Exercise Training Modulates the Effects of Lipoproteins on Acetylcholine-Induced Endothelial Calcium Signaling in Rat Aortas

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Exercise training improves vascular endothelial functions, while oxidized low-density lipoproteins (oxLDLs) impede them. We proposed that exercise training might influence the endothelial sensitivity to lipoprotein-induced vascular changes. Male Wistar rats either exercised on a leveled treadmill for 8 weeks or remained sedentary as the control. The endothelial intracellular calcium level (EC $[Ca^{2+}]_i$) in vitro was examined using dissected aortic segments treated with different lipoproteins, including native low-density lipoprotein (nLDL), various oxLDLs, and high-density lipoprotein (HDL). Our results indicated that i) none of the various lipoproteins directly evoked EC $[Ca^{2+}]_i$ elevation; ii) the acetylcholine-evoked EC $[Ca^{2+}]_i$ elevation in the control group was increased by nLDL and progressively suppressed by oxLDLs with increasing degrees of oxidation; iii) exercise training ameliorated the oxLDL-induced suppressive effects on acetylcholine-evoked EC $[Ca^{2+}]_i$ elevation; iv) HDL potentiated the acetylcholine-evoked EC $[Ca^{2+}]_i$ elevation in vessel segments from exercised rats but not those from control rats; and v) when HDL was present, the suppressive effects of extensively modified oxLDLs were reduced. Furthermore, comparing with the effects of various lipoproteins on EC calcium signaling, the lipoprotein effects on endothelium-dependent vasorelaxing response appeared to be similar but less pronounced. Taken together, one of the beneficial effects of exercise training on vascular functions might be to make blood vessels more resistant to oxLDLs and more sensitive to HDL. Exp Biol Med 234:323–331, 2009

Key words: endothelium; LDL; oxLDL; HDL

Introduction

An elevated plasma level of cholesterol and low-density lipoprotein (LDL) is a major risk factor for atherosclerosis (17, 29, 36). This type of alteration in blood lipid profile has been postulated to cause vascular endothelial dysfunction and chronic inflammation in the early stage of atherosclerosis (34). Moreover, some forms of modified LDL are believed to accelerate the advancement of atherosclerosis as well, because they are capable of inducing the expression of endothelial adhesion molecules, altering the structure of endothelial cytoskeleton, and activating platelets (1, 5, 35). Therefore, it is crucial to investigate whether and how various lipoproteins affect endothelial function. Endothelial cells respond to various stimuli by elevating their intracellular calcium level (EC $[Ca^{2+}]_i$) followed by releasing NO, prostacyclin, platelet activating factor, tissue plasminogen activator, etc. (30). In cultured endothelial cells, it has been reported that EC $[Ca^{2+}]_i$ elevation can be induced by either native LDL (nLDL) or oxidized LDL (oxLDL) (1, 13, 42). Previous in vitro studies have also shown that oxLDL decreases the expression of NO synthase mRNA, inhibits the synthesis of nitric oxide, or inactivates NO (7, 14, 26).

Despite the clear association between exercise and a reduced risk of cardiovascular disease, the precise mechanisms responsible for this association remain unclear at present. On the one hand, abundant evidence supports that exercise can exert direct effects on vascular physiology, especially the improvement of vascular endothelial functions (for reviews, see references 16, 22). On the other hand, it has been postulated that one of the beneficial effects of regular exercise is to reduce the plasma level of LDL, or "bad cholesterol," and to increase the plasma level of high-density lipoprotein (HDL), or "good cholesterol" (9). Although oxLDL inhibits acetylcholine (ACh)-induced vasorelaxation (8), whether various lipoproteins also affect ACh-evoked EC $[Ca^{2+}]_i$ elevation has not been reported. Efforts in resolving this issue are usually hampered by two

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difficulties: it is technically demanding to monitor this parameter *in situ* (10, 31) and cultured endothelial cells lack ACh receptors (37). We have developed a calcium imaging method with single-cell resolution to examine the EC $[Ca^{2+}]_i$ elevation response in isolated vascular segments (18, 19). Our previous studies have shown that the vascular EC $[Ca^{2+}]_i$ elevation response is a sensitive parameter that is suppressed by hypercholesterolemia-induced atherosclerosis in rabbits (23, 24). Moreover, localized alterations in this parameter occur in the endothelium covering or immediately surrounding miniature fatty streaks in early-stage atherosclerotic rabbit arteries (5). In the current study, this technique was applied to investigate i) whether various lipoproteins would directly induce vascular EC $[Ca^{2+}]_i$ elevation or indirectly alter the ACh-evoked EC $[Ca^{2+}]_i$ elevation in isolated rat aortas, and ii) whether exercise training would modulate the lipoprotein effects on vascular EC $[Ca^{2+}]_i$ signaling. Finally, we also examined whether lipoproteins exert similar effects on endothelium-dependent and endothelium-independent vasorelaxing responses.

Materials and Methods

Lipoprotein Preparation. This study conforms with the principles outlined in the Declaration of Helsinki for the use of human plasma. Native LDL and HDL were prepared from fresh human plasma of male healthy donors by the sequential ultracentrifugal flotation method as described in a previous study (3). LDL was oxidized by $CuSO_4$ (5 μM) for 2, 4, or 8 h, stopped by adding EDTA (0.3 mM), and dialyzed against phosphate buffered saline to produce various oxLDLs, i.e., oxLDL-2, oxLDL-4, or oxLDL-8. Minimally modified LDL (mmLDL) was prepared similarly except that the mild oxidation was accomplished by incubating nLDL with 5 mM $FeSO_4$ for 48 h instead of being oxidized by $CuSO_4$ (40). The oxidative modification in various LDLs was ascertained by measuring the amount of malondialdehyde (thiobarbituric acid-reactive substances, or TBAR) and conjugated dienes ($UV_{234\ nm}$ absorbance) (33). The protein concentration in various lipoproteins was measured and the samples were diluted to 1 mg protein/ml accordingly. All lipoproteins samples were filled with nitrogen and stored at 4°C for no longer than 2 weeks before use.

Animals and Exercise Protocol. 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 (NIH publication number 80–23), and the procedures followed were in accordance with institutional guidelines. Four- to five-week-old male Wistar rats were allowed to get familiarized with the treadmill (Model T408E, Diagnostic and Research Instruments Co., Taoyuan, Taiwan) by running at a low speed of 9 m/min for 10 min each day for 5 days. They were then randomly divided into exercise and control groups. Animals in the exercise group were trained for 8 weeks (5 days/week) at an intensity of about 60% of maximal oxygen consumption. Briefly, the training began at 12 m/min with increasing duration from 20 min to 60 min in the first two weeks, and increased 3 m/min every four weeks afterwards. No electric shock was applied to avoid possible stress effects. Rats in the sedentary control group were placed in the treadmill without running for 10 min/day. At the end of the experiments the rats were sacrificed by CO_2 euthanasia to obtain thoracic aorta for EC $[Ca^{2+}]_i$ or vasorelaxation measurements and soleus muscle for citrate synthase activity determination. To avoid the acute exercise effects of exercise, animals in the exercise group were sacrificed 3 days after the last running episode.

Vessel Preparation and Measurement of Endothelial $[Ca^{2+}]_i$. After cleaning, the thoracic aortas were cut into rings (5 mm long) and fluorescently labeled by incubating with 10 μM fura-2 AM at room temperature in the presence of 0.025% pluronic F-127. As the dye only stained the rat aortic endothelium in our hands, this method allowed the quantification of EC $[Ca^{2+}]_i$ *in situ* with little interference from the smooth muscle cells underneath (18). Briefly, fura-2 labeled vessel rings were longitudinally opened and pinned to the base plate of a tissue flow chamber with the proximal end facing the flow inlet. The chamber was mounted on an inverted microscope with epifluorescence attachments. The excitation wavelengths of 340 and 380 nm were provided, and the fluorescence images were recorded at 510 nm. The fura-2 fluorescence ratio images of endothelium were acquired and analyzed off-line using commercial software (MetaFluor, Universal Imaging, West Chester, PA, USA). The average value of endothelial $[Ca^{2+}]_i$ in each preparation was calculated by monitoring a large area, covering more than 200 cells. At the end of each experiment, the calcium concentration was calibrated by applying ionomycin (5 μM) in the presence of 5 mM EGTA, followed by 10 mM $CaCl_2$. All signals were corrected for autofluorescence determined by exposing the tissue to 5 mM manganese to quench cytosol fura-2 fluorescence. Endothelial $[Ca^{2+}]_i$ was estimated after subtracting background and autofluorescence. All experiments were conducted at room temperature. After the vascular endothelial cells had been focused properly, fresh Krebs-Ringer buffer (118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 24 mM $NaHCO_3$, 0.03 mM Na_2-EDTA, 2.5 mM $CaCl_2$, 11 mM glucose) was perfused through the chamber at a flow rate of 0.05 ml/min. At the same flow rate, dose responses of ACh-induced $[Ca^{2+}]_i$ elevation were determined by subsequent application of ACh (from 10^{-8} to 10^{-6} M). Between each application, the chamber was washed with fresh buffer to recover the basal $[Ca^{2+}]_i$ level. The specimen was subsequently exposed to one of the various lipoproteins (100 μg/ml) for 20 min. Twenty minutes of Krebs-Ringer buffer perfusion served as a control. Finally, the dose responses to ACh were repeated after buffer washing.

The effects of buffer and lipoproteins on EC calcium signaling were expressed as the relative ACh-evoked EC

Table 1. Effects of Exercise on Body Weight, Muscle Citrate Synthase Activity and Serum Lipid Profile[a]

	Body weight (g)	Citrate synthase activity (μmole/min/g wet weight)	Total cholesterol (mg/dL)	Triglyceride (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)
Control	443 ± 13	1.14 ± 0.01	65.8 ± 3.3	71.4 ± 4.9	25.1 ± 1.1	10.6 ± 1.4
Exercise	364 ± 14*	1.35 ± 0.04*	58.6 ± 2.0	62.0 ± 3.9	24.1 ± 1.0	8.9 ± 0.6

[a] Data are expressed as mean ± SE ($n = 9$).
* $P < 0.05$, exercise vs. control.

$[Ca^{2+}]_i$ elevation (R) between post-treatment ($\Delta[Ca^{2+}]_i$') and pre-treatment ($\Delta[Ca^{2+}]_i$) when treated with 1×10^{-7} M ACh, i.e., $R = (\Delta[Ca^{2+}]_i' / \Delta[Ca^{2+}]_i) \times 100\%$. Please refer to Figure 2 for actual data tracings.

Vessel Preparation and Evaluation of Vasodilating Responses. The methods used were slightly modified from a previous study using rabbit aortas (41). The isolated aortic rings (5 mm long) were mounted on force transducers and submerged in organ chambers containing Krebs-Ringer buffer bubbled with 95% O_2–5% CO_2 at 37°C. They were stretched to the optimal passive tension at which the phenylephrine-evoked contraction was maximal. During the experiment, the specimens were equilibrated, pre-constricted with phenylephrine (10^{-7} M), and exposed to various concentrations of ACh (10^{-10}–10^{-6} M) to evoke endothelium-dependent vasorelaxation. The dilating responses were expressed as percentage of the precontractile force. The endothelium-independent vasorelaxation was measured by adding various concentrations of sodium nitroprusside (10^{-10}–10^{-7} M), a nitric oxide donor, to the endothelium-denuded vessel rings.

Determination of Citrate Synthase Activity in Soleus Muscles. An increase in citrate synthase activity (reflecting an improved aerobic metabolism in mitochondria) in the skeletal muscle is commonly used to confirm the exercise training effect. Therefore, the citrate synthase activity in soleus muscles was measured using a method described previously (23). Briefly, the homogenized muscle specimens were centrifuged at 17,000 × g at 4°C for 15 min and the supernatant was collected. After adding DTNB, acetyl CoA and oxaloacetate to the sample, the mitochondrial citrate synthase activity was determined spectrophotometrically by measuring the time-dependent absorbance changes at 412 nm. Results were expressed in units of μmol/min/g wet weight.

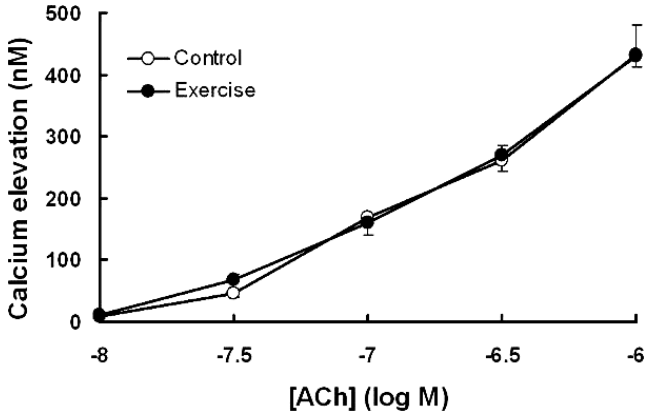

Figure 1. The effects of exercise training on ACh-evoked EC $[Ca^{2+}]_i$ elevation in rat aortas. This set of experiments was carried out in the absence of lipoproteins. The differences between exercise and control groups were insignificant at any tested ACh concentration ($n = 10$).

Determination of Serum Lipid Profile. Two days after the last running episode, a separate group of rats were fasted overnight for blood sampling. Biochemical indices in serum lipid profile (total cholesterol, triglyceride, LDL-C and HDL-C) were measured using an automatic analyzer (model 747, Hitachi, Japan).

Chemicals. All chemicals for preparing Krebs-Ringer solution were purchased from Merck (Darstadt, Germany). Other reagents were obtained from Sigma (St. Louis, MO, USA).

Statistical Analysis. Results were expressed as mean ± SE. Sample sizes were indicated by "n". Differences among groups were analyzed by analysis of variance, further by unpaired Student's t test with $P < 0.05$ considered statistically significant.

Results

Effects of Exercise Training on Citrate Synthase Activity, Serum Lipid Profile, and ACh-Evoked EC $[Ca^{2+}]_i$ Elevation. Treadmill exercise for 8 weeks in rats reduced the body weight and increased the citrate synthase activity in their soleus muscle (Table 1), indicating that the exercise training was effective. However, the same exercise paradigm did not significantly alter the indices in the serum lipid profile (Table 1). Moreover, although ACh reversibly evoked vascular EC $[Ca^{2+}]_i$ elevation in a dose-dependent manner, confirming our previous report (18), this parameter was unaltered by exercise training (Fig. 1).

Characterization of nLDL, mmLDL and Various oxLDLs. Table 2 shows the extent of oxidative modification in nLDL, mmLDL and various oxLDLs. It was noticed that i) nLDL showed the lowest oxidation level; ii) mmLDL showed an elevated level in the conjugated dienes with an unaltered malondialdehyde level; iii) the oxidation level in various oxLDLs was oxidation time-dependent—higher

Table 2. The Extent of Oxidative Modification in nLDL, mmLDL, and Various oxLDLs[a]

	nLDL	oxLDL-2	oxLDL-4	oxLDL-8	mmLDL
Pre-dialysis malondialdehyde (nmole/mg)	2.19 ± 0.50	7.05 ± 1.19	26.7 ± 3.8	28.4 ± 4.2	2.79 ± 0.30
Post-dialysis malondialdehyde (nmole/mg)	2.28 ± 0.17	4.49 ± 0.75	6.78 ± 0.20	7.14 ± 0.83	2.89 ± 0.28
Pre-dialysis conjugated dienes ($A_{234\ nm}$)	0.44 ± 0.07	0.71 ± 0.07	1.40 ± 0.11	1.31 ± 0.08	1.25 ± 0.09
Post-dialysis conjugated dienes ($A_{234\ nm}$)	0.22 ± 0.06	0.67 ± 0.09	1.03 ± 0.12	0.98 ± 0.10	1.10 ± 0.02

[a] Data are expressed as mean ± SE ($n = 5$).

oxidation level with increasing oxidation time; iv) the formation of malondialdehyde and conjugated dienes reached a plateau value in 4 h by copper-oxidation; and v) a large portion of malondialdehyde was dialyzable.

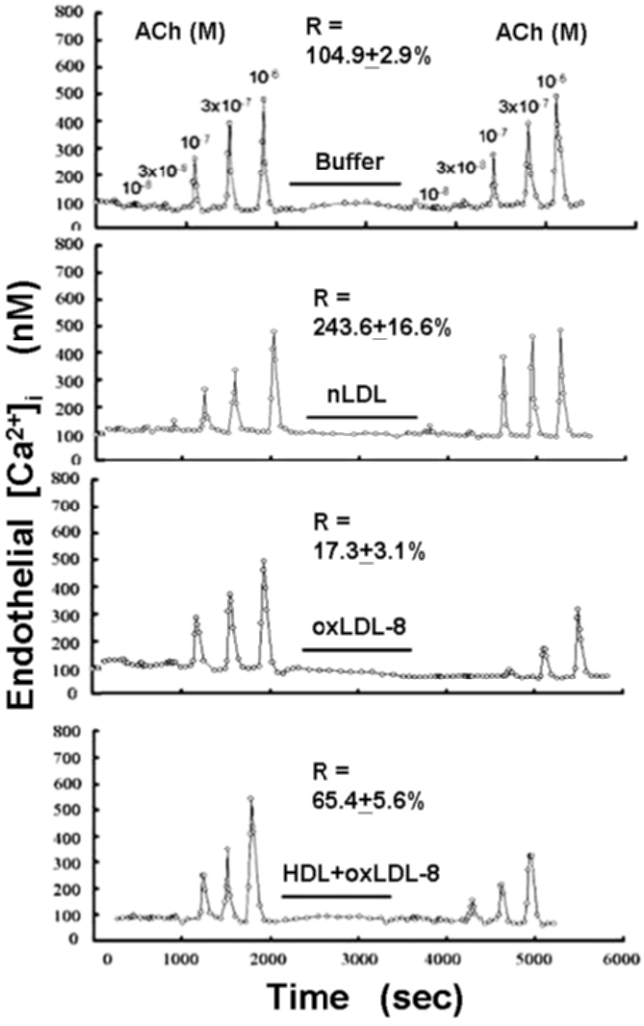

Figure 2. Typical tracings of ACh-evoked EC $[Ca^{2+}]_i$ elevation before and after exposure to various lipoproteins. The aortic segments were isolated from control rats. Note that various lipoproteins at 100 μg/ml did not acutely induce EC $[Ca^{2+}]_i$ elevation. The effects of buffer and lipoproteins were expressed as the relative ACh-evoked EC $[Ca^{2+}]_i$ elevation (R) between post-treatment ($\Delta[Ca^{2+}]_i$') and pre-treatment ($\Delta[Ca^{2+}]_i$) when treated with 1×10^{-7} M ACh, i.e., $R = (\Delta[Ca^{2+}]_i' / \Delta[Ca^{2+}]_i) \times 100\%$. Note that the ACh-evoked responses were unchanged after 20 min of Krebs-Ringer buffer perfusion.

Effects of Lipoproteins on ACh-Evoked EC $[Ca^{2+}]_i$ Elevation. Figure 2 shows the tracings of endothelial $[Ca^{2+}]_i$ in response to ACh and some lipoproteins in the sedentary group. The ACh-evoked responses remained the same after 20 min of buffer perfusion (Fig. 2, top panel). Surprisingly, none of the various lipoproteins (nLDL, oxLDLs, mmLDL, HDL) tested in our system showed any direct effect on the EC $[Ca^{2+}]_i$, i.e., this parameter remained unchanged during the 20-min lipoprotein incubation period. Instead, the treatment of various lipoproteins drastically influenced the subsequent ACh-evoked EC $[Ca^{2+}]_i$ elevation, either potentiating or suppressing the responses (Fig. 2, lower panels). In order to quantify the potentiation or suppression effects of various lipoproteins, the 10^{-7} M ACh-evoked EC $[Ca^{2+}]_i$ elevation was chosen as the standard assay condition and its pre-treatment value was used to normalize the corresponding post-treatment values.

When treatments of various LDLs were compared in either control or exercise groups (Fig. 3), the summarized results clearly showed that i) nLDL potentiated the ACh-evoked EC $[Ca^{2+}]_i$ elevation; ii) the nLDL-induced potentiation effects were largely diminished when the nLDL treatment was replaced by exposing the specimens to mildly modified mmLDL or oxLDL-2; and iii) they even changed to suppressive effects when more extensively modified oxLDL-4 or oxLDL-8 was applied instead. Moreover, although specimens from either exercised or control animals showed similar ACh-evoked responses in the presence of nLDL, the former appeared to be more resistant to the suppressive effects from extensively oxidized LDLs (Fig. 4). The exercise effects were negligible in the presence of either nLDL or oxLDL-2. However, exercise exerted significant ameliorating effects when oxLDL-4 or oxLDL-8 was applied (for oxLDL-4, 90.0 ± 5.4% vs. 62.7 ± 4.0%; for oxLDL-8, 60.5 ± 4.8% vs. 17.3 ± 3.1%; exercise vs. control, respectively, $P < 0.05$, $n = 5$).

Furthermore, although the HDL treatment exerted minimal influence on the ACh-evoked responses in sedentary control animals, it showed a significant potentiation effect in exercise trained animals (Fig. 3; 113.4 ± 4.4% and 249.7 ± 31.5% in control and exercise groups, respectively; $P < 0.05$, $n = 5$). Like exercise, the addition of HDL to extensively oxidized LDLs (oxLDL-4 or oxLDL-8) would also reduce the latter's suppressive effects (Fig. 5). However, the statistical analysis indicated that there were no interactions between the effects of exercise and HDL, i.e.,

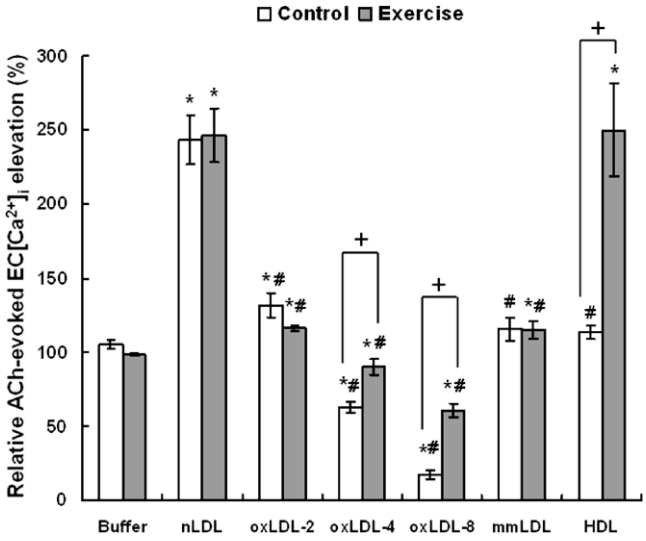

Figure 3. The effects of various LDLs and HDL on relative ACh (10^{-7} M)-evoked EC $[Ca^{2+}]_i$ responses in control (top panel) or exercise (bottom panel) rats. The relative ACh-evoked EC $[Ca^{2+}]_i$ elevation was defined as $(\Delta[Ca^{2+}]_i' / \Delta[Ca^{2+}]_i) \times 100\%$ (see Fig. 2). Data were presented as mean ± SE and analyzed by one-way ANOVA. * $P < 0.05$, compared to respective buffer-treated values in either control or exercise groups ($n = 5$); # $P < 0.05$, compared to respective nLDL-treated values in either control or exercise groups ($n = 5$); + $P < 0.05$, control vs. exercise ($n = 5$).

exercise and HDL were independent factors in reducing the suppressive effects of oxLDL-4 or oxLDL-8 on ACh-evoked EC $[Ca^{2+}]_i$ elevation.

Effects of Lipoproteins on Endothelium-Dependent and Endothelium-Independent Vasorelaxation. In order to examine the effects of lipoproteins on vasorelaxing responses, we have used aortic rings obtained from an additional set of rats. Summarized results about endothelium-dependent vasorelaxation are shown in Figure 6. The 10^{-7} M ACh-evoked endothelium-dependent vasorelaxation was impaired by oxLDL-8 in the sedentary group (64.0 ± 4.4% vs. 96.3 ± 2.5%, oxLDL vs. buffer, $P < 0.05$, $n = 7$), but not in the exercise group (78.8 ± 7.7% vs. 92.9 ± 5.6%, oxLDL vs. buffer, $P > 0.05$, $n = 7$). However, neither nLDL nor HDL significantly affected the ACh-evoked endothelium-dependent vasorelaxation. Although HDL seemed to ameliorate the oxLDL-8's suppressive effects in the exercise group, it did not reach statistically significant difference level (90.9 ± 10.8% vs. 78.8 ± 7.7%, HDL + oxLDL vs. oxLDL, $P > 0.05$, $n = 7$). As a whole, comparing with the effects of various lipoproteins on EC calcium signaling, the lipoprotein effects on endothelium-dependent vasorelaxing response appeared to be similar but less pronounced. Furthermore, we carried out similar experiments using sodium nitroprusside to stimulate endothelium-denuded vessel rings. It was clear that lipoproteins did not alter endothelium-independent vasorelaxation in either sedentary or exercised rats (Fig. 7).

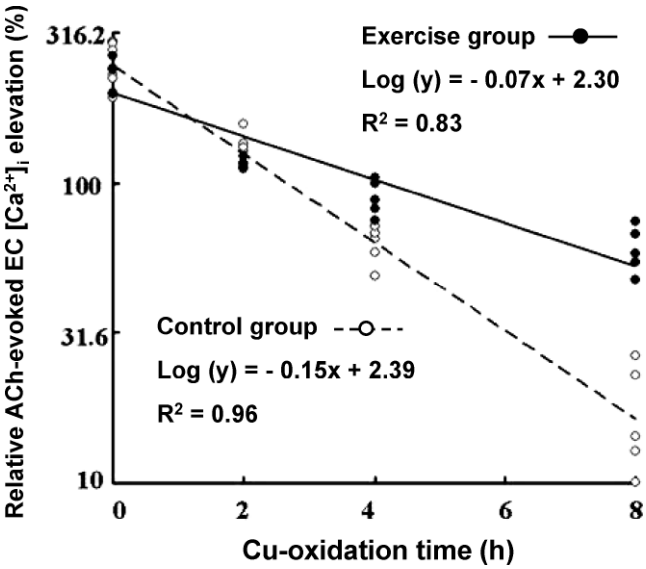

Figure 4. The effects of exercise on ACh-evoked EC $[Ca^{2+}]_i$ elevation in vessels treated with nLDL and various oxLDLs. Note that the suppressive effects of various oxLDLs depended on the copper oxidation time and that exercise made blood vessels more resistant to the challenge from extensively oxidized oxLDLs. Data were taken from Figure 3.

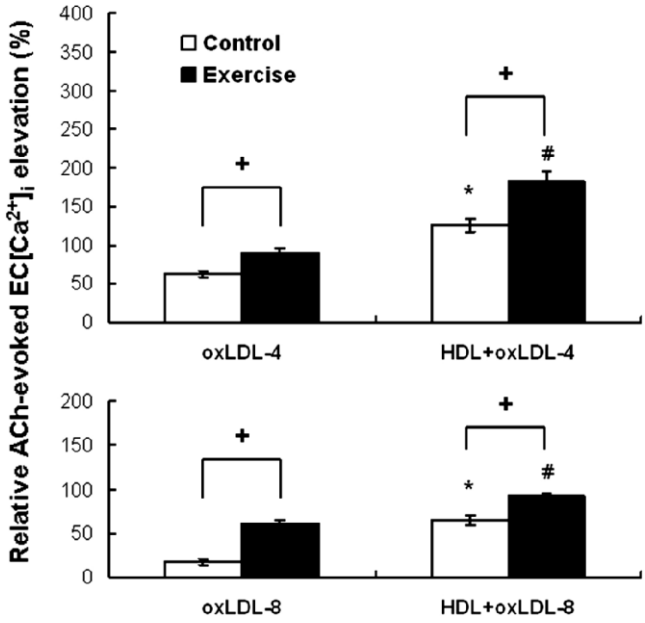

Figure 5. The effects of exercise and HDL on ACh-evoked EC $[Ca^{2+}]_i$ elevation in vessels treated with extensively oxidized LDLs. The relative ACh (10^{-7} M)-evoked EC $[Ca^{2+}]_i$ elevation was defined as $(\Delta[Ca^{2+}]_i' / \Delta[Ca^{2+}]_i) \times 100\%$ (see Fig. 2). Data were presented as mean ± SE and analyzed by two-way ANOVA. * $P < 0.05$, compared with the absence of HDL in control group; # $P < 0.05$ compared with the absence of HDL in exercise group; + $P < 0.05$ compared with the respective control groups; $n = 5$.

Discussion

By real-time measuring the endothelial $[Ca^{2+}]_i$ level in the rat thoracic aorta, we found that i) EC $[Ca^{2+}]_i$ elevation was not acutely induced by nLDL, HDL or any form of modified LDL; ii) nLDL pretreatment increased ACh-

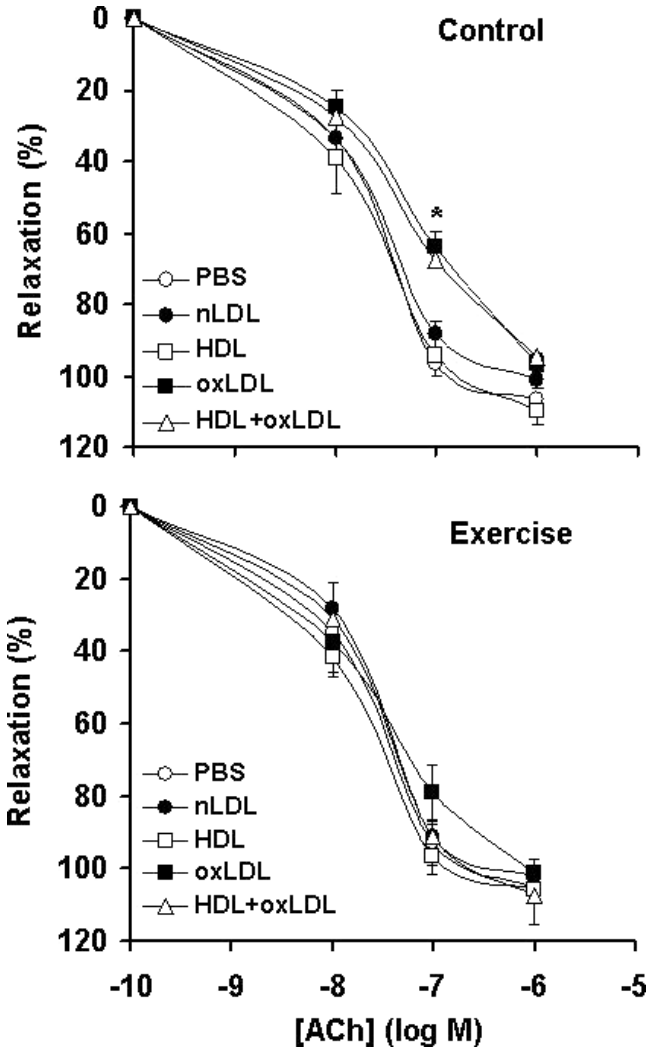

Figure 6. The effects of lipoproteins on endothelium-dependent vasorelaxation. ACh-induced vasorelaxation in aortic rings from control (top panel) or exercised (bottom panel) rats were measured in the presence of various lipoproteins. Data were presented as mean ± SE and analyzed at ACh (10^{-7} M) by unpaired Student's *t* test. * $P < 0.05$, oxLDL-8 compared with buffer in the control group; $n = 7$.

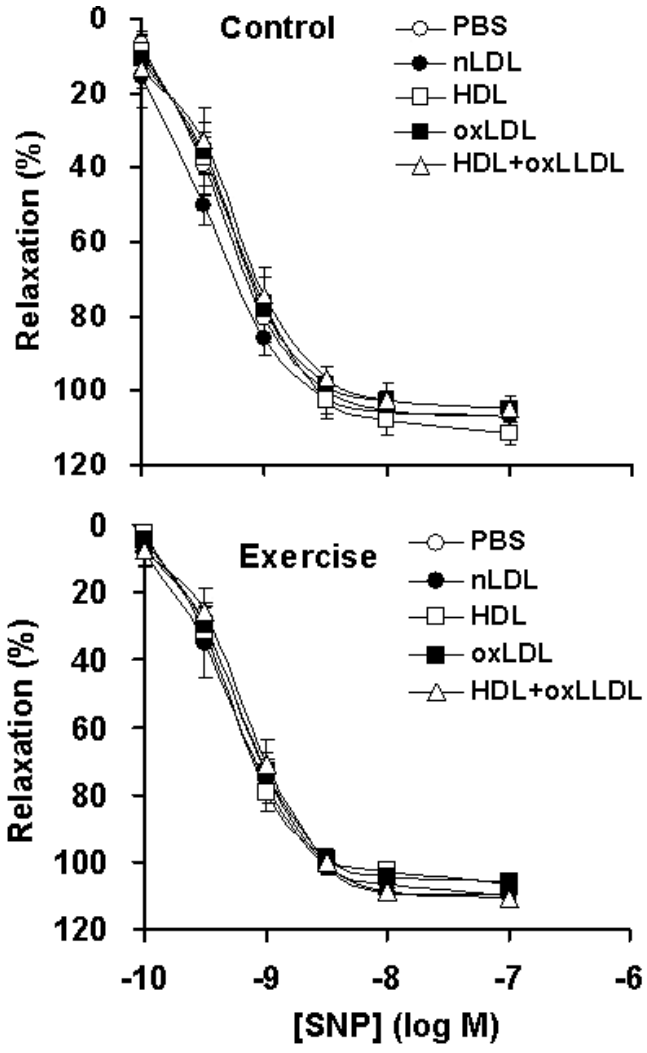

Figure 7. The effects of lipoproteins on endothelium-independent vasorelaxation. Sodium nitroprusside-induced vasorelaxation in endothelium-denuded aortic rings from control (top panel) or exercised (bottom panel) rats were measured in the presence of various lipoproteins. Data were presented as mean ± SE; $n = 7$.

evoked endothelial $[Ca^{2+}]_i$ elevations; iii) HDL pretreatment potentiated ACh-evoked responses in the exercise group, but not in the control; iv) pretreatment with mildly modified LDLs showed relatively minor effects, while pretreatment with extensively modified LDLs showed pronounced suppressive effects; v) compared with the control specimens, the exercise specimens were relatively resistant to the suppressive effects from extensively modified oxLDLs; and vi) exercise and HDL independently ameliorated the suppressive effects of oxLDL-8. Therefore, exercise training made vascular tissues more sensitive to HDL's protective effects and more resistant to oxLDL's adverse effects.

As the serum lipid profile remained unchanged in our exercise animal model (Table 1), the fact that the vascular sensitivity to various lipoproteins can be modulated by exercise deserves special attention. One of the major beneficial effects of exercise on cardiovascular diseases is to improve endothelial function in healthy subjects or patients (6, 15, 21). Here we provide another line of evidence in support of this notion. It is generally believed that hypercholesterolemia is a causative factor in atherosclerosis-related cardiovascular diseases, which often involve oxidative stress-induced chronic inflammation in vascular tissues (27, 34). Among the many risk factors for atherosclerosis, hypercholesterolemia and modified lipoprotein are thought to be the major triggers for inflammation in atherogenesis. In this regard, exercise may reduce the risk of atherosclerosis either by minimizing the assault (total amount of cholesterol and various modified LDLs in the blood) or by strengthening the vascular tissue's ability to cope with the challenge from the same assault. While the former is often postulated to be part of the underlying mechanism explaining the atheroprotective effects of

exercise, the latter has rarely been examined. Our previous studies have shown that treadmill exercise training in rabbits for ~2–8 weeks ameliorates the hypercholesterolemia-induced atherosclerosis and the impaired ACh-evoked EC $[Ca^{2+}]_i$ elevation (23, 24). Similar to the current study using rats, results from previous studies have also shown that exercise training in rabbits does not alter the serum lipid profile under normal or hypercholesterolemic conditions. As rats rarely develop atherosclerosis, it would be very interesting to examine whether or not exercise would exert atheroprotective effects in rabbits via making their vessels more resistant to modified LDLs.

Contrasting to studies using cell culture systems, we did not observe the direct effect of either nLDL or oxLDL on the endothelial $[Ca^{2+}]_i$ level in rat aortic segments. In our hands, nLDL showed pronounced potentiation effects on ACh-evoked EC $[Ca^{2+}]_i$ elevation; and this nLDL-induced potentiation effect was completely absent when using its mildly modified forms mmLDL or oxLDL-2 instead. As nLDL is an endogenous component in the blood, the endothelial functional response to various stimuli, such as ACh, may be maximized in its presence. It is plausible to assume that when nLDL is oxidatively modified, the vascular endothelium would not function optimally. Although previous reports have shown that nLDL or oxLDL induced endothelial $[Ca^{2+}]_i$ elevation in cultured human or bovine endothelial cells (1, 13, 42), the physiological significance of these results is uncertain. It has been reported that endothelial cells in culture behave differently from the vascular endothelium in many ways, e.g., after a few passages they express much reduced amounts of muscarinic ACh receptors and endothelial NO synthase (25, 37). Thus different origins of endothelial cells could explain this discrepancy. Moreover, the quality of nLDL and oxLDL might also play a role in causing different results. We stored freshly isolated nLDL at 4°C in nitrogen-filled vials for less than 2 weeks. The concentrated nLDL specimens were dialyzed and used to treat vascular tissues within 3 days. Our pilot experiments indicated that nLDL specimens stored in concentrated form for longer than 2 weeks or stored in dialyzed form for as short as 5 days rapidly lost their potentiation effects on ACh-evoked endothelial calcium responses. The difficulty in maintaining the biological activity of nLDL might partially explain the inconsistent results in the literature; some reports show that both nLDL and ox-LDL suppressed the endothelium-dependent vasorelaxation and the endothelium-derived nitric oxide availability (14, 39), while others often use nLDL as the control for ox-LDL (12, 26).

One of the major findings in this study was that oxLDL decreases ACh-evoked calcium responses in an oxidation-time dependent manner (Fig. 3). It should be noted that although mmLDL and oxLDL-2 were only mildly modified from nLDL, both showed significant increases in the level of conjugated dienes and neither could exert significant potentiation effects like nLDL did. Nevertheless, the observed suppressive effects in various modified LDLs did not seem to correlate with either the level of malondialdehyde or the level of conjugated dienes, as malondialdehyde was largely removed by dialysis and the levels of conjugated dienes were similar in mmLDL, oxLDL-4 and oxLDL-8 (Table 2). It is generally accepted that the ACh-induced vasorelaxation is mainly mediated by nitric oxide and that the synthesis of nitric oxide is calcium-dependent. Thus the oxLDL-induced decrease of ACh-evoked endothelial calcium responses would inevitably cause a reduction in ACh-induced vasorelaxation. Results in the literature consistently show that oxLDL exerts inhibitory effects on endothelial function, notably the reduction in NO availability (32). Although the underlying mechanisms are still unknown, part of the oxLDL's suppressive effects on endothelium-dependent vasorelaxation is mediated by its binding to some receptors located on the endothelial cell membrane. As a matter of fact, oxLDL binding to the lectin-like oxLDL receptor 1 causes rapid production of superoxide, which quenches the endothelium-derived NO (7, 12). Numerous products from oxidative modification of nLDL have been proposed to be responsible for the adverse effects of oxLDL. For example, a previous study has identified a cholesterol derivative in oxLDL that inhibits ACh-induced vasorelaxation in rabbit aortic segments (8). Lysophosphatidylcholine, a well-known lipid derivative in oxLDL and atherosclerotic arteries, inhibits a G protein-dependent pathway by disrupting the receptor-G protein interactions in porcine endothelial cells (11). Moreover, it has been shown that lysophosphatidylcholine inhibits ACh-evoked EC $[Ca^{2+}]_i$ elevation, which quantitatively correlates with its inhibitory effects on vasorelaxation in either rabbit or rat aortas (20, 31). Therefore, a portion of oxLDL's lipid component might be initially metabolized to lysophosphatidylcholine, which subsequently interfered with the ACh-induced calcium elevation cascade that ultimately lead to suppressed ACh-evoked vasorelaxation.

Interestingly, HDL could potentiate the ACh-evoked calcium signaling in vessels from the exercise group but not in those from the control group. Whether or not exercise would alter the HDL receptor or post-receptor signal transduction pathways is unknown at present. Our results also showed that like exercise, HDL was capable of reducing the inhibitory effects of oxLDL on ACh-evoked EC $[Ca^{2+}]_i$ elevation; and that the effects of exercise and HDL were additive. HDL exerts vascular protective effects not only via its classical cholesterol-removing mechanism but also via some less-appreciated characteristics, such as its anti-inflammatory and anti-oxidation capacity (2). Moreover, HDL also reverses the inhibitory effect of oxLDL on vascular functions, including the endothelium-dependent vascular relaxation (28) and the NO synthase relocation and activation (38).

In conclusion, the beneficial effects of exercise training on vascular function may be partially attributed to improved sensitivity to HDL and augmented resistance to oxLDL, at least in terms of ACh-evoked EC $[Ca^{2+}]_i$ elevation.

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