Enhanced removal from the plasma of LDL-like nanoemulsion cholesteryl ester in trained men compared with sedentary healthy men

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Vinagre CG, Ficker ES, Finazzo C, Alves MJ, de Angelis K, Irigoyen MC, Negráo CE, Maranhão RC. Enhanced removal from the plasma of LDL-like nanoemulsion cholesteryl ester in trained men compared with sedentary healthy men. J Appl Physiol 103: 1166–1171, 2007. First published July 12, 2007; doi:10.1152/japplphysiol.01176.2006.—The objective of this study was to evaluate the effects of exercise training on plasma removal of a cholesterol-rich nanoemulsion (LDE) that mimics low-density lipoprotein (LDL) lipid structure and binds to LDL receptors. LDE-cholesteryl ester plasma kinetics was studied in 24 exercise-trained and 20 sedentary male subjects. LDE labeled with [14C]cholesteryl ester was injected intravenously, and plasma samples were collected over a 24-h period to determine radioisotope decay curves. LDL cholesterol concentration was similar in both groups. Fractional clearance rate (FCR) of the nanoemulsion label was greater in the exercise-trained group compared with the sedentary group (0.138 ± 0.152 and 0.026 ± 0.03 h⁻¹, respectively). A positive correlation was found (r = 0.60, P < 0.01) between FCR and peak O2 consumption in trained subjects. Circulating oxidized LDL levels were lower in trained subjects compared with the sedentary group (9.0 ± 2.0 and 16.0 ± 3.0 μM). LDE was also injected into control and LDL receptor gene knockout mice submitted and not submitted to training. Muscle LDE uptake percentage was increased in the trained mice compared with the untrained mice (1.1 ± 0.8 and 0.2 ± 0.1, respectively, P < 0.0001) in the control group but not in the knockout animals, indicating that the LDL receptor is involved in the increased uptake elicited by exercise. These results show that exercise training increases LDE plasma removal, which in turn suggests that it also increases LDL receptors or LDL receptor activity.

The effects of exercise training on plasma lipoprotein profile have been well investigated. Exercise training usually tends to minimally decrease the concentration of fasting plasma triacylglycerols that mostly reflect the status of very-low-density lipoprotein (VLDL) and tends to increase the antiatherogenic high-density lipoprotein (HDL) fraction (17–20, 33). With regard to low-density lipoprotein (LDL), one of the most important lipid risk factors for atherogenesis, exercise training does not substantially change the lipoprotein concentration. It may, however, qualitatively change the lipoprotein particles. Exercise training decreases the small, dense LDL subfraction (40), as well as the susceptibility to oxidation (7, 36) and oxidized LDL concentration (7, 40). These effects are recognized as consistently antiatherogenic. The determination of the kinetics of LDL removal from the plasma is crucial in understanding the metabolic events and the quantitative and qualitative changes the lipoprotein undergoes while in the circulation. LDL plasma concentration is determined by the balance between the lipoprotein production rate and the lipoprotein removal rate from the plasma. LDL production comprises the hepatic synthesis of VLDL, LDL precursor lipoprotein, and VLDL catabolism by lipoprotein lipase action, with the generation of LDL. LDL uptake by body tissues is mediated through cell membrane receptors that recognize apolipoprotein (apo) B-100, the vast majority of protein in LDL (5).

In previous studies, we showed that artificially made nanoparticles that resemble the LDL lipidic structure and were termed LDE may be used to explore the LDL intravascular metabolism. LDE lacks protein, but when it is injected into the bloodstream, it acquires several small-molecular-weight apolipoproteins, including apo E (22). Apo E allows LDE particles to bind to the LDL receptors, since those receptors recognize not only the apo B present in LDL but also apo E that is not found in the LDL fraction (42). This approach was validated in animal and human studies and has been shown useful to disclose alterations of LDL metabolism in several clinical conditions (14, 22, 24–26, 32, 37). Conceivably, and differently from the metabolism of native LDL, genetic defects in the apo B structure affecting LDL binding to the receptors do not affect the removal from the plasma of LDE (11).

In this study, to determine whether exercise training alters the LDL intravascular metabolism, we determined the kinetics of LDE labeled with cholesteryl ester in a group of exercise-trained and in sedentary subjects. Also sought were relationships between lipoprotein kinetics and changes in the LDL status by simultaneously measuring the concentration of oxidized LDL in the plasma. The possibility that trained muscle can take up more LDE-cholesteryl ester than the untrained muscle was examined in control and LDL receptor knockout mice submitted or not submitted to exercise.

METHODS

Study Population

Study subjects were healthy male volunteers aged ≤50 yr. All were nonobese, were nonsmokers, did not have alcoholism, were nondiabetic, were under no treatment with lipid-lowering drugs, and had no arterial hypertension. All were submitted to a clinical and cardiopulmonary examination.
monary exercise evaluation before beginning the study. The two study groups were as follows.

**Exercise-trained group.** The exercise-trained group comprised 24 amateur cyclists (mean age 38 ± 7 yr; minimum 18 yr, maximum 49 yr), with an average training load of ~2-h sessions, three to four times per week. All trained individuals were oriented to abstain from any exercise training or other physical activities for at least 2 days before testing as exercise can acutely alter the plasma volume (39). Only subjects that practiced one sport modality were included in this group because of the well-known sport modality-dependent peak O2 consumption (V˙O2 peak) variability (34).

**Untrained group.** The untrained group comprised 20 healthy sedentary men (mean age 38 ± 6 yr; minimum 19 yr, maximum 50 yr). Their jobs and dietary habits did not substantially differ from those of the exercise-trained group. They were also oriented to abstain from any sort of physical activity that would alter their usual sedentary routine for at least 2 days before testing.

Table 1 shows that age and body mass index were similar for the two groups. The experimental protocol was approved by the Ethical Committee of the University of São Paulo Medical School Hospital. An informed written consent was obtained from each participant.

**V˙O2 peak**

Maximal exercise capacity was determined by means of a maximal progressive exercise test on an electromagnetically braked cycle ergometer (SensorMedics, Ergometrics 800, SensorMedics, Yorba Linda, CA), with work rate increments of 20 and 40 W every minute at 60 rpm until exhaustion for exercise-trained individuals and sedentary normal controls, respectively. Oxygen uptake (V˙O2) was determined by means of gas exchange on a breath-by-breath basis in a computerized system (VMAX, SensorMedics). The cycle ergometer was calibrated every 3–4 mo. V˙O2 peak was defined as the maximum attained V˙O2 at the end of the exercise period, which was determined by the moment in which the subject could no longer maintain the cycle ergometer velocity at 60 rpm and achieved two of the following criteria: 1) a plateau or leveling-off in the oxygen uptake with increase of oxygen uptake of less than 1.5 ml·kg⁻¹·min⁻¹ or successive values within 5% of each other (30) despite progressive increases in exercise intensity every 60 s, 2) respiratory exchange rate of 1.1 or above (15), and 3) heart rate above 95% of the age-related maximum (27).

### Table 1. Physical characteristics, plasma lipids, oxidized LDL, and fractional clearance rate of the nanoemulsion 14C-labeled cholesteryl ester in exercise-trained and sedentary subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>Untrained (n = 20)</th>
<th>Exercise (n = 24)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>38 ± 6</td>
<td>31 ± 7</td>
<td>0.09</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>25 ± 2</td>
<td>23 ± 2</td>
<td>0.20</td>
</tr>
<tr>
<td>V˙O2 peak, ml·kg⁻¹·min⁻¹</td>
<td>31 ± 8</td>
<td>56 ± 6*</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cholesterol, mg/dl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>180 ± 39</td>
<td>171 ± 29</td>
<td>0.40</td>
</tr>
<tr>
<td>LDL</td>
<td>120 ± 30</td>
<td>105 ± 24</td>
<td>0.10</td>
</tr>
<tr>
<td>HDL</td>
<td>41 ± 8</td>
<td>50 ± 11*</td>
<td>0.03</td>
</tr>
<tr>
<td>VLDL</td>
<td>20 ± 7</td>
<td>17 ± 10</td>
<td>0.49</td>
</tr>
<tr>
<td>Triacylglycerols, mg/dl</td>
<td>97 ± 35</td>
<td>86 ± 48</td>
<td>0.51</td>
</tr>
<tr>
<td>Ox-LDL, mg/dl</td>
<td>16.0 ± 3.0</td>
<td>9.0 ± 2.0*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>[14C]CE-FCR, h⁻¹</td>
<td>0.026 ± 0.023</td>
<td>0.138 ± 0.152*</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**LDE Preparation**

LDE was prepared from a lipid mixture composed of 40.0 mg cholesteryl oleate, 20.0 mg egg phosphatidylcholine, 1.0 mg triolein, and 0.5 mg cholesterol. For the plasma kinetic studies in subjects, 14C-labeled cholesteryl ester (cholesteryl [14C]oleate) (70 kBq) was added to the mixture. For the animal experiments, the lipid mixture was labeled with [3H]cholesteryl ether. The lipidic mixture was then submitted to an ultrasonic irradiation in aqueous media, dried through nitrogen flux, and purified by a procedure of two-step ultracentrifugation, according to Ginsburg et al. (10) modified by Maranhão et al. (22). The final lipid composition of LDE was 64% phospholipids, 33% cholesteryl oleate, 2% triacylglycerols, and 1% cholesterol. LDE was dialyzed against a saline solution and sterilized by passage through 0.22-µm filter. The entire nanoemulsion preparation procedure was performed in a laminar flux. All glassware used in this study was made pyrogen free by exposure to dried steam at 180°C for 2 h and sterilized by wet steam at 120°C for 30 min. All plastic materials were sterilized by ultraviolet light exposition. The risk due to the nanoemulsion injection is considered minimal and related to the consequences inherent to venous puncture. The risk of infection is minimum as well, since preparations were tested for sterility and absence of pyrogen. Furthermore, the injected nanoemulsion volume was extremely small, which reduces the chance of pyrogenic reactions.

**Radiological Protection**

The radioactive dose used in the intravenously injected labeled lipid experiments with the patients was in strict accordance with the International Commission on Radiological Protection (ICRP) (38), as described in our previous study (23). The equivalent dose induced by the injected radioactivity dose was 0.03 mSv, well below the permitted 50.0 mSv maximum dose.

**Kinetic Studies**

The test began at approximately 9 AM with all participants fasting for a 12-h period. One hundred microliters of LDE containing 37 kBq 14C-labeled cholesteryl ester and a total of 1 mg cholesteryl ester was intravenously injected in a bolus. Plasma samples were collected during 24 h, in intervals of 5 min and 1, 2, 4, 6, 8, and 24 h after injection. The subjects were allowed to eat low-fat meals, which included grilled chicken and fish, vegetables, fruits, and fruit juices on the evening before the test day and after the first blood collection and at about 1:00 PM, since it has been demonstrated that low-fat meals do not interfere with plasma removal of the LDE nanoemulsion (22). During the first 8 h of the sample collection, all the participants were resting in the laboratory test room and remained seated. They were then allowed to return home and come back to the laboratory the next day to collect the last (24 h) blood sample. They were oriented to rest at home.

Radioactivity in aliquots of 1.0 ml of plasma was quantified in a scintillation solution (PPO:dimethyl POPOP:Triton X-100:toluene, 5:0.5:0.5 g:333 ml:667 ml) using a liquid scintillation analyzer (Packard beta spectrometer, model 1600 TR).

The FCR of the LDE 14C-labeled cholesteryl ester was calculated by compartmental analysis, using a computational program (28) (Fig. 1).

**FCR Estimation of the Radioisotope**

For each patient the kinetic activity-time curve was fitted to the mathematical model (19) defined by the sum of two exponential functions, i.e., \( y = A_1 e^{-t/T_1} + A_2 e^{-t/T_2} \). The model consists of two discrete pools, one intravascular pool in dynamic equilibrium with an extravascular pool. This model assumes that all input or exit of the
**EXTRAVASCULAR**

![Diagram](image)

Fig. 1. Compartmental model used to analyze the kinetics of cholesterol-rich nanoemulsion (LDE) cholesteryl ester (CE). The model consists of two discrete compartments for $[^{14}C]CE$ label. All compartments are in the intravascular space ($1_{CE}$ and $2_{CE}$). LDE $[^{14}C]CE$ was injected intravenously in a bolus (arrow with asterisk) into compartment $1_{CE}$. A fraction $k_{1,0_{CE}}$ of the labeled lipid is removed to the extravascular space. Competitively, fraction $k_{1,2_{CE}}$ of the injected lipid is converted into compartment $2_{CE}$ due to the incorporation of apolipoproteins available in the plasma. Subsequently, the materials of this compartment is transferred to the extravascular space following the $k_{2,0_{CE}}$ route. The sampling, represented by triangle, corresponds to the indiscriminate combination of compartments $1_{CE}$ and $2_{CE}$.

radiolabeled lipid occurs from the intravascular pool. The fractional clearance rate of the radiolabeled lipid was estimated as

$$F_{CR} = \frac{A_1 + A_2}{\int_0^\infty [A_1 e^{-\alpha_1 t} + A_2 e^{-\alpha_2 t}] \, dt}$$

which is essentially the inverse of the area under the activity-time curve. The compartment model used is illustrated in Fig. 1.

**Plasma Lipid Measurements**

Starting at approximately 9 AM, venous blood samples were collected of the participants after 12-h fast to determine plasma lipid levels. Total cholesterol (Boehringer-Mannheim, Penzberg, Germany) and triacylglycerols (Abbott Laboratories) were determined by commercial enzymatic methods, using an automatic instrument (Cobas Mira Plus, Roche, Basel, Switzerland). HDL cholesterol was determined by the same method used for total cholesterol after lipoprotein chemical precipitation. VLDL cholesterol and LDL cholesterol were calculated by the Friedewald formula (9).

**Oxidized LDL Plasma Concentration**

An EDTA-blood sample was collected to measure circulating levels of plasma oxidized LDL by enzyme-linked immunosorbent assay (ELISA) kit, using monoclonal antibody 4E6 (12) (Mercodia AB, Uppsala, Sweden).

**Tissue LDE Uptake in Control and LDL Knockout Mice**

Twenty-four male C57/6J and 11 LDL knockout mice (25–30 g) were obtained from the breeding facility of the Heart Institute. The mice received standard laboratory feed and tap water ad libitum and were housed in temperature-controlled rooms (22°C) under a 12:12-h dark-light cycle. Control and LDL knockout mice were randomly assigned to nontrained and trained groups.

Exercise training was performed on a motor treadmill at low-moderate intensity (~50–70% maximal running speed) for 1 h each day, 5 days/wk for 2 wk, with gradual increase in speed from 0.3 to 1.2 km/h. All animals were adapted to the procedure (10 min/day; 0.3 km/h) for 1 wk before the beginning of the exercise training period. After the adaptation, the animals assigned to the untrained group were placed on the stationary treadmill three times per week to provide a similar environment to that of the trained animals.

Untrained and trained mice were submitted to a maximal treadmill test as described in detail in a previous study (6). The tests were made at the beginning and at the end of the training protocol. The purpose was to determine in both mice groups aerobic capacity and exercise training intensity.

After the training session, LDE labeled with $[^{3}H]$cholesteryl ether was injected into the jugular vein through a polyethylene-tipped Tygon cannula. Twenty four hours following the injection of radioactive emulsion, the animals were killed. Fragments of the biceps femoris and the liver were removed, washed in an ice-cold saline solution, dried in filter paper, and weighed. Lipids of 1 g of tissue were extracted with chloroform-methanol (2:1 vol/vol) (8) and separated by thin-layer chromatography (TLC). Radioactivity in the TLC bands corresponding to cholesteryl ether was measured using a liquid scintillation analyzer (Packard beta spectrometer, model 1600 TR).

This protocol was approved by the Experimental Animal Use Committee of the University of São Paulo.

**Statistical Analysis**

The Kolmogorov-Smirnov test was applied to verify the data distribution. The Student $t$-test was used for normally distributed data, and the Mann-Whitney test for those with non-Gaussian distribution. The Student $t$-test was used to evaluate the differences in plasma concentrations of total, LDL, VLDL, and HDL cholesterol, triacylglycerols, and in decay curves of nanoemulsion cholesteryl ester between trained and sedentary subjects, as well as the differences in the percentage of hepatic and skeletal muscle LDE uptake per gram of tissue between trained and untrained mice. The Mann-Whitney test was used to compare the FCR of the emulsion label. Statistical significance was set at the $P < 0.05$ level, and values were expressed as means ± SD.

The plasma decay curves of nanoemulsion cholesteryl ester of athletes and sedentaries were analyzed by two-way ANOVA with repeated measures. When significance was found, post hoc Scheffé’s comparison was used to test the differences between conditions.

Spearman correlation coefficients were used to evaluate the correlation between the cholesterol $[^{14}C]olate$-FCR and total and LDL cholesterol.

For all comparisons, probability values of $< 0.05$ were considered statistically significant.

**RESULTS**

High $VO_2$ peak values displayed in Table 1 confirm better physical capacity of the trained individuals. Table 1 shows that the concentration of total and LDL cholesterol and plasma triacylglycerol concentrations did not differ between exercise-trained individuals and sedentary individuals. On the other hand, HDL cholesterol was higher in the trained individuals. In Table 1, plasma concentration of oxidized LDL values are also shown. There was roughly two times more oxidized LDL in the plasma of the sedentary group than the trained group.

Figure 2 shows the decay curves of the nanoemulsion cholesteryl ester for both groups. The two-way ANOVA with
repeated-measure test followed by Scheffé’s post hoc comparisons showed that there was a significant difference between the curves of both groups. The nanoemulsion cholesteryl ester plasma decay was faster in athletes compared with sedentaries (P = 0.0038). Table 1 shows the FCR values of the LDE label obtained from the curves. Indeed, LDE cholesteryl ester FCR is pronoucnedly greater in trained individuals than in sedentary individuals. No correlation was found between the FCR of the cholesteryl ester of LDE and plasma concentration of total and LDL cholesterol.

Tables 2 and Table 3, show the uptake of LDE cholesteryl both by the muscle and the liver of control and of LDL receptor knockout mice submitted to and not submitted to exercise training. Exercise training in control mice increased the muscle uptake of LDE. This training effect was nullified in LDL receptor knockout mice: in those animals, there is no difference in tissue uptakes between the trained and the untrained group. Whereas the LDE liver uptake was decreased by training in the controls, in the LDL receptor knockout mice, training did not change the hepatic uptake.

**DISCUSSION**

In this study, the removal from the plasma of LDE was markedly accelerated in the exercise-trained group compared with the sedentary group. Since LDE is removed mostly by the LDL receptors (26), the mechanism underlying this effect is presumably through the induction by exercise of increased activity of LDL receptors. As long as we injected a standard preparation into the subjects, the effects on LDE FCR can only be ascribed to the mechanisms of LDL removal from the plasma and not to eventual differences in LDL composition that could affect the removal of the native lipoprotein (42). We have shown that the radioactively labeled cholesteryl ester moiety of LDE behaves very similar to the labeled apo B of native LDL, although it is removed faster from the plasma than apo B (14, 28, 42).

It is worthwhile to point out that the removal of LDE from the plasma was greater in the exercise-trained subjects despite their LDL cholesterol values being similar to those of the sedentary group. This suggests that the increased removal of LDE and for extension of LDL could be compensated by an increased input of the lipoprotein into the plasma compartment. Therefore, exercise training can elicit increased LDL turnover, i.e., the LDL plasma pool is renewed more rapidly.

Jacobs et al. (16) and Altena et al. (1) examined the effects of exercise training on LDL cholesterol before and after a 9-wk and a 4-wk endurance training applied to sedentary subjects, respectively. In the first study (16), LDL cholesterol decreased by only 7%, whereas in the second study training had no effect whatsoever (1). It is worthwhile to point out that the plasma volume may be expanded in trained subjects (39), which could also tend to diminish the LDL-cholesterol concentration.

The oxidation of LDL is involved in coronary artery disease (CAD) (12, 13). Although scavenger receptors are the main mechanism for oxidized LDL uptake, depending on the degree of oxidation, oxidized LDL may still be recognized by the LDL receptors (4). This study showed that the oxidized LDL levels in the exercise-trained were two times smaller than in the sedentary subjects. Regarding this issue, Sanchez-Quesada et al. (35) found that aerobic exercise increased the LDL susceptibility to oxidation, while Bergholm et al. (3) reported that the susceptibility was unchanged. However, subsequent studies showed that either susceptibility to oxidation (7, 36) or oxidized LDL concentration (7, 40) are decreased in exercise-trained subjects, as did the present study. These findings do not appear to be directly mediated by increased antioxidant status (40) or by the ability of HDL to inhibit LDL oxidation (2). Thus, in view of our results, the hypothesis can be raised that the smaller concentration of oxidized LDL can be due, at least in part, to the increased removal from the plasma and the increased turnover of the lipoprotein in the plasma compartment of exercise-trained subjects, as assessed by the LDE approach. It is conceptually valid to assume that in subjects with accelerated removal and increased turnover of LDL, the lipoprotein would be less prone to undergo deleterious changes such as increased plasma lipid oxidizability and peroxidation.

Our results suggest that the greater LDL exposure to oxidation by the increased generation of reactive oxygen species during an exercise session (21) would largely be compensated...
by the increased renewal of the plasma pool of the lipoprotein. The overall result would be a net reduction of oxidized LDL in exercise-trained subjects.

The presumable increase in LDL receptor expression in exercise-trained subjects could be taking place either in the liver, which is the main uptake tissue for both the native lipoprotein and for LDE (24), or in peripheral tissues, such as muscle, that is directly affected either mechanically or metabolically by exercise training.

The current experiments show that the increase in LDL uptake by the skeletal muscle in trained control mice no longer persists in LDL receptor-knockout trained mice. It follows that this effect on the LDL uptake can be reasonably ascribed to an increase in LDL receptor activity due to exercise. This assumption is confirmed by the finding of decreased hepatic uptake of LDE in trained controls, whereas in the LDL receptor knockout mice the hepatic uptake was unaffected by training. This is an additional indication that in controls, LDE is deviated to the muscle because of the increase in LDL receptor activity by training. The results from our animal experiments strongly suggest that the positive effect of the exercise training on the LDE plasma clearance observed in our study subjects is due to increased uptake of the nanoparticles by the muscle.

It has been well established that a sedentary lifestyle is an important independent risk factor for CAD (31). Furthermore, it has been suggested that exercise training is related to an attenuated progression of atherosclerotic lesions (29). LDL receptor function has been considered a major antiatherogenic factor. Therefore, our results offer a new mechanism to explain the role of exercise training in the prevention of cardiac disease. By increasing the LDL removal from the plasma, exercise training may promote the removal of the lipoprotein by the physiological LDL receptor endocytic pathway, avoiding the scavenger receptor-mediated macrophage uptake.

In conclusion, this study shows that sedentary subjects have greater difficulty in removing a nanoemulsion that binds to LDL receptors than do exercise-trained subjects, although the LDL concentration in the plasma shows no difference. The compared results of control and LDE-receptor knockout mice suggest that the exercise-induced increase in LDL clearance observed in the cyclists was mediated by the LDL receptors. Consequently, it is possible that the LDL turnover in sedentary subjects is diminished in the absence of exercise training, thus exposing the lipoprotein to greater oxidative transformation. These observations may furnish an additional mechanism whereby a sedentary lifestyle is an independent risk factor for CAD and exercise training a protection against the development of the disease.

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Use of LDE for drug-targeting purposes is protected as in U.S. Patent 5,578,583.

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EXERCISE AND CHOLESTEROL-RICH NANOEMULSION KINETICS

1171


