Short-Term Exercise Training Improves Vascular Function in Hypercholesterolemic Rabbit Femoral Artery

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Abstract

Chronic exercise in healthy or hypercholesteremic animals for at least two months improves their vascular functions. This study is to examine whether short-term exercise training protocols can correct early-stage vascular dysfunction induced by high-cholesterol diet feeding. Male New Zealand White rabbits were fed for 2, 4 or 6 weeks with rabbit chow with or without the addition of 2% (w/w) cholesterol. They were further divided into control and exercise groups. Animals in exercise groups ran on a leveled treadmill for the same time periods as diet intervention. At the end of experiments, femoral arteries were dissected, loaded with fura 2-AM, and mounted in a tissue flow chamber. Phenylephrine-precontracted vessel specimens were exposed to acetylcholine. The endothelial intracellular calcium elevation and vasorelaxation were determined simultaneously under an epifluorescence microscope with ratio imaging capability. *En face* oil red O staining was used to evaluate fatty streak formation. Our results showed that 1) high-cholesterol diet feeding for ≥ 4 weeks caused lipid deposition, reduced the acetylcholine-evoked endothelial calcium signaling, and impaired both endothelium-dependent and endothelium-independent vascular responses in a time-dependent manner; 2) vasorelaxation at given levels of endothelial intracellular calcium elevation decreased in hypercholesterolemia; 3) concomitant exercise program had reverse effects. We conclude that high-cholesterol diet intervention for as short as 4 weeks induces vascular structural changes, impairs endothelial intracellular calcium signaling and vasodilatation in rabbit femoral arteries. Short-term exercise training in parallel completely eliminates these adverse effects so long as the diet intervention is no more than 6 weeks.

Key Words: acetylcholine, atherosclerosis, calcium, endothelium, vascular dysfunction

Introduction

Being a leading cause of mortality, atherosclerosis in the developed world is possibly exacerbated by a high-fat diet and a sedentary lifestyle (12). Risk factors of atherosclerosis, ie, smoking, hypertension, diabetes mellitus, elevated low-density lipoprotein cholesterol levels, and lack of exercise, are usually associated with vascular dysfunction (21). Moreover, the atherogenesis-related endothelial impairment occurs well before any detectable structural changes of the vessel wall (18). Normally, the vascular endothelium constitutively releases NO, which is considered to be an endogenous anti-atherosclerotic factor (1). Current consensus indicates that regular exercise causes the regression of cardiovascular symptoms and reduces the incidence of diseases and death (10, 12, 20). We and others have reported that exercise training increases agonist-stimulated NO release and enhances endothelium-dependent vasodilatation in vessels obtained from normal or hypertensive animals (5, 6, 9). How chronic exercise improves endothelial function in atherosclerosis has not been thoroughly examined. Our recent studies using hypercholesterolemic rabbits showed that 8 weeks of 2% cholesterol diet feeding caused severe vessel damage in aortas and moderate

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dysfunction in the femoral arteries (15, 23). The endothelium-dependent vasorelaxation was impaired in both vessels.

We hypothesize that the agonist-evoked endothelial intracellular calcium (EC $[Ca^{2+}]_i$) signaling is involved in the exercise-induced vascular adaptation. Many receptor-mediated agonists, such as acetylcholine (ACh), affect cellular function *via* generating intracellular calcium signals (3, 19, 22). Endothelial NO synthase (eNOS), the key enzyme responsible for endothelium-dependent vasorelaxation, is a calcium-dependent enzyme (17). Our previous studies have demonstrated that high-cholesterol diet feeding for 8 weeks reduces, whereas chronic exercise increases, ACh-evoked EC $[Ca^{2+}]_i$ elevation response in hypercholesterolemic rabbits and normal rats (8, 15). The aim of this study was to verify the time course of high-cholesterol diet-induced vascular dysfunction, and the duration necessary for exercise training to become effective. We evaluated diet or exercise effects on ACh-evoked EC $[Ca^{2+}]_i$ signaling and concomitant vasorelaxation in rabbit femoral arteries with mild atherosclerosis.

Materials and 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 ~1 kg at the beginning) were divided into 4 groups: the normal diet control (N) group, the normal diet with exercise training (NE) group, the high-cholesterol diet control (H) group, and the high-cholesterol diet with exercise training (HE) group. The control diet groups were fed normal rabbit chow, whereas the high-cholesterol diet groups were fed a 2% (w/w) high-cholesterol diet (PMI Feeds Inc, Richmond, IN, USA) for 2, 4, or 6 weeks. They were housed in an environmentally controlled room.

Exercise Protocol

A moderate, progressive exercise training protocol similar to that in our previous studies (15, 23) was used. In brief, animals in exercise groups ran on a leveled treadmill (model Q55, Quinton Instrument Co, Seattle, WA, USA) at the speed of 0.88 km/h for up to 40 minutes per day, 5 days per week for 2, 4, or 6 weeks. The exercise training and diet intervention in HE group began at the same time. The soleus muscles from all exercised animals showed increases in citrate synthase activity (data not shown), indicating that our training protocols for various time periods were effective. 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 (25 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 artery rings (5 mm long) were prepared and loaded with fura 2-AM as described in previous studies (13, 15). In brief, they were fluorescently labeled with 10 μmol/l of fura 2-AM at 22°C for 1 hour in Krebs-Ringer solution (95% O_2/ 5% CO_2, pH 7.4). We believed that the endothelial fluorescence signals in rabbit femoral arteries were free of contaminated signals from the smooth muscle layer underneath for the following two reasons: First, the fluorescence intensity in a stained vascular segment was undetectable in areas devoid of endothelial cells. Second, the fura fluorescence ratio value (an index for local calcium ion concentration) drastically changed when the specimen was exposed to the endothelial agonist, ACh, but not to the smooth muscle agonist, phenylephrine (PE).

Simultaneous Measurements of ACh-Induced EC $[Ca^{2+}]_i$ Responses and Vasorelaxation in PE-Precontracted Vascular Segments

Methods for simultaneous measurements of vascular EC $[Ca^{2+}]_i$ and smooth muscle contraction have been developed previously (14, 16). 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 vessel segment was fixed 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 in response to vasoactive reagents. After mounting, the flow chamber was placed on the stage of an inverted microscope with epifluorescence attachments. The fura-2 fluorescence ratio image of endothelium was used to calculate the EC $[Ca^{2+}]_i$ level, whereas the concomitant image movements indicated the vascular contraction/ relaxation. To evaluate the contribution of NO to the effects of exercise, some specimens were pretreated with 10^{-4} mol/l of N^{ω}-nitro-L-arginine (L-NNA) for 15 minutes to block NO synthesis (5). In addition, we also evaluated the vascular responses either to 2×

Table 1. Comparison of the areas of lipid deposit in the femoral arteries between HE and H groups after high-cholesterol diet feeding for 2, 4, or 6 weeks.

Group	Lipid Deposit (% surface area)		
	2 W	4 W	6 W
H	0.4±0.3 (2/6)	3.6±0.9 (7/7)	11±2 (7/7)
HE	0.4±0.3 (2/6)	1.7±0.8 (4/7)	4±2* (7/7)

*$P < 0.05$ (HE *vs.* H). The data in parentheses indicate the number of animals that have lipid deposits on femoral arteries over the total number of animals in that group. Abbreviations: W- week; H- high-cholesterol diet control; HE- high-cholesterol diet with exercise.

10^{-8} mol/l of A23187 (a calcium ionophore that induces endothelium-dependent vasodilatation without receptor activation), or to 10^{-6} mol/l of sodium nitroprusside (SNP, an endothelium-independent vasodilator) (6).

En Face Oil Red O Staining of Blood Vessels

En face oil red O staining was used to evaluate lipid deposition on the inner surface of vessel segments. The extent of lipid deposition was analyzed 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 means ± SEM. Sample sizes, indicated by "n", represented the number of animals used in each group. 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 Scheffe F test. If only 2 groups were compared, the Student *t* test was applied. Differences were considered at $P < 0.05$. To evaluate the correlation between ACh-evoked vasorelaxation and the corresponding EC $[Ca^{2+}]_i$ elevation, the logarithmically fitted curves were plotted by using data from four groups with 6 weeks of intervention.

Results

Oil Red O Staining

Confirming with our previous study (24), high-cholesterol diet feeding for ≥ 2 weeks significantly elevated serum levels of total cholesterol, high-density and low-density lipoproteins (data not shown). Two weeks of high-cholesterol diet feeding induced little fat deposits in rabbit femoral arteries, whereas the diet intervention for more than 4 weeks caused scattered lipid deposition in a time-dependent manner (Table 1). Furthermore, the concomitant exercise program reduced the area of lipid deposits, especially in 6 week-intervention groups. In contrast, there was no lipid deposition in either N or NE groups.

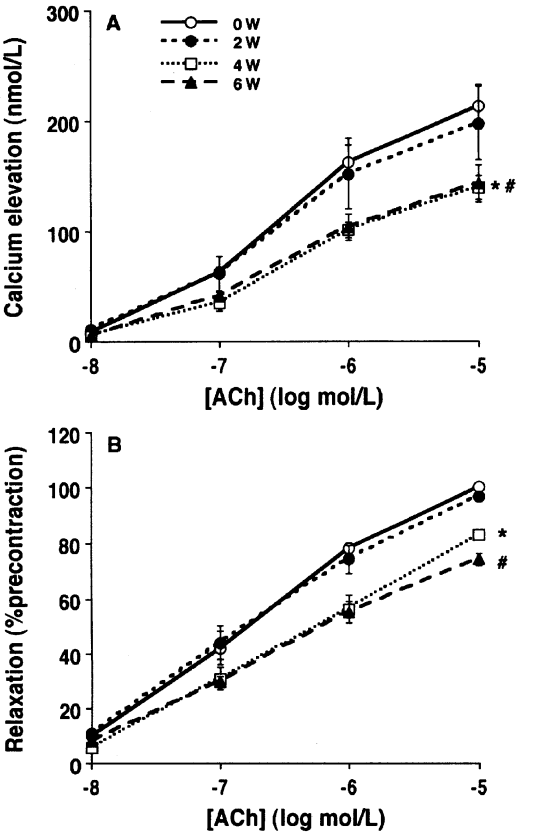

Fig. 1. Comparison of ACh-evoked EC $[Ca^{2+}]_i$ elevation (A) and vasorelaxation (B) at different time points of high-cholesterol diet feeding (i.e., 2, 4 or 6 weeks) with the normal control (i.e., 0 week). W- week. *$P < 0.05$ (4W *vs.* 0W), #$P < 0.05$ (6W *vs.* 0W). n = 5-7.

ACh-Induced EC $[Ca^{2+}]_i$ Responses and Vasorelaxation in PE-Precontracted Vascular Segments

The functional performance of rabbit femoral arteries, as accessed by simultaneously measuring ACh-evoked EC $[Ca^{2+}]_i$ elevation and vasorelaxation, was identical in normal diet control animals of three different time points. Two weeks of high-cholesterol diet intervention did not affect the ACh-evoked vascular responses, whereas diet feeding for 4 or 6 weeks significantly reduced them (Fig. 1). Short-term exercise training in parallel with the diet intervention enhanced these responses to nearly

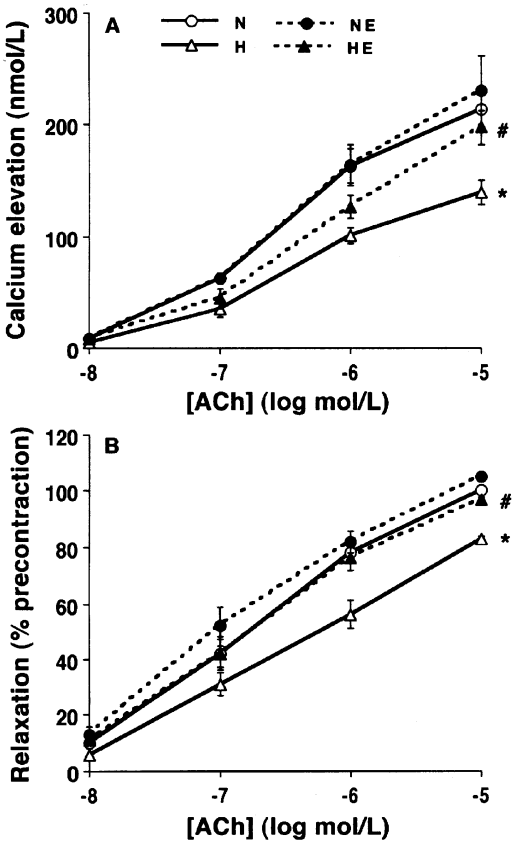

Fig. 2. Comparison of dose-response relations of ACh-induced EC $[Ca^{2+}]_i$ elevation (A) and vasorelaxation (B) after 4-week diet/exercise intervention. It was noticed that 4 weeks of diet intervention reduced ACh responses (*P < 0.05, H *vs*. N, n = 5-6), whereas concomitant exercise training enhanced these responses in hypercholesterolemic groups (#P < 0.05, HE *vs*. H, n = 5), but not in normal diet groups (P > 0.05, NE *vs*. N, n = 5-6).

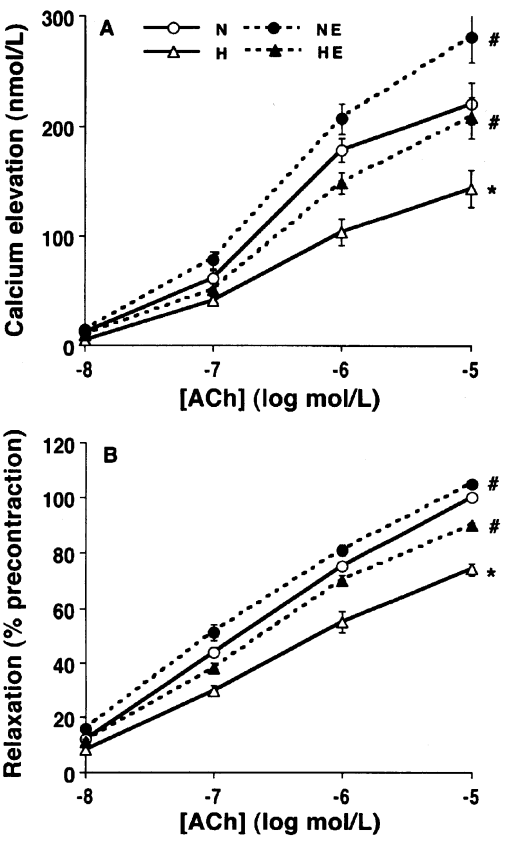

Fig. 3. Comparison of dose-response relations of ACh-induced EC $[Ca^{2+}]_i$ elevation (A) and vasorelaxation (B) after 6-week diet/exercise intervention. It was noticed that 6 weeks of diet intervention significantly reduced ACh responses (*P < 0.05, H *vs*. N, n = 7), whereas concomitant exercise training enhanced these responses in either hypercholesterolemic or normal groups (#P < 0.05, HE *vs*. H, or NE *vs*. N, n = 6-7).

normal levels (Fig. 2 and 3). In contrast, the vascular functions in normal diet groups could be further improved only after longer time periods (6 weeks) of exercise intervention. When NO synthesis was blocked by L-NNA pretreatment, the ACh-evoked vascular responses in all four groups were largely inhibited, along with the diminished diet/exercise effects (Fig. 4). When the ACh-evoked vasorelaxation was plotted against the corresponding EC $[Ca^{2+}]_i$ elevation by using data from four groups with 6 weeks of intervention, we found that these two parameters were significantly correlated (r^2 = 0.855 and 0.845 for N and H groups, respectively), and that the quantitative relationship was significantly altered by the high-cholesterol diet feeding for 6 weeks (Fig. 5). Similar alterations, but to a less extent, were observed in animals with diet intervention for 4 weeks (data not shown). In normal diet groups, 6 weeks of chronic exercise extended the curve to the higher-level range without altering the curve shape (Fig. 5A; r^2 = 0.855 and 0.918 for N and NE groups, respectively; EC_{50}~52 nmol/l for both groups). In high-cholesterol diet groups, however, 4 or 6 weeks of concomitant exercise training not only extended the curve to nearly normal ranges but also elevated the slope of the relation curve (Fig. 5B for 6 weeks of intervention; r^2 = 0.845 and 0.900, EC_{50}: 64 and 55 nmol/l, for H and HE groups, respectively).

Vascular Responses to SNP or A23187

SNP or A23187 was used to evoke vasorelaxation *via* either endothelial-independent or endothelial-dependent but non-receptor mediated mechanisms (15). The results are summarized in Table 2. In accordance with our previous report (6), exercise in the normal diet group did not significantly alter these vascular responses (P > 0.05, NE *vs*. N). However, high-cholesterol diet feeding for 4 or 6 weeks, but not for 2 weeks, impaired the SNP- or A23187-evoked

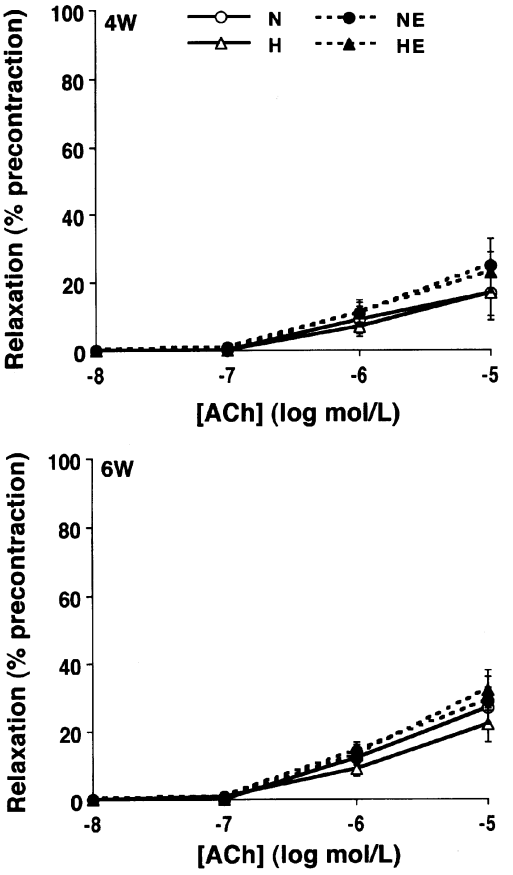

Fig. 4. The ACh-induced vasorelaxation in the presence of L-NNA after 4 weeks (upper panel) or 6 weeks (lower panel) of diet/exercise intervention. The results showed that L-NNA completely abolished diet/exercise effects.

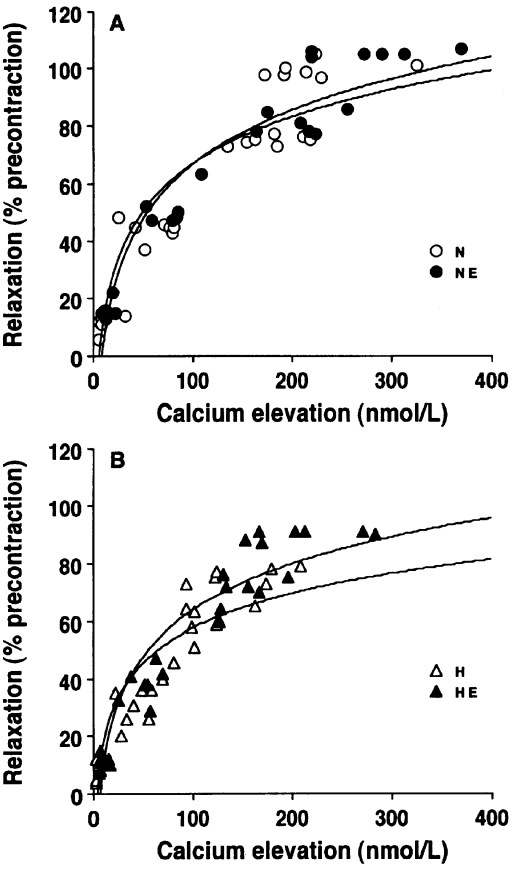

Fig. 5. Effects of 6-week exercise on the relationship between ACh-evoked EC $[Ca^{2+}]_i$ elevation and vasorelaxation. Panel A shows data for normal diet groups, and panel B is from high-cholesterol diet groups. It was noticed that in high-cholesterol diet groups, chronic exercise not only had higher maximal values of EC $[Ca^{2+}]_i$ and vasorelaxation, but also elevated the curve to nearly normal.

vasorelaxation ($P < 0.05$, H *vs*. N), and this impairment was almost reversed by short-term exercise training ($P < 0.05$, HE *vs*. H).

Discussion

Our results indicated that 1) 2% high-cholesterol diet feeding for more than 2 weeks induced scattered lipid deposition in femoral arteries in a time-dependent manner; 2) 6 weeks of short-term exercise training significantly reduced lipid deposition; 3) high-cholesterol diet feeding for ≥ 4 weeks reduced the responses of ACh-evoked EC $[Ca^{2+}]_i$ elevation and agonist-induced vasorelaxation, whereas parallel exercise reversed them; 4) the effects of diet/exercise on ACh-induced EC $[Ca^{2+}]_i$ elevation and vasorelaxation were abolished by L-NNA pretreatment, indicating that NO was the major contributing factor for these effects; 5) ACh-induced vasorelaxation was well associated with EC $[Ca^{2+}]_i$ elevation in all groups; and 6) high-cholesterol diet feeding for ≥ 4 weeks reduced the extent of vasorelaxation at a certain level of EC $[Ca^{2+}]_i$ elevation, whereas exercise training completely reversed it to normal. These new findings expand the current knowledge beyond what has been reported previously using an 8-wk exercise/diet intervention protocol (15). The present results imply that short-term high-cholesterol diet feeding reduced the sensitivity of ACh-evoked vasodilation to a given level of EC $[Ca^{2+}]_i$ elevation in rabbit femoral arteries in a duration-dependent manner, and that exercise training could ameliorate the relationship between vasorelaxation and EC $[Ca^{2+}]_i$ elevation nearly back to normal.

Regional difference in the micro-vasculature is well known among different vascular beds, e.g., microvessels in the kidney, in contrast to those in the brain, are relatively permeable to blood substances. However, functional differences between major arteries have not been well investigated yet. In the present study, significant atherosclerotic lesions and endothelial dysfunction in femoral arteries were observed only after 4 weeks of high-cholesterol diet feeding in

Table 2. Comparison of SNP- or A23187-evoked vasorelaxation among four groups.

Group	Vasorelaxation (% precontraction)		
	2 W	4 W	6 W
SNP:			
N	40±6	39±2	37±1
NE	47±6	39±1	42±3
H	39±3	30±2*	30±2*
HE	42±8	37±2#	33±2
A23187:			
N	60±5	44±2	46±3
NE	59±7	45±1	50±2
H	50±5	36±2*	33±2*
HE	51±7	41±2#	43±3#

*$P < 0.05$ (H *vs*. N), #$P < 0.05$ (HE *vs.* H). n = 4-6
Abbreviations: N- normal diet control; NE- normal diet with exercise; others are the same as in Table 1.

rabbits. In comparison, atherosclerotic changes were apparent as early as 2 weeks of diet intervention in rabbit aortas (24). Moreover, in rabbits fed with 8 weeks of high-cholesterol diet, the aortic lesions at proximal locations were more severe than at distal locations (23). Thus the atherosclerotic changes in rabbits appear to develop progressively downstream along the major arterial tree. We have reported that in normal rats the ACh-evoked EC $[Ca^{2+}]_i$ signaling shows pronounced regional heterogeneity between the endothelium of aorta and that of the adjacent intercostal orifice (13). Similar type of endothelial regional difference may exist among rabbit major arteries, which could explain why the femoral artery is not as atherosclerosis-prone as the aorta.

Interestingly, atherosclerosis affected rabbit femoral artery functions not only at the calcium signaling level in the endothelium (an upstream signaling component of endothelium-dependent vascular responsiveness), but also at the contractility level in smooth muscles (a downstream signaling component). The vasodilating responses to A23187 (a receptor-independent agonist) or SNP (an endothelium-independent agonist) were also impaired after 4 or 6 weeks of diet intervention (Table 2). These data coincide with our observation that high-cholesterol diet feeding alters the relationship between EC $[Ca^{2+}]_i$ elevation and vasorelaxation (Fig. 4). In contrast, high-cholesterol diet feeding did not change vasorelaxing responses to SNP or A23187 in rabbit aortas (11, 24). Therefore, both endothelial and smooth muscle functions were reduced in femoral arteries of hypercholesterolemic rabbits, whereas only the former was affected in aortas. A recent study has reported that hypercholesterolemia inhibits coronary smooth muscle calcium channel activity in miniature swine (2). Whether similar ion-channel dysfunction occurs in hypercholesterolemic rabbit smooth muscle cells or not needs to be further studied.

It is noted that exercise improves vascular functions by altering both endothelial and smooth muscle performances in high-cholesterol diet fed animals, and by altering only endothelial function in normal animals. According to the current study and our previous study (15), short-term exercise training for more than 6 weeks in normal diet groups increases the ACh-evoked EC $[Ca^{2+}]_i$ elevation and vasorelaxation without altering the relationship between these two parameters. Moreover, exercise in rabbits fed a normal diet enhances 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 contrast, the exercise training in high cholesterol-fed animals apparently prevents and/or attenuates vascular dysfunction by a variety of means. First, concomitant exercise training for 4 or 6 weeks in hypercholesterolemia not only enhances the ACh responses, but also improves the relationship between vasorelaxation and EC $[Ca^{2+}]_i$ elevation in rabbit femoral arteries. Second, 4 or 6 weeks of short-term exercise training improved SNP- or A23187-induced vasorelaxation, and reduced lipid deposition in hypercholesterolemic rabbit femoral arteries, indicating that the structure and function as a whole were improved by short-term exercise training in these diseased vessels. Since endothelial receptors in rats are upregulated by exercise (4, 7), similar mechanisms may partially explain the endothelium-dependent part of exercise effects seen in hypercholesterolemic rabbits.

In conclusion, our results provide direct evidence that exercise can completely reverse the diet-induced adverse effects on vascular functions in the early stage of atherosclerosis.

Acknowledgments

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