of light per 24 h period. The diet and water were available *ad libitum*. Food and water intake and body weight were monitored weekly. After one week of adaptation, mice were fed a normal chow diet (NCD, $n = 15$) or a WD ($n = 26$) for 8 weeks.

Thereafter, 4 μmol MMP-2/9 probe (synthesized as described before [8]) in 150 μL PBS was intravenously injected into 14 randomly selected mice (6 animals fed NCD, 8 animals fed WD). Four hours after fasting and 24 h after tail vein injection of the probe, blood was collected from the retro-orbital plexus and was centrifuged to measure total cholesterol, low density lipoprotein (LDL) + very low density lipoprotein (VLDL) cholesterol, high density lipoprotein (HDL) cholesterol, and glucose levels using a commercial assay kit (EHDL-100 and DIGL-200, BioAssay-Systems, Hayward, CA). The animals were euthanized, and the aortas, after being inspected and photographed using a digital camera attached to a surgical microscope (S6E-zoom-stereomicroscope, Leica-Microsystems, Heerbrugg, Switzerland), were carefully excised. In all animals, we confirmed the presence of atherosclerotic plaques in the aorta or branch arteries (Fig. 1).

The remaining animals ($n = 27$) were fed the same NCD (NCD group; $n = 9$) or WD ($n = 18$) for additional 10 weeks; during this period, half of the animals on a WD were trained to run on a treadmill (WD + exercise, WD + E group; $n = 9$). A control cohort also remained on the WD but did not exercise (WD group; $n = 9$). At the end of the study period, all animals underwent digital photography and MMP-2/9 NIRF reflectance imaging of the aortas. After the imaging, the aortas were embedded in paraffin for histology including Masson-Trichrome (MT) staining and immunohistochemistry for macrophages (Mac-3), MMP-2, and MMP-9, while saving the aortic root tissue for the measurement of lipid peroxidation (LPO) as indicator of oxidative stress using commercially available assay kits (Cayman, Ann Arbor, MI) after homogenization of the tissue.

In the second set of experiments, we studied eight-week-old $ApoE^{-/-}$ mice ($n = 27$; 9/group) for 19 weeks under the same protocol described above. Additionally, we performed 1) oil-red-o staining of the entire aorta and 2) Luminex bead-based multiplexing assay (Millipore, Billerica, MA) of serum MMP-9 and cytokines including soluble vascular cell adhesion molecule-1 (sVCAM-1), IL-1β, IL-6, IL-10, adiponectin, resistin, leptin, MCP-1, plasminogen activator inhibitor-1 (PAI-1), and tumor necrosis factor (TNF-α).

2.2. Exercise training

The animals in the WD + E group were trained on a treadmill with a rubber belt driven at a controlled speed (DJ-344, Daejong, Korea) for 30 min/day, 5 days/week. For the exercise acclimation, the treadmill speed was 7 m/min on the first day, and then it was increased by 1 min each day until the maximum speed of 17 m/min was reached at the end of the second week; thereafter, the speed was kept constant. All mice tolerated the exercise protocol well throughout the study, and they did not show any signs of exhaustion.

2.3. NIRF imaging and quantification of MMP activity

Ex vivo NIRF imaging and lesion quantification were performed as reported previously [8,9]. The excised aortas were washed with PBS three times and imaged *ex vivo* by using a NIRF imaging device with a charge-coupled device camera (CoolSnap-EZ, Roper-Scientific, Tucson, Az). White light and Cy5.5 NIRF images (excitation/emission, 675/690 nm; 1-second acquisition) were acquired. After normalization [8], the mean NIRF signal intensities of the entire aorta or subdivisions were quantified using the histogram function of Adobe Photoshop CS-3-Extended (Adobe Systems, San Jose, CA).

2.4. Measurement of plaque size

Quantification of atherosclerotic lesion size ($n = 9$ animals/group) was performed by using five equidistant longitudinal aortic sections (4 μm-thick) stained with MT: one section at midline and four sections at 40 or 80 μm apart from the midline, anteriorly and posteriorly [9]. In the second set of experiments, atherosclerotic lesion size was determined ($n = 5$ randomly selected animals/group) using the en face method after oil-red-o staining [9]. Adobe Photoshop was used to segment lesion areas and to calculate the percentage of the aortic tissue covered by atherosclerotic lesions.

2.5. Immunohistochemistry and quantification of Mac-3 or MMP-positive areas

Immmunohistochemistry for Mac-3, MMP-2 or MMP-9 was performed using the avidin-biotin-peroxidase method as previously reported [8]. Immuno-positive areas were quantified by using three equidistant longitudinal aortic sections: one section at midline and two sections at 80 μm apart from the midline, anteriorly and posteriorly. The extent of brown-colored immuno-positive areas was quantified with the color range function and measured using the histogram function of Adobe Photoshop. Values are reported as a percentage of the aortic tissue covered by the lesion divided by the total area of the aortic tissue.

2.6. Data analysis

Student's t-tests, one-way analysis of variance (ANOVA) with Dunnett's post hoc tests, or Kruskal–Wallis ANOVA with