Enhanced Cholesterol Efflux Promotion in Well-Trained Soccer Players

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It is widely accepted that aerobic physical activity is associated with a less atherogenic lipid and lipoprotein profile and, consequently, with a reduced cardiovascular risk. Both cross-sectional studies and prospective-interventional trials show that the most frequent modification observed consists of a slight but significant increase in high-density lipoprotein cholesterol (HDL-C) levels. Nevertheless, only few studies made an attempt to elucidate if this quantitative modification was accompanied by an improvement in any of HDL antiatherogenic functions. The purpose of this study was to evaluate the main steps of reverse cholesterol transport, the best known antiatherogenic function performed by HDL, in a group of well-trained soccer players (n = 35) in comparison to sedentary controls (n = 15). Average HDL-C levels were 12.5% higher in the sportsmen, in large part because of greater HDL_2-C concentration. No statistically significant differences were observed in the other lipid- and lipoprotein-related parameters. The capacity to promote cholesterol efflux from Fu5AH cells was significantly higher in the soccer players than in the control individuals (20.5% ± 0.4% *v* 15.9% ± 1.2%, respectively, $P < .001$). However, lecithin:cholesterol acyltransferase (LCAT; 2.6 ± 0.9 *v* 1.4 ± 0.3%/mL · h, respectively) and cholesteryl ester transfer protein (CETP; 69.5 ± 8.3 *v* 62.7 ± 14.8%/mL · h, respectively) activities did not reach statistically significant difference between both groups. Correlation analysis showed that cholesterol efflux induced by serum samples was directly related to HDL-C ($r = 0.59$, $P < .001$), HDL_2-C ($r = 0.37$, $P < .01$), and lipoprotein (Lp)A-I ($r = 0.44$, $P < .05$). On the other hand, negative correlations were observed with waist/hip ratio ($r = -0.36$, $P < .05$), low-density lipoprotein cholesterol (LDL-C; $r = -0.33$, $P < .05$), apolipoprotein B (apo B; $r = -0.42$, $P < .05$), and LpA-I;A-II ($r = -0.51$, $P < .005$). In conclusion, the well-known cardioprotective benefit of regular exercise could be based, at least in part, on a less atherogenic lipid and lipoprotein profile and an enhanced cellular cholesterol efflux.

A SEDENTARY LIFESTYLE is associated with increased atherosclerotic cardiovascular disease, while physical activity has been proved to decrease atherogenic risk.[1] Although exercise has been reported to be cardioprotective, the mechanisms of this benefit have yet to be fully elucidated.

On one hand, it has been shown that regular exercise improves the antioxidant status by increasing hydrosoluble, liposoluble and enzymatic antioxidant defenses, probably in response to the oxidative stress caused by higher oxygen consumption.[2,3] On the other hand, it is well documented that short- or long- term exercise can modify lipoprotein metabolism, independently of secondary factors such as weight loss, dietary modification, or smoking cessation.[4-6] Sustained physical activity has been shown to decrease plasma triglyceride levels by enhancing lipoprotein lipase activity,[7] to cause relatively small improvements in low-density lipoprotein cholesterol (LDL-C) levels,[4] and to increase high-density lipoprotein cholesterol (HDL-C) concentrations.[5]

Early observations of higher HDL-C levels associated with physical activity led to the suggestion that exercise could also enhance reverse cholesterol transport.[8] This metabolic pathway is responsible for the movement of excess cholesterol from peripheral tissues to the liver for lipoprotein recycling or excretion and could be defined as a progression of closely interconnected events.[9] Among them, 4 steps are pointed out as the most relevant: (1) free cholesterol efflux from extrahepatic cells and its uptake by initial acceptors[10]; (2) free cholesterol esterification by lecithin:cholesterol acyltransferase (LCAT)[11]; (3) transfer of newly synthesized cholesteryl esters from HDL to apolipoprotein (apo) B–containing lipoproteins and interchange with triglycerides, performed by the cholesteryl ester transfer protein (CETP)[12]; and (4) hepatic uptake of cholesteryl esters so formed.[13]

The aim of the present study was to explore the first 3 steps of reverse cholesterol transport and especially the capacity to promote cholesterol efflux from cultured cells in relation to regular physical activity. For this purpose, we evaluated a group of well-trained soccer players in comparison to sedentary controls, both groups with similar anthropometric parameters. We also characterized the lipoprotein, apolipoprotein, and lipoprotein particle environment concerned in this antiatherogenic pathway.

MATERIALS AND METHODS

Subjects and Samples

Two groups comprising 35 sportsmen and 15 sedentary control subjects were studied (Table 1). All were young lean men. The sportsmen were soccer players engaged in a controlled physical training program that consisted of 20 hours of training and 6 soccer matches per week for at least 1 year. Sport was practiced at sea level. The control subjects were medical students who led a sedentary lifestyle and did not practice any sport regularly. The sportsmen and the sedentary controls were frequency matched by age and anthropometric parameters (body mass index and waist/hip ratio). All of the subjects selected were

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Table 1. Anthropometric Characteristics of Sportsmen (n = 35) and Sedentary Controls (n = 15)

	Sportsmen	Controls
Age (yr)	18.2 ± 0.2	18.5 ± 0.3
Body mass index (kg/m^2)	22.9 ± 0.2	24.1 ± 0.9
Waist/hip	0.81 ± 0.01	0.81 ± 0.01

NOTE. Values are means ± SEM.

healthy without familial or personal history of diabetes, dyslipidemia, bulimia, or anorexia nervosa, and presented normal thyroid, hepatic, and renal functions. None of the subjects was taking any drug known to affect the lipid and lipoprotein metabolism. Special attention was paid to exclude subjects who were taking anabolic drugs, vitamins, or other antioxidants, or who were smokers. Ethanol intake was considerably less than 50 g per week in all participants. None of the subjects was following a special diet. A face-to-face interview was conducted with each participant and a detailed food frequency questionnaire was completed to obtain information about their dietary habits. The quality, quantity, and frequency of consumption of red meat, chicken, fish, eggs, vegetables, fruits, milk products, and soft drinks was similar in all of the subjects. They followed a typical Argentine diet, which mainly consisted of: red meat 7 times per week, chicken 3 times per week, fish once per week, and floury foods 3 times per week. Vegetables in low quantities and 1 fruit were consumed at each meal. The sportsmen and the controls were all sampled within the same 2 weeks during spring. The soccer players refrained from physical activity during the week preceding the evaluation to avoid the effect of an acute exercise bout because the purpose of the present study was to evaluate the effect of regular exercise. After a 12-hour overnight fast, venous blood was drawn from the antecubital vein. The samples were collected into dry tubes and maintained in a cold chamber (4°C) for 1 hour. Serum was separated by centrifugation at 1,500 × *g* for 15 minutes at 4°C and immediately used for determination of general parameters, lipids, lipoproteins, and apolipoproteins. Aliquots were stored at −80°C for the other determinations. The study protocol was in accordance with the policy statements of the American College of Sports Medicine and was approved by the Ethical Committee of the School of Pharmacy and Biochemistry, University of Buenos Aires. Informed consent was obtained from all participants.

Analytical Determinations

Creatine kinase activity, triglycerides, and total cholesterol were quantified by standardized methods (Boehringer Mannheim, Mannheim, Germany) in a Hitachi 717 autoanalyzer (Tokyo, Japan). Within-run coefficients of variation were 1.10%, 1.11%, and 1.32%, respectively. Between-day coefficients of variation were 1.21%, 1.52%, and 2.44%, respectively. Laboratory bias values were 0.8%, −1.7%, and 1.1%, respectively. HDL was isolated in the supernatant obtained after precipitation of apo B–containing lipoproteins with 20 g/L dextran sulfate (50 kd) and 1.0 mmol/L $MgCl_2$.[14] HDL_3 was separated by precipitation of the supernatant containing total HDL with 40 g/L dextran sulfate (50 kd) and 2.0 mmol/L $MgCl_2$.[14] Cholesterol in total HDL and HDL_3 fractions was determined by standardized enzymatic methods (Boehringer Mannheim). Within-run and between-day coefficients of variation for HDL-C were 3.20% and 3.80%, respectively. Laboratory bias was −2.0%. HDL_2-C was calculated as the difference between HDL-C and HDL_3-C. Triglyceride concentrations in total HDL were determined by standardized enzymatic methods (Boehringer Mannheim), while the phospholipid measurement in the same fraction was performed following the Bartlett method.[15] Non–HDL-C was calculated as the difference between total cholesterol and HDL-C. The LDL-C level was determined as the difference between total cholesterol and the cholesterol contained in the supernatant obtained after selective precipitation of LDL with 10 g/L poly(vinyl sulfate) in poly(ethylene glycol) (600 d; 2.5% wt/vol; pH 6.7).[16] Within-run and between-day coefficients of variation were 4.70% and 5.00%, respectively. Very-low-density lipoprotein cholesterol (VLDL-C) was calculated as the difference between the cholesterol measured in the supernatants obtained after precipitation with poly(vinyl sulfate) (VLDL + HDL) and dextran sulfate (HDL). Apo B, apo A-I, apo A-II, and apo A-I contained in lipoprotein (Lp)A-I particles were measured by electroimmunodiffusion (Hydragel; SEBIA, Issy-les-Moulineaux, France) in serum samples from the sportsmen and the control subjects. The procedure was performed according to the manufacturer's instructions and had previously been validated.[17] Apo A-I contained in LpA-I;A-II particles was calculated as the difference between plasma levels of apo A-I and apo A-I in LpA-I. HDL proteins were estimated as the summatory of apo A-I and apo A-II. Quality control was performed under the surveillance of Dr R. Warnick's Laboratory using the Riqa program (Crumlin, Ireland).

Cholesterol Efflux From Fu5AH Cells

Cellular cholesterol efflux was determined using Fu5AH rat hepatoma cells following the procedure described by de la Llera Moya et al.[18] Briefly, the cells were maintained in minimal essential medium containing 5% fetal calf serum. A total of 25,000 Fu5AH cells/mL were plated on a 24-mm multiwell plates (Inbro; Polylabo, Strasbourg, France) using 2 mL per well. Two days after plating, cellular cholesterol was labeled during a 72 hour-incubation with ^{3}H-cholesterol (NEN, Dupont de Nemurs, Paris, France) (1 μCi/well). To allow equilibration of the label, the cells were washed and incubated for 24 hours in minimal essential medium with 0.5% wt/vol bovine serum albumin. Then the cells were washed with phosphate-buffered saline (PBS) and incubated at 37°C for 3 hours with 2.5% vol/vol diluted serum. At the end of the incubation, the medium was removed and centrifuged; the monolayer cells were washed 3 times with PBS and harvested with 0.5 mL of 0.1 mol/L NaOH. Radioactivity was then measured in both medium and cells, and percentage of cholesterol efflux calculated. Results were corrected by the protein content of each cellular fraction as determined by the method of Lowry et al.[19] All efflux values were averages of 3 determinations.

LCAT Activity

LCAT activity was determined according to the exogenous substrate method modified by Chen and Albers.[20] Briefly, an artificial proteoliposome substrate was prepared containing apo A-I, lecithin, unlabeled cholesterol, and ^{14}C-cholesterol at a molar ratio of 0.8:250:7.5:5. The LCAT activity assay was performed by incubation of 10 μL of the proteoliposome substrate with 100 μL of serum from the patients and the controls at 37°C during 60 minutes. The esterification was linear during this time. The enzymatic reaction was then stopped and lipids were extracted with chloroform:methanol (1:1). Free cholesterol and cholesteryl esters were separated by thin-layer chromatography and the radioactivity of the bands was counted. Results were expressed as percentage of ^{14}C-cholesteryl esters formed, per hour, per milliliter of plasma. Total coefficient of variation for this determination was 6.50%. All samples were tested for LCAT activity using the same proteoliposome substrate preparation. All LCAT values were averages of 2 determinations.

CETP Activity

CETP activity was determined in serum samples according to the general procedure previously described.[21] Briefly, the capacity of serum samples to promote the transfer of tritiated cholesteryl esters from a tracer amount of biosynthetically labeled HDL_3 (^{3}H-cholesteryl ester

Table 2. Lipid and Lipoprotein Levels in Sportsmen (n = 35) and Sedentary Controls (n = 15)

	Sportsmen	Controls
Triglycerides	89 ± 6	95 ± 11
Total cholesterol	164 ± 4	170 ± 6
HDL-C	48 ± 1*	42 ± 2
HDL_2-C	12 ± 1*	8 ± 2
HDL_3-C	37 ± 1	34 ± 2
HDL-phospholipids	70 ± 5	75 ± 5
HDL-triglycerides	14 ± 2	12 ± 1
Non–HDL-C	116 ± 4	128 ± 7
LDL-C	95 ± 4	108 ± 7
VLDL-C	21 ± 1	22 ± 2

NOTE. Values are means ± SEM (mg/dL).
*$P < .05$ *v* controls.

[CE]-HDL[3]) towards serum apo B–containing lipoproteins was evaluated. Serum samples (25 μL) were incubated with ^{3}H-CE-HDL_3 (2.5 nmol of cholesterol) and iodoacetate (75 nmol) in a final volume of 50 μL, during 3 hours at 37°C in a shaking water bath. Since CETP activity is negligible at 0°C, each sample incubated at this temperature served as control. Incubations were stopped by placing the tubes on ice for about 15 minutes; they were centrifuged for 5 minutes at low speed to remove condensed water, and apo B–containing lipoproteins were separated by ultracentrifugation. Incubation mixtures (45 μL) were added to 2 mL of a potassium bromide (KBr) solution (density = 1.070 g/mL) and then ultracentrifuged for 4 hours at 4°C and 250,000 × *g* in a TLA-100.4 rotor in a TL-100 ultracentrifuge (Beckman, MN). Both supernatant (containing the VLDL, intermediate-density lipoprotein [IDL], and LDL fractions) and subnatant (containing the HDL fraction) were recovered and radioactivity was measured in both fractions. Results were expressed as percentage of ^{3}H-CE transferred from HDL_3 to apo B–containing lipoproteins, per hour, per milliliter of plasma. Within-run and between-day coefficients of variation were 4.90% and 6.00%, respectively. All samples were tested for CETP activity using the same ^{3}H-CE-HDL_3 preparation and all values were averages of 2 determinations.

Data and Statistical Analysis

The sample size required to detect significant differences between groups was estimated for the main parameters at a significance level of .05 and 80% power.

Data are presented as the mean ± SEM. Differences between groups were tested using unpaired Student's *t* test or Mann-Whitney *U* test as appropriate. Correlations between all variables were assessed by the Pearson or Spearman test. Differences were considered significant at $P < .05$ in the bilateral situation.

RESULTS

In accordance with the selection criteria, the soccer players and the sedentary controls did not differ in age, body mass index, or waist/hip ratio (Table 1). The activity of creatine kinase, an enzyme of muscular location, was significantly increased in the soccer players in comparison to the control subjects (227 ± 25 *v* 118 ± 22 IU/L, respectively, $P < .005$).

Lipid and lipoprotein levels are listed in Table 2. No significant differences were found in triglyceride, total cholesterol, HDL-phospholipid, HDL-triglyceride, non–HDL-C, LDL-C, and VLDL-C concentrations. Average HDL-C levels were 12.5% higher in the sportsmen, in large part because of greater HDL_2-C concentration. No statistically significant differences were observed in apolipoprotein or lipoprotein particle levels (Table 3).

Figure 1 shows the results of reverse cholesterol transport assays. The capacity to promote cholesterol efflux from Fu5AH cells was significantly higher in the soccer players than in the control individuals (20.5% ± 0.4% *v* 15.9% ± 1.2%, respectively, $P < .001$). However, LCAT (2.6 ± 0.9 *v* 1.4 ± 0.3%/mL · h, respectively) and CETP (69.5 ± 8.3 *v* 62.7 ± 14.8%/mL · h, respectively) activities did not reach statistically significant difference between both groups.

Correlation analysis showed that cholesterol efflux induced by serum samples was directly related to HDL-C ($r = 0.59$, $P < .001$), HDL_2-C ($r = 0.37$, $P < .01$), and LpA-I ($r = 0.44$, $P < .05$). On the other hand, negative correlations were observed with waist/hip ratio ($r = -0.36$, $P < .05$), LDL-C ($r = -0.33$, $P < .05$), apo B ($r = -0.42$, $P < .05$), and LpA-I;A-II ($r = -0.51$, $P < .005$).

DISCUSSION

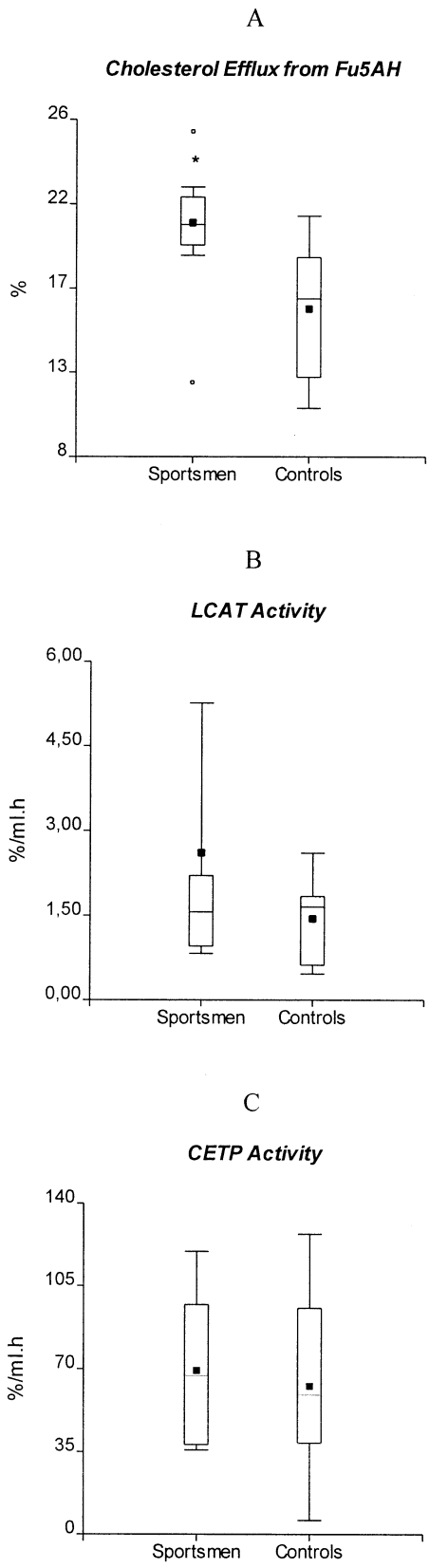
It is widely accepted that aerobic physical activity is associated with a less atherogenic lipid and lipoprotein profile and, consequently, with a reduced cardiovascular risk.[22] In several cross-sectional studies where sportsmen were compared with sedentary controls as well as in prospective-interventional trials where both trained and sedentary individuals were subjected to exercise training, the most frequent modification observed in lipid and lipoprotein profile consisted of a slight but significant increase in HDL-C levels.[23,24] Nevertheless, only a few studies have attempted to elucidate whether this quantitative modification was accompanied by an improvement in any of HDL antiatherogenic functions. To gain further insight into this topic, we evaluated the main steps of reverse cholesterol transport, the best known antiatherogenic function performed by HDL, in a group of well-trained soccer players in comparison to sedentary controls, both groups with similar anthropometric parameters. It must be noted that the selection of lean sportsmen and sedentary controls matched for body mass index and waist/hip ratio could have led to an underestimate of the beneficial effects of exercise training, because a sedentary lifestyle is typically associated with an increase in obesity and worsening of metabolic profiles.

The effect that physical activity exerts on different biochemical parameters seems to be controversial. The contradictory results reported in the literature may derive from important variations observed in the frequency, duration, and intensity of physical activity.[25] The present study focused on a group of well-trained soccer players who could reflect the benefits of

Table 3. Apolipoprotein and Lipoprotein Particle Levels in Sportsmen (n = 35) and Sedentary Controls (n = 15)

	Sportsmen	Controls
Apo B	65 ± 3	68 ± 5
Apo A-I	117 ± 4	115 ± 8
Apo A-II	16 ± 1	15 ± 2
LpA-I	51 ± 2	47 ± 3
LpA-I;A-II	67 ± 4	76 ± 10

NOTE. Values are means ± SEM (mg/dL).



regular training. Soccer is a popular sport and it mainly stresses the aerobic but also the anaerobic pathways. As it was expected, creatine kinase, an enzyme of muscular location, showed significantly higher activity in the sportsmen than in the sedentary controls, a finding that has been reported in other sports.[3,26] Creatine kinase activity may be considered an indicator of the difference in training level between sportsmen and sedentary subjects, although it does not allow elucidating between anaerobic and aerobic training.

Evaluation of lipid, lipoprotein, and apolipoprotein profiles revealed a 12.5% increase in HDL-C levels in the sportsmen in comparison to the sedentary subjects, which reflects the higher aerobic capacity of trained individuals. This increment was due to HDL_2 subfraction, while HDL-phospholipids, HDL-triglycerides, and HDL main apolipoproteins did not show relevant variations between both groups. Metabolic studies using autologously labeled HDL showed that while apo A-I and apo A-II synthetic rates were not different in trained subjects in comparison to sedentary controls, fractional catabolic rates were significantly decreased in sportsmen.[23,27] Nevertheless, it must be noted that this reduction was always associated with a significant diminution in triglyceride levels. In contrast, in our study, both groups of subjects showed similar triglyceride levels. In conclusion, our results could indicate that there is no change in HDL particle number in association with physical activity, and that the HDL fraction is cholesterol-enriched. LpA-I and LpA-I;A-II levels were not different between the sportsmen and the sedentary controls. In contrast, Wilund et al[28] found that plasma levels of LpA-I, the most antiatherogenic subfraction of HDL, significantly increased after 6 months of endurance exercise training in 39 sedentary women and men. These results differ from those reported by Frey et al,[29] who showed that HDL enhancement in 19 endurance-trained sportsmen was selectively due to the LpA-I;A-II subpopulation when compared with 26 sedentary individuals.

The first step of reverse cholesterol transport, cellular cholesterol efflux, was significantly enhanced in the soccer players in comparison to the sedentary controls evaluated in this study. We also found positive and significant correlations between cholesterol efflux capacity and HDL-C, HDL_2-C, and LpA-I subfraction concentrations. These correlations and the fact that HDL-C and HDL_2-C levels were significantly higher in soccer players in comparison to sedentary controls could represent both the cause and the consequence of the increment in cholesterol efflux capacity. Our results are in agreement with those reported by Jafari et al,[30] who showed that acute exercise significantly increased plasma levels of pre–β_1-HDL, generally known as the first acceptor of free cholesterol effluxed from cells. Even if we did not analyze the distribution of apo A-I–containing particles by 2-dimensional electrophoresis, we could

Fig 1. Box plots showing (A) cholesterol efflux promotion, (B) LCAT activity, and (C) CETP activity in sportsmen and sedentary controls. The boxes represent the interquartile ranges of values and contain 50% of values. The line across each box indicates the media. The error bars indicate the highest and lowest values when outliers and extremes are excluded. The square indicates outliers (values between 1.5 and 3 times the box length from the upper or lower edge of the box). $^{*}P < .001$ ***v* controls.**

speculate that the sportsmen evaluated in this study also had higher pre–β_1-HDL levels. Moreover, sportsmen have been shown to present an improved general antioxidant status and, mainly, higher HDL antioxidant capacity attributed to the activity of its associated enzyme paraoxonase.[2,3] This process has been described to preserve different HDL physiologic functions, including cholesterol efflux induction.[31] It is also interesting to note the negative association between cholesterol efflux and LpA-I;A-II subpopulation, which would highlight the fact that LpA-I, and not LpA-I;A-II, is the main HDL antiatherogenic subfraction. Furthermore, negative correlations were observed with 2 atherogenic markers, LDL-C and apo B, and with waist/hip ratio. The latter could be caused by the well-known inverse relationship between abdominal adiposity and HDL-C levels.[32] For cholesterol efflux experiments, we incubated whole diluted serum from the sportsmen and the controls with Fu5AH rat hepatoma cells, a model widely employed to study cholesterol efflux promotion preferentially mediated by scavenger receptor class B type 1 (SR-B1).[33] In a small number of runners and sedentary controls, Campaigne et al[34] found that samples from sportsmen were less efficient to promote cholesterol efflux, even if they presented higher HDL-C levels. Nevertheless, when the subjects exercised for 30 minutes, only the runners showed a significant increase in cholesterol efflux promotion.

In the present study, no statistically significant differences were detected in the second and third steps of reverse cholesterol transport, which are mediated by LCAT and CETP, respectively. Accordingly, Zhang et al[35] found no variation in LCAT and CETP activities after a bout of acute exercise during 1 hour, while only lipoprotein lipase activity significantly increased. Other studies compared LCAT and/or CETP activities in sportsmen versus sedentary controls or in trained and untrained subjects before and after an exercise program. Those studies found either unchanged,[29,36,37] decreased,[24] or even increased[29,38] enzymatic activities in sportsmen.

To our knowledge, only one study reported in the bibliography simultaneously explored the 3 main steps of reverse cholesterol transport in trained subjects ($n = 9$) in comparison to sedentary individuals ($n = 9$).[39] Nevertheless, as mentioned by the authors, a weakness of the study was that selection criteria were not rigorous enough and even subjects who smoked were included as controls. When these investigators evaluated the first step of reverse cholesterol transport, they found no differences in cholesterol efflux promotion, but they reported significantly higher net mass of free cholesterol transported out of cultured human fibroblasts into the athletes' serum than that for controls. Even if these results are in agreement with our findings, it must be noted that values were highly heterogeneous. As regards LCAT and CETP, the previous investigators detected significantly higher activities in sportsmen than in controls.

In conclusion, the well-known cardioprotective benefit of regular exercise could be based, at least in part, on a less atherogenic lipid and lipoprotein profile and an enhanced cellular cholesterol efflux.

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