Chronic Exercise Improves Endothelial Calcium Signaling and Vasodilatation in Hypercholesterolemic Rabbit Femoral Artery

Chaoying J. Jen, Hung-Pin Chan, Hsiun-ing Chen

Objective—This study was to investigate the effects of chronic exercise on vasodilatation and endothelial intracellular calcium (EC [Ca$^{2+}$]) signaling in atherosclerotic animals.

Methods and Results—For 8 weeks, male New Zealand White rabbits were fed rabbit chow with or without the addition of 2% cholesterol. They were further divided into control and exercise groups. Animals in the exercise groups ran on a leveled treadmill at 0.88 km/h for 10 to 60 minutes gradually for 5 days per week for a total of 8 weeks. At the end of experiments, femoral arteries were dissected, loaded with fura 2-AM, and mounted in a tissue flow chamber. PE-precontracted vessel specimens were exposed to acetylcholine (ACh). The EC [Ca$^{2+}$], elevation and vasorelaxation were determined simultaneously under an epifluorescence microscope equipped with a ratio-imaging capability. Our results showed the following: (1) high cholesterol diet feeding caused lipid deposition on vascular surface, reduced the ACh-evoked EC [Ca$^{2+}$] elevation, and impaired endothelium-dependent and endothelium-independent vascular responses, but chronic exercise had the opposite effects; (2) ACh-induced vasorelaxation was associated with EC [Ca$^{2+}$] elevation in all groups; and (3) vasorelaxation at high levels of EC [Ca$^{2+}$] elevation decreased in hypercholesterolemia.

Conclusions—Our data suggest that hypercholesterolemia induces vascular structural changes and impairs EC [Ca$^{2+}$] signaling and vasodilatation, whereas chronic exercise partially reverses these adverse effects. (Arterioscler Thromb Vase Biol. 2002;22:1219-1224.)

Key Words: chronic exercise • high cholesterol diet • nitric oxide • calcium signaling • vasodilators

Atherosclerosis is the leading cause of mortality in the developed world, and it is possibly caused by a high-fat diet and a sedentary lifestyle.1 Risk factors of atherosclerosis, ie, elevated LDL cholesterol levels, diabetes mellitus, hypertension, homocystinemia, and smoking, are associated with vascular dysfunction, such as monocyte adhesion and invasion, smooth muscle proliferation and migration, platelet activation, and extracellular matrix formation.2 Moreover, the atherogenesis-related endothelial impairment occurs well before any detectable structural changes of the vessel wall.3 Normally, the vascular endothelial cells release NO.4 NO relaxes vascular smooth muscle, inhibits platelet adhesion/aggregation, inhibits monocyte adhesion and migration, reduces the production of superoxide, inhibits oxidation of LDL, and inhibits smooth muscle proliferation and migration. Therefore, it is considered to be an endogenous antiatherosclerotic factor.5 It is likely that the impairment of endothelium-derived NO activity is a cause, not a consequence, of atherosclerosis.

Current consensus indicates that regular exercise reduces the incidence of cardiovascular diseases and death or causes a regression of symptoms.1,6–8 Several possible mechanisms of these exercise effects have been proposed, such as an increase in HDL cholesterol level,9,10 a decrease in the oxidation of LDL cholesterol or total cholesterol levels,10,11 a decrease in the production of atherogenic cytokines (eg, interleukin-1α, tumor necrosis factor-α, and interferon), and an increase in the production of atheroprotective cytokines (eg, interleukin-4 and transforming growth factor-β1).12 We and other investigators have reported that exercise training increases agonist-stimulated NO release and enhances endothelium-dependent vasodilatation in vessels obtained from normal or hypertensive animals.13–15 Whether the improvement of endothelial function by chronic exercise occurs in atherosclerosis has not been proved yet. Therefore, the first purpose of the present study was to investigate the effect of chronic exercise on endothelium-dependent vasodilating responses in rabbits made atherosclerotic by a high cholesterol diet.

Many receptor-mediated agonists, such as acetylcholine (ACh), affect cellular function via generating intracellular calcium signals.16–18 Besides, endothelial NO synthase (eNOS) is known to be a calcium-dependent enzyme.19 It is likely that agonist-evoked endothelial intracellular calcium (EC [Ca$^{2+}$]) signaling is involved in the exercise-induced vascular adaptation. Our previous study20 has demonstrated that chronic exercise increases the ACh-evoked EC [Ca$^{2+}$] elevation response in normal rats. Therefore, the second

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purpose of the present study was to evaluate the role of ACh-evoked EC [Ca\(^{2+}\)], signaling in the effects of exercise in hypercholesterolemic rabbits.

### Methods

#### Animals and Diet

The present study was conducted in conformity with the policies and procedures detailed in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health, and the procedures followed were in accordance with institutional guidelines. Male New Zealand White rabbits (weighing \(\sim 1\) kg at the beginning) were divided into 4 groups: the normal diet control (NC) group, the normal diet with exercise training (NE) group, the high cholesterol diet control (CC) group, and the high cholesterol diet with exercise training (CE) group. The control groups were fed normal rabbit chow, whereas the high cholesterol diet groups were fed a 2% high cholesterol diet (PMI Feeds Inc). They were housed in an environmentally controlled room.

#### Exercise Protocol

A training protocol similar to that discussed in our previous study \(^{14}\) was used. After 1 week of familiarization, the exercise training groups ran on a leveled treadmill (model Q55, Quinton Instrument Co) at a speed of 0.88 km/h for 10 minutes for the first week. On subsequent training weeks, the running time was extended 5 to 10 minutes each week until they could run for 60 minutes per day. They were trained for 5 days per week for a total of 8 weeks. According to our previous study, this training intensity was \(\sim 70\%\) of their maximal exercise capacity. In contrast, the sedentary groups were placed on the treadmill for 10 minutes each day without receiving any exercise training.

At the end of experiments, rabbits were anesthetized by injecting ketamine (30 mg/kg) and pentobarbital (20 mg/kg) into their ear veins. Blood samples were drawn from the inferior vena cava for lipid profile determination. Femoral arteries were then isolated for various experiments described below.

#### Vessel Preparation and Fura 2 Loading

The femoral arteries were excised and cut into rings (5 mm long), which were stored in an organ chamber containing Krebs-Ringer solution bubbled with 95% O\(_2\)/5% CO\(_2\) (22°C, pH 7.4). This solution had the following composition (in mmol/L): NaCl 118.0, KCl 4.8, CaCl\(_2\) 2.5, MgSO\(_4\) 1.2, KH\(_2\)PO\(_4\) 1.2, NaHCO\(_3\) 24, Na\(_2\)-EDTA 0.03, and glucose 11.0. Vessel rings were fluorescently labeled by incubation with 10 \(\mu\)mol/L fura 2-AM and 0.025% Pluronic F-127 in Krebs-Ringer solution for 1 hour. \(^{21}\) Extracellular fura 2-AM was washed away afterward. As in rat aortas, our preliminary results indicated that the endothelial fluorescence signals in rabbit femoral arteries were basically free of contaminated signals from the smooth muscle layer underneath.

#### Simultaneous Measurements of ACh-Induced EC [Ca\(^{2+}\)] Responses and Vasorelaxation in Phenylephrine-Precontracted Vascular Segments

To allow simultaneous measurements of vascular EC [Ca\(^{2+}\)], and smooth muscle contraction, the vessel segment was mounted as described previously. \(^{22,23}\) In brief, fura 2-loaded vessel rings were opened longitudinally and pinned onto the base plate of a tissue flow chamber. One side of the longitudinally opened vessel segment was fixed in the direction of blood flow with insect pins. The corners on the opposite side were passively stretched and pinned onto the base plate. This arrangement allowed free movement of the central portion of the specimen when vasoactive chemicals were added. After vessel mounting, the tissue flow chamber was mounted on an inverted microscope with epifluorescence attachments (Diaphot 300, Nikon) and perfused with Krebs-Ringer buffer at 30°C under a constant flow rate of 0.7 mL/min for an equilibration period of 1 hour.

For the measurement of EC [Ca\(^{2+}\)], the 510-nm fluorescence images excited at 340 or 380 nm were recorded by a high-sensitivity SIT camera (model C2400-08, Hamamatsu). Axon image workbench software (Axon Instruments) was used to acquire, digitize, and store the experimental results for offline processing. The average value of EC [Ca\(^{2+}\)], was calculated by monitoring a large area in the mainstream region of an opened femoral segment, covering \(\sim 0.15 \text{ mm}^2\) of tissue surface, or >200 cells. At the end of each experiment, the calcium concentration was calibrated by the established methods. \(^{24}\) In brief, the calcium concentration was calibrated by applying ionomycin (5 \(\mu\)mol/L) in the presence of 5 mmol/L EGTA, followed by 10 mmol/L CaCl\(_2\). Finally, the background autofluorescence was determined by exposing the tissue to 5 mmol/L manganese to quench cytosolic fura 2 fluorescence. EC [Ca\(^{2+}\)], was calculated after subtracting the background autofluorescence by using the following equation: \[ [\text{Ca}^{2+}] = K_h (R - R_{min})/(R_{max} - R) \]\(,\) where \(K_h\) is the dissociation constant (\(\sim 224 \text{ mmol/L}\)), \(R\) is the ratio of 340 to 380 nm during measurements, \(R_{max}\) is the 340/380 ratio in the presence of saturating calcium levels, \(R_{min}\) is the 340/380 ratio in calcium-free solution, and \(B\) is the ratio of the fluorescence at 380 nm in calcium-free solution to the fluorescence at 380 nm in saturated CaCl\(_2\) solution.

The dose responses of ACh-induced EC [Ca\(^{2+}\)], elevation and vascular displacement were determined in the phenylephrine (PE, 5 \(\times 10^{-7}\) mol/L)–precontracted vessel segment by subsequent exposure to cumulative ACh (10\(^{-9}\) to 10\(^{-3}\) mol/L). The relative movement of endothelial cells was used as an index of vascular tone, whereas fluorescence ratio images from fura 2–labeled endothelial cells provided quantitative information of EC [Ca\(^{2+}\)]. \(^{25}\) To calculate the vascular displacement, MetaMorph software (Universal Imaging Corp) was used to trace and analyze a series of images. The fluorescence image was focused sharply, and a particular endothelial cell in the initial image was selected as a location marker. In the presence of PE, this image was shifted inward during experiments. After the marker had reached its final equilibrium location, the total displacement represented the extent of vascular contraction induced by PE. When the preparation was subsequently exposed to ACh, the marker moved back toward its initial location, indicating vasorelaxation. The percentage changes in vascular tone were normalized by the PE precontraction value.

To evaluate the contribution of NO to the effects of exercise, some specimens were pretreated with indomethacin (10\(^{-5}\) mol/L) and tetraethylammonium (TEA, 10\(^{-3}\) mol/L) for 15 minutes to restrain the effects from prostacyclin (PGI\(_2\)) and endothelium-derived hyperpolarization factor (EDHF). \(^{14,25}\)

#### Vascular Responses to A23187 or SNP

In normal rabbit aortas, chronic exercise does not alter the vascular responses either to A23187, a calcium ionophore that induces endothelium-dependent vasodilatation without receptor activation, or to sodium nitroprusside (SNP), an endothelium-independent vasodilator. \(^{4}\) It is unknown whether these responses are impaired in hypercholesterolemic rabbits. If they are, it is interesting to investigate whether the impaired responses can be reversed by exercise. Therefore, the vascular responses to A23187 (10\(^{-6}\) mol/L) or SNP (10\(^{-7}\) mol/L) were evaluated in some PE (5 \(\times 10^{-7}\) mol/L)–precontracted femoral arteries. The level of vasorelaxation was expressed by the percentage of the PE precontraction value.

#### Determination of Serum Lipid Profile

Blood samples were collected from the inferior vena cava when the animals were under general anesthesia. The serum lipid profiles, including total cholesterol, triglycerides, HDL, and LDL, were determined by using an automatic analyzer (model 747, Hitachi Ltd).

#### Citrate Synthase Activity Assay

It is generally accepted that exercise training increases oxidative enzyme activity. An increase in citrate synthase activity is a common biochemical method to confirm the exercise training effect. \(^{14}\) Therefore, we examined citrate synthase activity of the soleus muscle for
all animal groups. Mitochondrial citrate synthase activity was measured from whole muscle homogenates by using the method of Sere27; ie, the citrate synthase activity was determined spectrophotometrically at 412 nm. The activity was expressed as micromoles of substrate used per minute per gram of tissue.

Histological Studies of Blood Vessels
Standard hematoxylin/eosin staining was performed on frozen sections of vessel segments (5 μm thick) for analysis of morphological details by light microscopy. In addition, en face oil red O staining was used to evaluate lipid deposition on the inner surface of longitudinally opened vessel segments (5 mm long). The image analysis of lipid deposition was performed by using Image-Pro Plus (Media Cybernetics), and the results of stained areas were normalized against the total measured surface area.

Statistical Analysis
Data are expressed as mean±SEM. Sample sizes are indicated by the letter n. Dose responses of ACh-induced EC [Ca^{2+}]_{i} elevation or vasorelaxation were analyzed by ANOVA with a repeated-measures design. Others were analyzed by ANOVA and further evaluated by the Scheffé F test. If only 2 groups were compared, the unpaired Student t test was applied. Differences were considered at P<0.05.

Results

Serum Lipid Profile
After 8 weeks of high cholesterol diet feeding, serum levels of total cholesterol, HDL, and LDL were significantly increased in the CC and CE groups (Table). These observations indicated that high cholesterol diet feeding indeed induced hypercholesterolemia in our animal models. Nonetheless, chronic exercise did not affect serum lipid profile in the present study (Table).

Citrate Synthase Activity of Soleus Muscle
Citrate synthase activities in soleus muscles of 2 exercise training groups (ie, NE and CE groups) were higher than the activities in their control groups (for normal diet, 1.32±0.02 versus 1.51±0.02 μmol/min per gram wet weight for the control and exercise groups, respectively; for high cholesterol diet, 1.33±0.02 versus 1.43±0.03 μmol/min per gram wet weight for the control and exercise groups, respectively; P<0.01, n=9). These results indicated that our training protocol was effective.

Oil Red O Staining and Histological Examination
All vessels in the CC group (n=8) had scattered lipid deposition, whereas only 3 of 8 in the CE group showed lipid deposition. Statistical analysis of the results of oil red O staining indicated that lipid deposition in the CC group was more severe than that in the CE group (11±3% versus 2±1% for CC versus CE groups, respectively; P<0.01). In contrast, there was no lipid deposition in either NC or NE groups. We also noticed that intimal thickening was observed in some vascular sections of the CC group but was not found in the CE group (Figure 1).

Comparison of ACh-Induced EC [Ca^{2+}]_{i} Responses and Vasorelaxation in PE-Precontracted Vascular Segments Between Control and Exercise Groups
When the specimen precontracted with PE (5×10^{-5} mol/L) was subsequently exposed to various concentrations of ACh, EC [Ca^{2+}]_{i} elevation and concomitant vascular relaxation happened in a dose-dependent manner. Dose responses of ACh-evoked EC [Ca^{2+}]_{i} elevation in PE-precontracted vessel segments are shown in Figure 2A. The results indicated that high cholesterol feeding reduced, but chronic exercise im-
proved, ACh-evoked EC $[\text{Ca}^{2+}]_i$ elevation ($P<0.05$). The ACh-induced vasorelaxation was expressed as percentage of PE-induced contractile displacement for normalization. When the dose responses of ACh-induced vasorelaxation were compared, it was also found that high cholesterol diet feeding reduced, whereas chronic exercise enhanced, these responses (Figure 2B, $P<0.05$). When PGI$_2$ and EDHF were inhibited, the previously mentioned effects of high cholesterol diet feeding and chronic exercise on ACh-induced EC $[\text{Ca}^{2+}]_i$ elevation and vasorelaxation largely remained (Figure 3). These results imply that high cholesterol diet feeding impairs NO release and that chronic exercise enhances it.

When the ACh-evoked vasorelaxation was plotted against the corresponding EC $[\text{Ca}^{2+}]_i$ elevation by using the data from Figure 2, the logarithmically fitted curves were almost identical in control and exercise groups whether they were fed the normal or the high cholesterol diet (Figure 4). Chronic exercise did not change the curved shape; rather, it extended the curve to the higher level range. To examine the effects of high cholesterol feeding on this EC $[\text{Ca}^{2+}]_i$–vasorelaxation relationship, results obtained from the NC and CC groups were compared in Figure 5. Interestingly, high cholesterol feeding altered this relationship when the EC $[\text{Ca}^{2+}]_i$ elevation was $>100$ nmol/L. That is, when EC $[\text{Ca}^{2+}]_i$ elevation was $>100$ nmol/L, vasorelaxation in the femoral arteries of hypercholesterolemic rabbits was less than that in normal rabbits. In addition, the EC $[\text{Ca}^{2+}]_i$–vasorelaxation relationship was unaltered by pretreatment of indomethacin and TEA (data not shown).

**Vascular Responses to A23187 and SNP**

The A23187 ($10^{-8}$ mol/L)–induced vasorelaxation in the NC group was $21\pm6\%$. In accordance with our previous report,$^{14}$ exercise in the normal diet group (ie, the NE group) did not significantly alter this response ($24\pm3\%$). However, high cholesterol diet feeding impaired the A23187-evoked vasorelaxation, and this impairment was reversed by chronic exercise (5±2% and 16±3% for CC and CE groups, respectively; $P<0.05$). Similar results were observed in SNP ($10^{-7}$ mol/L)–induced vasorelaxation (NC group, 47±4%; NE group, 48±4%; CC group, 28±2%; and CE group, 40±6%; $P<0.05$ for CC versus NC groups and for CE versus CC groups).

**Discussion**

Our results indicated that (1) 2% high cholesterol diet feeding for 8 weeks increased serum levels of total cholesterol, HDL, and LDL in rabbits and induced lipid deposition and intimal...
thickening in the rabbit femoral artery; (2) 8 weeks of chronic exercise reduced lipid deposition and the occurrence of intimal thickening in femoral arteries without significantly changing serum lipid profile in hypercholesterolemic rabbits; (3) high cholesterol diet feeding reduced, whereas chronic exercise enhanced, the responses of ACh-evoked EC [Ca\(^{2+}\)]

...remained when PGI\(_2\) and EDHF were both blocked, indicating that NO was the major contributing factor for these effects; (5) ACh-induced vasorelaxation was well associated with EC [Ca\(^{2+}\)], elevation in all the groups; and (6) chronic exercise did not change the relationship between vasorelaxation and EC [Ca\(^{2+}\)], elevation but extended the curve to a higher level range. However, high cholesterol feeding altered the relationship between these 2 parameters; ie, the vasorelaxation was impaired at the higher level range of EC [Ca\(^{2+}\)], elevation in hypercholesterolemic animals.

It has been reported that chronic exercise increases endothelium-dependent vasodilatation in the aortas of normal rabbits or rats. Our results suggested that 8-week chronic exercise improved endothelium-dependent vasodilating responses in the femoral arteries of hypercholesterolemic rabbits as well. In addition, the reduced lipid deposition and intimal thickening that occurred after chronic exercise indicated that our exercise protocol could ameliorate the progression of atherosclerosis. Endothelial dysfunction occurs in the early stage of atherosclerosis. In the present study, atherosclerotic lesion and endothelial dysfunction were observed after 8 weeks of high cholesterol diet feeding in rabbits. It would be interesting to examine the time course of the changes of these parameters by using animals on a high-cholesterol diet for shorter time periods.

To clarify whether the receptor-independent or endothelium-independent vasodilating responses were also affected by interventions of high cholesterol diet feeding or exercise, we examined vascular responses to A23187 or SNP. Our results indicated that vasodilating responses to these agonists were also impaired in femoral arteries of hypercholesterolemic rabbits. Previous reports demonstrated that 0.1% or 0.2% cholesterol diet feeding did not affect SNP-elicited vasorelaxation in rabbit aortas, whereas 1% cholesterol diet feeding caused a reduction in SNP responses.

In the present study, we fed rabbits an even higher dose (ie, 2%) cholesterol diet, which led to scattered lipid deposition in the femoral arteries of all nonexercised rabbits. Therefore, structural alterations in femoral arteries of hypercholesterolemic rabbits may impair endothelium-dependent and endothelium-independent vasorelaxation. This is consistent with our observation that high cholesterol feeding alters the relationship between EC [Ca\(^{2+}\)], elevation and vasorelaxation (Figure 5). Therefore, not only endothelium but also smooth muscle cells are affected by high cholesterol diet intervention.

Chronic exercise enhanced the ACh-evoked EC [Ca\(^{2+}\)], elevation and vasorelaxation without altering the relationship between these 2 parameters (Figure 4). On the basis of this observation alone, it seemed that the effects of exercise on vascular responsiveness were mainly on the endothelium, especially on EC [Ca\(^{2+}\)], signaling. However, we also noticed that chronic exercise reduced lipid deposition and the occurrence of intimal thickening and improved SNP- or A23187-induced vasorelaxation in hypercholesterolemic rabbits, indicating that the structural/functional alterations of femoral arteries in hypercholesterolemic rabbits were improved by chronic exercise as well. In comparison, exercise in rabbits fed a normal diet enhanced only ACh-induced, but not SNP- or A23187-induced, vasorelaxation. Thus, the exercise effects in normal animals are most likely focused on the endothelium, and they are receptor-mediated. In fact, previous studies using normal rats have provided evidence that endothelial receptors are upregulated by exercise. Similar mechanisms may also partially explain the endothelium-dependent part of the exercise effects seen in hypercholesterolemia. Taken together, it appears that the exercise effects in normal animals were mediated by improving endothelial function. In high cholesterol-fed animals, however, the exercise effects were due to improvements of endothelial function and vascular structure.

EC [Ca\(^{2+}\)], signaling has been shown to serve as an integrating signal for endothelium-dependent vasorelaxation in rat aortas. In the present study, we found that ACh-evoked EC [Ca\(^{2+}\)], elevation and vasorelaxation were impaired in hypercholesterolemic rabbit femoral arteries, whereas chronic exercise enhanced ACh-evoked EC [Ca\(^{2+}\)], response and improved the endothelium-dependent vasorelaxation. Because eNOS is a calcium-dependent enzyme, this increased EC [Ca\(^{2+}\)], signaling could be a key factor responsible for the enhanced NO-dependent vasodilation after chronic exercise. Because EC [Ca\(^{2+}\)], signaling has also been reported to be improved in normal rats given a single bout of exercise, there may be a common pathway or stimulus for these exercise effects, such as elevated blood flow or shear stress. Previously, we have shown that either flow pretreatment or exercise enhances ACh-evoked EC [Ca\(^{2+}\)], elevation by facilitating the calcium influx. Moreover, gadolinium, the mechanosensitive cationic channel blocker, reduces the calcium response to ACh in the exercise groups but has little effect in the control groups. This suggests that mechanosensitive cationic channels upregulated by flow during exercise may partially account for the enhanced EC [Ca\(^{2+}\)], signaling after chronic exercise.

It is interesting to note that endothelium-derived NO is not just a downstream effector of EC [Ca\(^{2+}\)], but that these 2 intracellular messengers may interact with each other. Existing evidence not only supports the notion that eNOS is a Ca\(^{2+}\)-dependent enzyme and that ACh-induced vasorelaxation is coupled to EC [Ca\(^{2+}\)], elevation, but also indicates that NO is capable of inducing EC [Ca\(^{2+}\)], elevation in culture. Our previous study also suggests that a high concentration of ACh-stimulated NO after exercise is capable of evoking EC [Ca\(^{2+}\)], elevation. Therefore, large amounts of ACh-stimulated endothelial NO release in the exercise-trained animals may promote the EC [Ca\(^{2+}\)], response, which further activates eNOS and enhances endothelium-dependent vasorelaxation in a positive-feedback manner. In addition, eNOS gene upregulation by chronic exercise may be partially responsible for the elevated vascular responses as well. Recent studies suggest that exercise training upregulates NO
synthase gene expression in normal animals.35–37 In contrast, eNOS expression is impaired in atherosclerosis.38 It is plausible to assume that exercise-induced NO synthase gene expression may compensate for the defect of eNOS gene expression in hypercholesterolemic rabbits. However, this viewpoint needs to be further clarified.

In conclusion, long-term high cholesterol diet feeding induces vascular structural alterations and impairs EC [Ca \textsuperscript{2+}] signaling and vasodilatation, whereas chronic exercise partially reverses these adverse effects.

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