Effects of Omega-3 Fatty Acid Supplementation and Exercise on Low-Density Lipoprotein and High-Density Lipoprotein Subfractions

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The purpose of this study was to examine the effect of combining exercise with omega-3 fatty acids (n-3fa) supplementation on lipoprotein subfractions and associated enzymes. Subjects were 10 recreationally active males, aged 25 ± 1.5 years (mean ± SE), who supplemented n-3fa (60% eicosapentaenoic acid [EPA] and 40% docosahexaenoic [DHA]) at 4 g/d for 4 weeks. Before and after supplementation, subjects completed a 60-minute session of treadmill exercise at 60% Vo_2max. Following a 24-hour diet and activity control period, blood was collected immediately before and after the exercise session to assess lipid variables: high-density lipoprotein cholesterol (HDL-C) and subfractions, low-density lipoprotein cholesterol (LDL-C) and subfractions and particle size, lecithin:cholesterol acyltransferase (LCAT) activity, and cholesterol ester transfer protein (CETP) activity. Supplementation with n-3fa alone increased total HDL-C and HDL_2-C, while exercise alone increased total HDL-C, HDL_3-C, and total LDL-C. LDL subfractions, particle size, and LCAT and CETP activities were not affected by supplementation. Combination treatment resulted in an additive effect for HDL_3-C only and also increased LDL_1-C versus baseline. LCAT and CETP activities were not affected by treatments. These results suggest that n-3fa supplementation or an exercise session each affect total HDL-C and subfractions but not LDL-C or subfractions. In addition, the combination of n-3fa and exercise may have additional effects on total HDL-C and LDL-C subfractions as compared to either treatment alone in active young men.

THE SCIENTIFIC LITERATURE provides consistent evidence that omega-3 fatty acids (n-3fa) supplementation is a useful strategy for the primary and secondary prevention of atherosclerosis.[1,2] Several mechanisms for this beneficial effect have been hypothesized, including anti-adhesion and anti-arrhythmic properties, as well as lipoprotein-altering effects.[1,2]

There is considerable evidence linking small, dense low-density lipoprotein (LDL) to the risk of coronary heart disease (CHD).[3-6] On the other hand, total high-density lipoprotein cholesterol (HDL-C), as well as subfractions HDL_2-C and HDL_3-C, has been associated with decreased risk of myocardial infarction.[7,8] The impact of n-3fa on LDL and HDL subfractions and particle size may provide valuable information regarding the role of these fatty acids on disease risk.

Investigators consistently have observed the reducing effect of moderate doses of n-3fa supplementation on triglycerides (TG) and postprandial lipemia (PPL).[9-11] On the other hand, the effects of n-3fa on total cholesterol,[11,12] HDL-C,[13-15] and LDL-cholesterol (LDL-C)[9,14-16] have not been as consistent. Furthermore, the existing literature on the impact of n-3fa supplementation on lipoprotein subfractions and particle size also is not consistent for HDL[15,17] or LDL.[3,13,18,19] Griffin[3] reported that the shift to less dense LDL particle size came in spite of a nonsignificant increase in total LDL-C. This result warrants confirmation because a shift in particle size may counter the impact on CHD risk of increased total LDL-C, which sometimes is observed with n-3fa supplementation.[3]

Like n-3fa, exercise may have beneficial effects on lipoproteins and subfractions. Although the impact may be delayed, a single session of aerobic exercise has been shown to have effects similar to n-3fa supplementation on TG concentrations[20] and PPL.[21] A session of exercise has been shown to beneficially affect the lipoprotein profile by increasing total HDL-C and usually HDL_3-C.[22] The effect of acute exercise on total LDL-C concentration is less consistent,[23] but a single exercise session has been shown to change LDL subfractions and particle size toward a larger, less dense fraction.[24-26] Epidemiological studies suggest that when repeated over periods of weeks or months, exercise is associated with chronic changes in the HDL and LDL profile.[27] Therefore, it may be beneficial to combine n-3fa and exercise treatments to enhance the effect of either treatment alone on HDL and LDL subfraction profiles and thus lower risk for CHD.

The purpose of this study was to examine the effect of combining exercise with n-3fa supplementation on HDL and LDL subfractions and associated enzymes. We hypothesized that a session of exercise alone would increase HDL-C subfractions and shift LDL concentrations toward the larger subfractions and that n-3fa supplementation would be additive to the exercise effect.

MATERIALS AND METHODS

Subjects

Participants for the study were 10 recreationally active males, aged 21 to 40 years, who participated in 3 to 5 hours of physical activity per week (Table 1). We used active individuals because we previously had observed that n-3fa and exercise were additive in reducing PPL in active individuals (unpublished observations) but were not additive in sedentary individuals.[28] Each subject was informed of the risks associated with the study and signed informed consent as approved by the University of Missouri Health Sciences Institutional Review Board.

A subject was excluded if he presented with more than one major

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Table 1. Subject Characteristics at Baseline

Age (yr)	25.0 ± 1.5
Weight (kg)	76.61 ± 2.23
Body fat (%)	10.2 ± 1.0
BMI (kg/m^2)	23.2 ± 0.6
Waist/hip	0.80 ± 0.01
Vo_2max (mL/kg/min)	53.2 ± 1.7
HRmax (beats/min)	197 ± 3
RERmax	1.16 ± 0.03

NOTE. Values are means ± SE.

Abbreviations: BMI, body mass index; Vo_2max, maximal oxygen consumption; HRmax, maximum heart rate; RERmax, maximum, respiratory exchange ratio.

risk factor or any disease symptom,[29] or if he currently was taking an n-3fa supplement or any supplement that may affect lipoprotein metabolism.

Design

The general design of the study involved the measurement of plasma lipoproteins and enzymes before and after a session of aerobic exercise. The exercise session was administered before and then again after 4 weeks treatment with n-3fa supplementation. Immediately prior to the pre- and post-exercising testing sessions, a 3-day dietary record was completed by each subject to monitor potential dietary changes during the experimental period. Records were evaluated using the Food Processor 8.10 software (esha, Salem, OR).

Trials

Subjects completed a running VO_2max test as previously described.[21] Body composition also was measured by underwater weighing with directly measured lung volume as previously described.[30]

In preparation for blood collection prior to supplementation, each subject abstained from exercise for 48 hours, and on the day of exercise testing followed an individually standardized diet, including a 3-hour fast prior to the evening exercise session. The exercise session consisted of jogging on a treadmill for 60 minutes at 60%VO_2max as determined from the maximal test. Heart rate and VO_2 were monitored throughout the session. Blood was collected by venipuncture immediately before and immediately after the exercise session. Each sample was immediately separated by centrifugation at 4°C at 3,000 × *g* for 15 minutes, and the plasma was transferred to cryogenic vials and stored at −70°C until analysis. These procedures were repeated after the 4-week n-3fa supplementation period.

n-3fa Supplementation

Subjects ingested 8 soft gel capsules per day (Super EPA-500, Bronson Pharmaceutical, St Louis, MO). Each capsule contained 300 mg of eicosapentaenoic acid (EPA) and 200 mg of docosahexaenoic (DHA) (500 mg n-3fa/capsule) for a total of 4 g n-3fa per day. Subjects were instructed to take 2 capsules with each meal (breakfast, lunch, and dinner) and 2 before going to bed. Compliance was monitored by providing each subject with excess number of capsules for the week and then, when the subject returned for a refill, counting the capsules remaining. Only minor discrepancies were uncovered between expected and actual consumption and these were discussed with the subjects. In addition, plasma samples were assessed for n-3fa concentration by gas chromatography[31] before and after the supplementation period.

Blood/Plasma Assays

Hematocrit was measured using a microcentrifuge (Model MB, International Equipment Co) and a microhematocrit tube reader (Model CR, International Equipment Co, Needham Heights, MA). Hemoglobin was measured using a diagnostic kit (Procedure #525, Sigma Diagnostics, St Louis, MO). Percent changes in plasma volume (PV) were calculated according to Dill and Costill.[32]

For lipid-associated parameters, all samples from a given subject were analyzed in the same assay to eliminate interassay variability. In addition, a standard from a stock plasma pool was run with each assay for quality control. Plasma concentrations of HDL-C, HDL_2-C, and HDL_3-C analysis were measured enzymatically using a modified heparin-$MnCl_2$-dextran sulfate method as previously described.[21] After subfraction separation, cholesterol was assayed using a diagnostic kit (Infinity, Sigma Diagnostics). The coefficients of variation for HDL-C, HDL_2-C, and HDL_3-C were 0.85%, 1.0%, and 3.2%, respectively.

LDL-C subfractions were measured by Lipoprint (Quantimetrix, Redondo Beach, CA) according to manufacturer manual and as previously described in the literature.[33,34] Briefly, 25 μg of plasma was mixed with liquid loading gel which contained Sudan Black B dye, which binds proportionately to the relative amount of cholesterol in each lipoprotein.[34] The resulting mixture was added to the top of precast 3% polyacrylamide gel tubes. After photopolymerization at room temperature for 30 minutes, samples were electrophoresed for 1 hour. As the lipoprotein particles migrated through the separating gel matrix, they were resolved into lipoprotein bands according to their particle sizes from largest to smallest due to the sieving action of the gel. Densitometry was performed at 610 nm using a Helena EDC system (Helena Labs, Beaumont, TX) in a special holding device. Raw data generated by the densitometer and the electrophoretic scan patterns were analyzed using software furnished by the company to calculate the cholesterol in 7 LDL subfractions (LDL_1-C, LDL_2-C, etc). As designed by Quatimetrix and previously reported in the literature,[35] the software determined the relative area for each lipoprotein band, which was multiplied by the total cholesterol concentration of the sample to yield the cholesterol concentration for each band. The intra-assay coefficient of variability was 3% to 10% for the various subfractions. The data thus generated also were converted into LDL particle sizes,[33] and the ratio of large to small LDL particle areas, using 263Å cutoff, was calculated.[34] The original validation study also calculated LDL scores and then estimated phenotype as an estimate of CHD risk.[33] However, we have not found this phenotyping to discriminate when using apparently healthy, young men and women (unpublished observations).

Lecithin:cholesterol acyltransferase (LCAT) activity was measured using heat-treated plasma samples. Test plasma (0.1 mL) was added to 20 μL of 5′5′-dithio-bis(2-nitrobenzoic acid) (DTNB) solution and incubated at 37°C for 30 minutes; this was followed by adding labeled cholesterol-albumin emulsion and incubated at 37°C for 4 hours. Lipids from the mixture were extracted with 4 mL $CHCl_3$-MeOH (2:1 by volume) and after evaporation of the organic solvent under nitrogen, samples were applied to high-performance thin-layer chromatography (HPTLC) using silica gel G plates and developed in a solvent system consisting of hexane/diethyl ether/acetic acid (85:15:2 by vol). Cholesterol (migration ratio factor [Rf] = 0.3) and cholesterylester (Rf = 0.9) bands were clearly separated in the HPTLC plate and were individually transferred to scintillation vials for measurement of radioactivity in a scintillation counter. LCAT was expressed as percent radioactivity of cholesterol converted to cholesterylester and the coefficient of variation was 6.3%.

Cholesterol ester transfer protein (CETP) activity was measured enzymatically using a diagnostic kit (Roar Biomedical, New York, NY). Cholesterol esters (CE) were fluorescently labeled as nitrobenzooxadiazolfluorophor-CE (NBD-CE). Measurements were made using a fluorescence spectrometer (Spex CM1T11I, Edison, NJ) with known standards. CE donor particles (10 μL) and acceptor particles (10

Table 2. Results of 3-Day Dietary Record Analysis

	Presupplement	Postsupplement	*P* Value
Total kcal/d	3,077 ± 338	2,760 ± 342	.518
Carbohydrate (g)	358 ± 53	366 ± 43	.907
% Carbohydrate	50.8 ± 2.0	52.6 ± 2.7	.604
Protein (g)	135 ± 23	114 ± 15	.462
% Protein	18.2 ± 2.4	17.0 ± 2.1	.713
Fat (g)	108 ± 20	93 ± 20	.609
% Fat	29.9 ± 2.5	28.5 ± 2.0	.673
Saturated fat (g)	35 ± 7	30 ± 6	.612
% Saturated fat	10.0 ± 1.0	9.7 ± 0.8	.821
Monounsaturated fat (g)	20 ± 3	15 ± 3	.277
% Monounsaturated fat	5.7 ± 1.0	4.7 ± 1.1	.500
Polyunsaturated fat (g)	9 ± 2	7 ± 2	.293
% Polyunsaturated fat	2.7 ± 0.4	2.1 ± 0.4	.335

NOTE. Values are means ± SE. There were no significant differences between pre- and postsupplementation for any of the dietary measurements.

μL) were combined in 0.5 mL buffer (10 mmol/L tris, 150 mmol/L NaCl, 2 mmol/L EDTA,) at pH 7.4 at 37°C in a temperature-controlled, stirred cuvette while monitoring fluorescence (excitation wavelength 465 nm, emission wavelength 535 nm). After 1 minute, 3 μL of plasma was added to the cuvette and incubated for 15 minutes while monitoring the plasma CETP-facilitated transfer of NBD-CE from donor to acceptor particles. Data was expressed as percent fluorescence increase over baseline in 15 minutes. A blank control sample with no plasma transfer CETP increased 1.2 % in 15 minutes.

Means were analyzed using 2-way analysis of variance (ANOVA) with repeated measures. Significant differences ($P < .05$) were followed up using post hoc contrast comparisons with specifically designed error terms.

RESULTS

Calculated PV changes were minor (<4%), and statistical results were not affected by corrections for plasma volume. Therefore, the uncorrected values are reported in tables and figures. Subjects exercised at 60.7% ± 0.5 % of V_{O_2}max on the presupplement exercise test and 60.6% ± 0.4% on the postsupplement exercise test. Associated energy expenditure was 733 ± 26 kcal and 727 ± 26 kcal, respectively. No dietary macronutrient changed significantly pre- to postsupplement (Table 2).

As tabulated from capsule count, subjects ingested 96% ± 1.9% of the required number of capsules. In addition, the sum of plasma EPA and DHA in the phospholipid plus TG fractions increased from 2.5% (of total fa) prior to supplementation to 10.1% postsupplementation.

n-3fa

Fasting TG concentration decreased significantly from 156.8 ± 24.0 mg/dL pre to 116.0 ± 8.8 post n-3fa supplementation (Table 3). For total HDL-C and HDL_2-C, there was a main effect for n-3fa with combined postsupplement values significantly higher than presupplement (Fig 1A and B). However, supplementation had no effect on HDL_3-C (Fig 1C).

There was a trend toward increased total LDL-C concentra-

Table 3. Lipids and Subfractions.

	Presupplementation		Postsupplementation	
	Pre-exercise	Post-exercise	Pre-exercise	Post-exercise
Cholesterol	164.7 ± 9.3	169.9 ± 9.3	163.6 ± 8.0	174.2 ± 9.3
Triglycerides	156.8 ± 24.0[a]	161.7 ± 18.7[a]	116.0 ± 8.8[b]	121.9 ± 12.6[ab]*
VLDL-C	34.1 ± 3.4	35.1 ± 4.1	29.0 ± 1.5	29.8 ± 2.0
Total IDL-C	30.0 ± 2.9	32.4 ± 3.8	33.5 ± 3.1	35.3 ± 3.0
IDL_C-C	12.0 ± 1.3	12.0 ± 1.8	13.5 ± 1.2	13.9 ± 1.3
IDL_B-C	5.9 ± 1.0	8.1 ± 1.9	6.7 ± 1.0	7.7 ± 1.1
IDL_A-C	12.2 ± 1.1	12.3 ± 1.8	13.3 ± 1.4	13.7 ± 1.2
Total LDL-C	74.5 ± 6.4	76.9 ± 6.9	79.7 ± 6.8	84.6 ± 7.6#
LDL_1-C	26.7 ± 2.3[a]	24.3 ± 2.9[ab]	28.9 ± 2.8[ab]	30.6 ± 2.9[b]
LDL_2-C	16.3 ± 2.2	16.9 ± 2.5	14.8 ± 1.9	16.5 ± 2.0
LDL_3-C	2.3 ± 0.9	3.5 ± 1.3	1.4 ± 0.7	2.4 ± 1.2
LDL particle size	269.9 ± 0.9	269.0 ± 1.3	270.8 ± 0.7	270.1 ± 1.0
LDL ratio	1.8 ± 0.3	1.7 ± 0.5	2.0 ± 0.2	1.8 ± 0.2

NOTE. Values are means ± SE, mg/dL except particle size = angstroms. LDL ratio = large LDL/small LDL. Means in same row with different letters are significantly different, $P < .05$. *Significant main effect for n-3fa; #significant main effect for exercise. All other exercise and n-3fa main effects are not significant.

Abbreviation: IDL, intermediate-density lipoprotein.

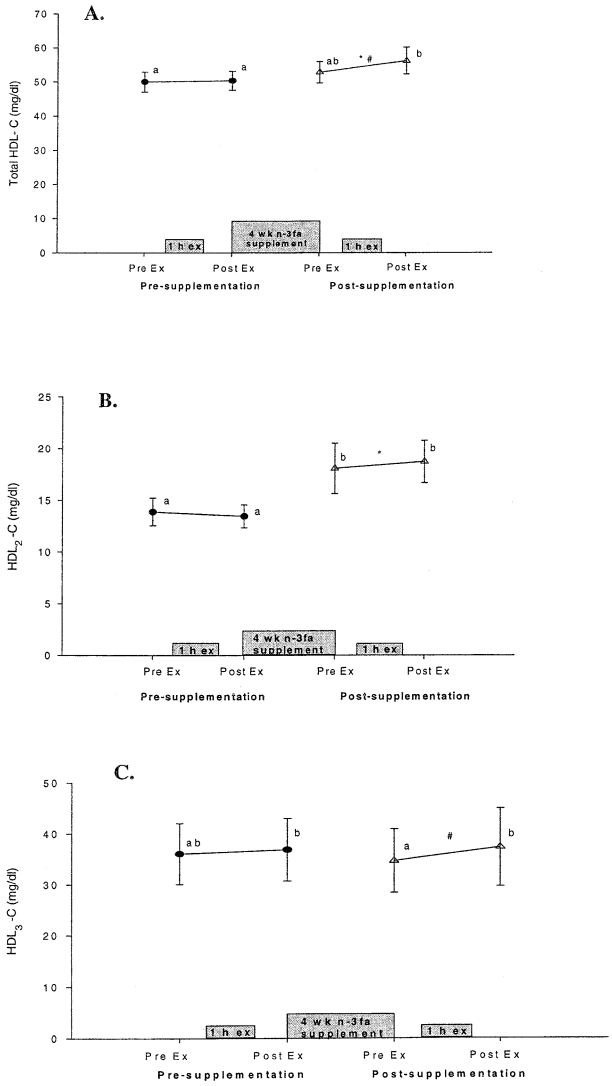

Fig 1. HDL-C and subfractions: (A) HDL-C, (B) HDL_2-C, and (C) HDL_3-C. Means with different letters are significantly different, $P < .05$. *Significant main effect for combined post n-3fa supplement *v* combined presupplement, $P < .05$. #Significant main effect for combined post-exercise *v* combined pre-exercise, $P < .05$. Other main effects for n-3fa or exercise are not significant.

tion with supplementation, but the effect was not significant ($P = .1$). Most of the increase was due to increased LDL_1-C concentration (Table 3). Supplementation with n-3fa did not affect LDL_2 or LDL_3-C concentrations or LDL size or ratio significantly (Table 3). Likewise LCAT and CETP activities were not affected by n-3fa supplementation (Table 4).

Exercise Session

There was no statistical main effect for the session of exercise on TG concentration (Table 3). A session of exercise produced a significant elevation in LDL-C concentrations, but did not affect LDL subfractions or LDL particle size (Table 3). There was no main effect of exercise session on LCAT or CETP activity (Table 4). For total HDL-C and HDL_3-C, there was a main effect of exercise with combined post-exercise values significantly higher than pre-exercise values (Fig 1A and C). In contrast, exercise alone had no effect on HDL_2-C (Fig 1B).

Combined Treatments

The combination treatments did not affect TG concentrations in an additive way (Table 3). The combination of exercise and n-3fa supplementation did not produce a significant additive effect on LDL-C or subfractions or particle size (Table 3). Likewise, the combined treatments did not produce additive effects on LCAT or CETP activities (Table 4). In contrast, HDL-C concentration for acute exercise plus n-3fa was higher than exercise alone and tended to be higher than n-3fa alone (Fig 1A). The combination treatment was not additive for HDL_2-C or HDL_3-C (Fig 1B and 1C).

DISCUSSION

As indicated by capsule count and n-3fa plasma concentrations, subjects appeared to ingest the amount of n-3fa as prescribed. The 4-fold increase in plasma EPA and DHA with 4 g/d n-3fa supplementation for 4 weeks in the present study was comparable to plasma changes reported by Rivellese et al[18] who used 2.4 g/d for 13 weeks and Adler and Holub[9] who administered 3.6 g/d for 12 weeks and were greater than changes reported by Finnegan et al[36] who gave 1.7 g/d for 3 months. Thus the plasma n-3fa changes appear to agree with previous literature and suggest that subjects were compliant to the prescription. Analysis of the 3-day dietary records (Table 2) suggests that changes in diet over the 4- to 5-week period between pre- and post-testing was not a confounder.

One of the primary findings of this study is that n-3fa supplementation did not significantly increase total LDL-C concentration. The trend for the increase in LDL-C was caused primarily by an increase in LDL_1-C concentration (Table 3). This finding agrees with the report by Griffin et al[3] in which LDL_1-C was increased in some patients while LDL-C was maintained with n-3fa supplementation. This is important information as previous results have raised concerns about pre-

Table 4. LCAT and CETP Results

Variable	Presupplementation		Postsupplementation	
	Pre-exercise	Post-exercise	Pre-exercise	Post-exercise
LCAT activity (% esterification)	$1.66 \pm .06^{ab}$	$1.75 \pm .07^{a}$	$1.64 \pm .07^{b}$	$1.66 \pm .06^{ab}$
CETP activity (% fluor/15 min)	24.1 ± 1.0	23.5 ± 1.5	23.4 ± 2.2	24.5 ± 2.1

NOTE. Values are means ± SE Means in same row with different letters are significantly different, $P < .05$. Main effects for n-3fa or exercise were not significant.

scribing n-3fa due to the potential to elevate LDL-C.[16,19] The trend toward increasing LDL_1-C suggests that the potential increase in total LDL-C induced by n-3fa supplementation may not increase the risk for CHD.

The lack of significant change in particle size and LDL ratio is in agreement with data reported by Patti et al[13] who observed no shift in LDL particle size although minor composition shifts within the subfractions were observed. Likewise, Sorensen et al[14] and Rivellese et al[18] reported no change in LDL particle size with n-3fa supplementation. Therefore, it appears that shifts in LDL particle size may not be a mechanism by which n-3fa protects against CHD.

A session of aerobic exercise increased total LDL-C, but not LDL_1-C. This finding contrasts with others who observed that exercise induced changes in the LDL pattern toward a less dense, larger profile.[27,37,38] However, previous observations were made in individuals who were habitually trained[27] or following severe endurance triathlons,[37,38] exercise which is not comparable to our single, moderate exercise session. Although there was no statistical main effect for either n-3fa or exercise to elevate LDL_1-C, the combination of these treatments raised LDL_1-C additively over baseline (Table 3).

The timing of blood collection may have affected the interpretation of our results. It is possible that collecting samples immediately after a session of aerobic exercise is not the most appropriate time to analyze the optimal effect of exercise on LDL subfractions. We also collected fasting blood at 12 hours after the exercise session on most of the subjects. We have not included these data in the statistical analysis because these samples could not be analyzed in the same assay run as the pre-/post-exercise samples and interassay variability is high with this procedure. However, the results of this analysis suggest that total LDL but not LDL_1 concentrations were approximately 8% lower at 12 hours post versus concentrations immediately after the exercise session both before and after n-3fa supplementation. This finding suggests that exercise may have a delayed effect in lowering LDL-C, a result that has been reported by others.[23]

Unlike the tenuous effect on LDL-C, supplementation with n-3fa caused significant increases in HDL-C and HDL_2-C concentrations (Fig 1A and B). This finding agrees with some observations in the literature[15] but not with others.[17,39] HDL is at the center of the putative reverse cholesterol transport system in which the HDL scavenges tissue cholesterol and delivers it to the liver for degradation.[40] Total HDL-C and each of the subfractions have been identified as independent protective factors against CHD.[7,8] Thus, increased concentrations of HDL-C and HDL_2-C may be one of the mechanisms whereby n-3fa improves the CHD risk profile.

A session of exercise raised HDL-C and HDL_3-C concentrations. The elevating effect of a session of exercise on these lipoproteins has been reported consistently in the literature.[23] Surprisingly, the combined n-3fa and exercise treatments were not additive for HDL-C or subfractions. Previously, we observed that in sedentary individuals the two treatments negated each other,[28] but this effect apparently does not carry over to HDL-C in active individuals. To our knowledge, no other researchers have examined the effects of acute aerobic exercise plus n-3fa supplementation on HDL-C.

When comparing the HDL results between n-3 fa supplementation and acute exercise, it appears that both treatments tend to elevate HDL-C, n-3fa by elevating HDL_2-C and exercise by elevating HDL_3-C. The reason for this difference in response to the treatments is unknown but may relate to differing effects on LCAT, CETP, or lipoprotein lipase (LPL). In this study, LCAT and CETP were not altered by either treatment, but LPL has been shown to be increased with a session of aerobic exercise.[42] On the other hand, n-3fa have been reported to reduce apolipoprotein B and very-low-density lipoprotein (VLDL) packaging in the liver.[16] Thus, differences in LPL and VLDL synthesis may account for the difference in lipoprotein effects between the two treatments.

LCAT binds CEs to HDL, which may enhance cholesterol uptake from tissues. Exercise has been shown to stimulate the activity of this enzyme, but the results have not been consistent.[41,42] The only previous report on n-3fa supplementation and LCAT indicated that supplementation for 3 weeks induced a reduction in enzyme activity.[43]

CETP catalyzes the exchange of TG from TG-rich lipoproteins with cholesterol from HDL, especially HDL_2-C. Elevated activity of this enzyme may increase CHD because of the potential for lowering HDL-C and HDL_2-C. CETP has been shown to be decreased by exercise training,[44] but previously we observed no change in the enzyme activity over 24 hours following a single aerobic exercise session.[42] We found no previous reports that have measured CETP following n-3fa supplementation. The negative findings related to LCAT or CETP activities suggest that any changes in lipoproteins induced by exercise or n-3fa are not stimulated by increased activity of these enzymes

These results suggest that n-3fa supplementation or a single session of exercise each affect lipoproteins, especially HDL and subfractions. In addition, the combination of treatments may provide additional effect on HDL_3-C and LDL_1-C. However, LCAT and CETP activities do not appear to mediate the adaptations of lipoproteins induced by n-3fa supplementation or exercise.

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