Evidence for altered positional specificity of LCAT in vivo: studies with docosahexaenoic acid feeding in humans

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Abstract The percentage of saturated cholesteryl esters (CEs) synthesized by human LCAT is several times higher than expected from the *sn*-2 acyl composition of plasma phosphatidylcholine (PC), whereas the synthesis of 20:4 CE and 22:6 CE is much lower than expected. To explain these discrepancies, we proposed that LCAT transfers some saturated fatty acids from the *sn*-1 position of PC species that contain 20:4 or 22:6 at *sn*-2. The present studies provide in vivo evidence for this hypothesis. We determined the composition and synthesis of CE species in plasma of volunteers before and after a 6 week dietary supplementation with docosahexaenoic acid (22:6; DHA). In addition to an increase in the DHA content of all plasma lipids, there was a significant (+12%; $P < 0.005$) increase of 16:0 CE, although there was no increase in 16:0 at *sn*-2 of PC. The increase of DHA in CE was much lower than its increase at *sn*-2 of PC. Ex vivo synthesis of CE species in plasma showed a significant (+24%; $P < 0.005$) increase in the synthesis of 16:0 CE after DHA supplementation, which correlated positively with the increase of 22:6, but not of 16:0, at *sn*-2 of PC. These results show that the positional specificity of human LCAT is altered when the concentration of 16:0-22:6 PC is increased by DHA supplementation.—Subbaiah, P. V., J. M. Sowa, and M. H. Davidson. **Evidence for altered positional specificity of LCAT in vivo: studies with docosahexaenoic acid feeding in humans. *J. Lipid Res.* 2004.** 45: **2245–2251.**

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Most of the cholesteryl esters (CEs) in the lipoproteins of human plasma are derived from the action of LCAT (1). Although this enzyme has been shown to be specific for the *sn*-2 position of phosphatidylcholine (PC), the composition of the CEs in human plasma does not match that of the *sn*-2 acyl group (1–4). For example, although 16:0 constitutes only ~2–3% of the PC *sn*-2 acyl groups, it accounts for 10–12% of plasma CE. On the other hand, although 20:4 constitutes ~16% of the *sn*-2 acyl groups of PC, only ~5% of the plasma CE is 20:4. Similarly, the concentration of 22:6 at *sn*-2 of PC (~5%) is much higher than that in CE (~0.4%). Although these discrepancies have been attributed to the preference of the enzyme for the PC substrates containing 16:0 at *sn*-2 (5), in vitro studies with synthetic substrates and isolated enzyme did not support this mechanism (6). Our studies on the use of PC species by LCAT in native plasma also showed that 16:0-16:0 PC, the major PC with 16:0 at *sn*-2, is not preferred over "average" PC in plasma. Based on our studies with synthetic PC substrates and isolated LCAT (7, 8), we proposed that human LCAT transfers significant amounts of *sn*-1 acyl group from the PC species containing 20:4 or 22:6 at the *sn*-2 position. This mechanism not only explains the synthesis of higher than expected percentages of saturated CE species but also accounts for the formation of lower than expected percentages of 20:4 and 22:6 CE. This alteration in positional specificity appears to be a function of the architecture of the enzyme's active site, because rat and mouse enzyme are not substantially affected by the presence of *sn*-2-20:4 PC (8) and because the human LCAT behaves like the mouse enzyme if its active site domain is replaced by the corresponding domain from mouse enzyme (4, 9).

Although we have provided multiple lines of evidence for the altered positional specificity of human LCAT in vitro, there is no direct evidence that this occurs in vivo. Previous studies of the effects of fish oil feeding in humans (10) and nonhuman primates (11, 12) reported an increase in the appearance of 16:0 CE in plasma. Because fish oil feeding results in an increase in 22:6 at *sn*-2 of plasma PC, we postulated that the observed increase in 16:0 CE was attributable to the utilization of 16:0 from the *sn*-1 position of 16:0-22:6 PC (7). However, because fish oil contains several other fatty acids, including 16:0, this increase also could be attributable to the increased forma-

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Abbreviations: CE, cholesteryl ester; DHA, docosahexaenoic acid; PC, phosphatidylcholine; TG, triacylglycerol.

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tion of *sn*-2-16:0 PCs or to the increased contribution of liver ACAT-2, which is known to be relatively nonspecific for the fatty acids (13, 14) and therefore can synthesize saturated CE. The interpretation of fish oil studies is also complicated by the fact that fish oils contain varying amounts of 20:5 and 22:6, and our in vitro studies showed that only *sn*-2-22:6 PCs cause altered positional specificity (8). The recent availability of highly enriched docosahexaenoic acid (DHA) oils from marine algae, containing virtually no 20:5, provided an opportunity to test our hypothesis in vivo in human subjects. As part of an ongoing study to determine the efficacy of DHA in reducing cardiovascular risk factors, we analyzed plasma samples from a subgroup of subjects for detailed fatty acid composition of various plasma lipids, before and after DHA supplementation. In addition, we studied the ex vivo synthesis of CE species by LCAT in the plasma, as affected by DHA enrichment. The results presented here show that specific enrichment of plasma PC with DHA leads to an increased synthesis of 16:0 CE without a significant change in 16:0 at *sn*-2 of PC. These results therefore support the hypothesis that the positional specificity of human LCAT is altered when the *sn*-2 position of PC is occupied by a very-long-chain fatty acid.

MATERIALS AND METHODS

Subjects

The study was conducted in a subgroup of subjects who were recruited by the Chicago Center for Clinical Research for a double-blind, controlled clinical study aimed at determining the effect of DHA-enriched marine lipids on cardiovascular risk factors. The study protocol was approved by an institutional review board (Schulman Associates IRB, Inc., Cincinnati, OH). The characteristics of the study subjects were as follows: equal number of males and females, ages 21–80 years, body mass index < 40 kg/m^2, total cholesterol < 300 mg/dl, triacylglycerol (TG) < 350 mg/dl, and HDL < 44 mg/dl for males and < 54 mg/dl for females. The average BMI for the subjects was 29.6 ± 0.9 kg/m^2. Subjects over 40 kg/m^2 were excluded. All subjects were on the National Cholesterol Education Program (NCEP) Step 1 diet for at least 4 weeks before the start of DHA supplementation and throughout the 6 weeks of supplementation. None of the subjects was on hypolipidemic medication or dietary supplementation with fish oil for at least 4 weeks before the start of the study. Frozen plasma samples from 15 randomly selected subjects on DHA supplement (out of a total of 27) were used for the present study, after the completion of the above study. Detailed effects of the supplement on lipid levels and other cardiovascular risk factors will be published separately.

Each subject received a dietary supplement (4 g/day) of marine lipid capsules (derived from *Schizochytrium* species; supplied by Martek Biosciences Boulder Corp.) containing 22:6, n-3 (38%), 22:5, n-6 (16%), and 20:5, n-3 (2%). Other fatty acids in the capsule were as follows: 16:0 (21%), 14:0 (8%), 18:2, n-6 (2%), 20:4, n-6 (2%), 18:0 (1%), and 18:1, n-9 (1%). The total amount of DHA in the supplement was 1.52 g/day, which amounted to 0.78% of the total calories. Blood samples were collected in 1 mM EDTA at baseline and after 6 weeks of dietary supplementation. Plasma was separated immediately by centrifugation and stored at −30°C until analysis.

Lipid analyses

Plasma cholesterol and TG were measured by enzymatic procedures on a Hitachi 747. HDL cholesterol was estimated by the formula of Friedewald, Levy, and Fredrickson (15). Lipoprotein subfractions were also analyzed by the Vertical Auto Profile (VAP II) (16).

Fatty acid analysis

Total lipids were extracted by the Bligh and Dyer procedure (17), and an aliquot of the lipid extract was used to measure the total fatty acid composition by gas chromatography after methylation with BF_3 in methanol (see below). The rest of the lipid extract was spotted on a silica gel TLC plate and separated using a two-step solvent system. First, the solvent (chloroform-methanol-water, 65:25:4, v/v) was run up to 10 cm from the bottom of the plate (step I). The plate was then air-dried for 5 min in a fume hood and developed in hexane-diethyl ether-acetic acid (70:30:1 v/v) up to 19 cm from the bottom (step II). Standards of PC, sphingomyelin, cholesteryl oleate, and TG were run in separate lanes for identification purposes. After covering the lanes containing the samples with a clean glass plate, the lanes containing the standards were exposed to iodine vapors, and the areas corresponding to PC, TG, and CE were scraped from the unexposed lanes. The CE and TG spots were methylated directly using 1 ml of BF_3 in methanol (Alltech) and heating at 90°C for 1 h under N_2. After adding 1 ml of water, the methyl esters were extracted twice with 2 ml of hexane. The PC spot was eluted by the Bligh and Dyer procedure (17) and subjected to hydrolysis with snake venom phospholipase A_2 in a screw-cap tube. The incubation mixture contained the eluted PC, 2.5 units of phospholipase A_2 from *Naja mossambica* venom, 20 mM $CaCl_2$, and 2 ml of diethyl

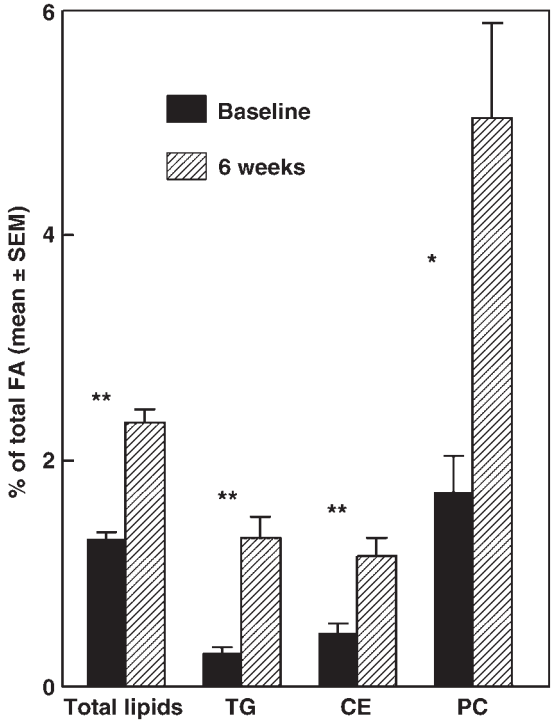

Fig. 1. Enrichment of plasma lipids with docosahexaenoic acid (DHA). The percentage of DHA in each lipid class was determined by gas chromatography as described in the text. The statistical significance of differences between baseline and 6 week values was determined by paired *t*-tests: * $P < 0.005$ and ** $P < 0.001$. CE, cholesteryl ester; PC, phosphatidylcholine; TG, triacylglycerol.

ether. After incubation for 1 h at room temperature, the ether was evaporated off under N_2, and the total lipids were extracted (17) and separated on a silica gel TLC plate with the solvent system of chloroform-methanol-water (65:25:4, v/v). Standards of lyso-PC, PC, and FFA were spotted on separate lanes and exposed to iodine vapors (after covering up the sample lanes with a clean glass plate) for identification purposes. Spots corresponding to FFA and lyso-PC were scraped and methylated with 1 ml of BF_3-methanol as described above and quantified by gas chromatography on a Shimadzu GC-17A gas chromatograph equipped with a flame ionization detector and an Omegawax (30 m × 0.25 mm; Supelco) column. Hydrogen was used as the carrier gas at 1.2 ml/min, with a split ratio of 50:1. The temperature programming was as follows: initial temperature of 175°C (8 min), increased to 200°C at a rate of 4.5°C/min, kept at 200°C for 8 min, then increased to 225°C at a rate of 6°C/min, and maintained at this temperature for 15 min. In the case of CE samples, an additional step was included at the end, in which the temperature was increased to 270°C (20°C/min) and maintained for 15 min, to remove free sterol and other contaminants that would otherwise interfere with the analysis of subsequent samples. Identification of the fatty acids was done using authentic standards, and the percentage composition was determined using EZChrom software (Scientific Software, Inc., San Ramon, CA).

Ex vivo synthesis of CE by LCAT

Plasma samples were incubated with a human serum albumin-[4-^{14}C]cholesterol complex (18) at 37°C in the presence of 1.3 mM DTNB to equilibrate labeled cholesterol with endogenous cholesterol. The samples were then incubated with 11.8 mM mercaptoethanol for 3 h at 37°C. The total lipids were extracted (17), and the composition of labeled CE species formed was analyzed by reverse-phase HPLC, as described before, using an online radioactivity detector (19). The mobile phase was acetonitrile-tetrahydrofuran-water (65:35:1.5, v/v) at a flow rate of 2.0 ml/min. The ratio of scintillation fluid to solvent was maintained at 2:1, and the peaks were quantified by using the computer program EZChrom (Scientific Software, Inc.).

RESULTS

Effect of DHA supplementation on plasma lipids

The following major changes were observed in plasma lipid and lipoprotein composition after 6 weeks of dietary supplementation with DHA (1.52 g/day). TG decreased by 25%, total cholesterol increased by 5.8%, and HDL cholesterol increased by 9.0%. The increase in HDL was mainly in HDL_2 (+14.0%), with only a modest change in HDL_3 (+1.7%). Total LDL increased by 12.0%, with an increase in large LDL subfractions but a decrease in small LDL subfractions. Detailed analyses of the lipids and other cardiovascular risk factors for the whole group of cohorts will be published separately.

Incorporation of DHA into plasma lipids

Figure 1 shows the enrichment of specific plasma lipids with DHA after dietary supplementation with the marine lipid concentrate for 6 weeks. The DHA content of total plasma lipids increased by 79%, whereas its concentration

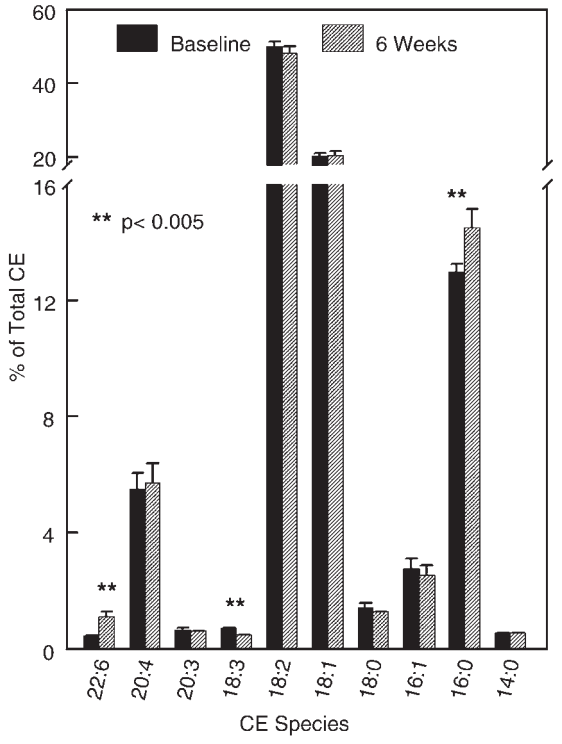

Fig. 2. Fatty acid composition of CE species. Plasma CE was separated by silica gel TLC, and its fatty acid composition was analyzed by gas chromatography, as described in the text. The statistical significance of differences between baseline and 6 week samples was determined by paired *t*-tests.

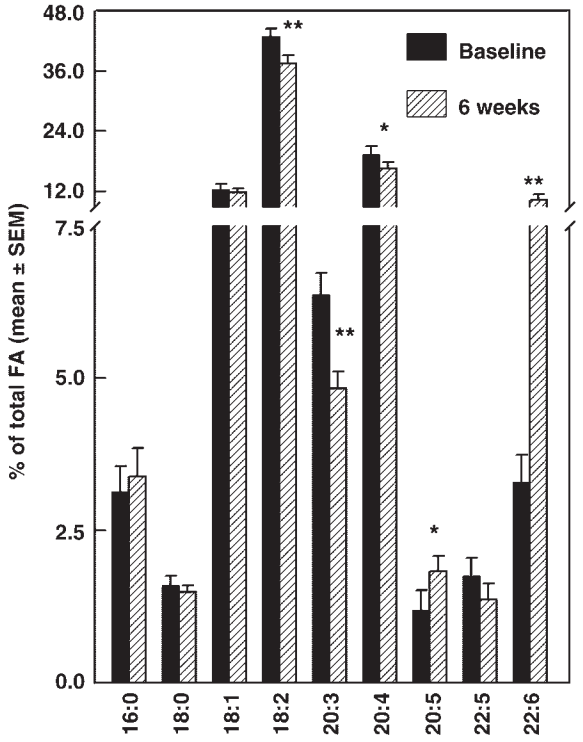

Fig. 3. Acyl composition of the *sn*-2 position of plasma PC. PC was first separated from other lipids by silica gel TLC, eluted, and subjected to snake venom phospholipase A_2 treatment, as described in the text. The liberated fatty acids were then analyzed by gas chromatography, as described in the text. * $P < 0.05$ and ** $P < 0.005$ by paired *t*-tests.

in TG was increased by 340%. The increase in CE was 147%, whereas the increase in PC was 192%. The DHA concentration of the PC after 6 weeks was 5.04% of the total fatty acids. Because DHA is found exclusively at the *sn*-2 position of PC, ~10% of all plasma PC molecules contained DHA after supplementation, whereas only ~1.2% of CE contained DHA. Thus, there was a disproportionate increase of DHA in PC, compared with CE, as reported in other studies in humans (10, 20, 21) and in experimental animals (22) after fish oil supplementation.

CE fatty acid composition

The detailed fatty acid composition of plasma CE at baseline and after 6 weeks of DHA supplementation is shown in **Fig. 2**. Compared with the baseline, significant increases occurred only in 22:6 CE (+149%) and 16:0 CE (+12%). There was a significant decrease in 18:3 (n-6) CE (−32%), a minor component. The major CE species in plasma, 18:2 CE, decreased by ~4%, but this decrease was not statistically significant. Thus, the increases in 22:6 and 16:0 CEs appear to have occurred at the expense of 18:3 and 18:2 CEs.

Effect on acyl composition of *sn*-2 and *sn*-1 of PC

The effect of DHA supplementation on the acyl group composition at the *sn*-2 position of PC is shown in **Fig. 3**. As expected, the 22:6 content of PC at *sn*-2 increased significantly (3.5-fold). There was also a small but significant increase in 20:5, but no increase in 22:5 (n-6), which was one of the major unsaturated fatty acids in the dietary supplement. Significant decreases occurred in 18:2, 20:3, and 20:4, indicating that 22:6 mainly replaced these fatty acids in PC. In contrast to the *sn*-2 position, there were no significant changes at the *sn*-1 position of PC. Only three fatty acids were found in the *sn*-1 position (16:0, 18:0, and 18:1), as expected from the published data on the molecular species composition of human plasma PC (20), and their composition did not change significantly after DHA supplementation (**Fig. 4**).

Ex vivo synthesis of CE

To investigate whether the changes in plasma CE could be attributed to the activity and/or specificity of LCAT, or to changes in the contribution of ACAT-2 activity of liver or intestine, we determined the synthesis of labeled CE from labeled free cholesterol after incubation of the whole plasma at 37°C. The labeled free cholesterol was first equilibrated with endogenous cholesterol by incubation of the plasma in the presence of DTNB (to inhibit LCAT) for 4 h at 37°C. The inhibition was then released by the addition of excess mercaptoethanol, and the synthesis of CE was allowed to take place for 3 h at 37°C. The labeled CE species formed were quantified by reverse-phase HPLC as described in Materials and Methods. The composition of labeled CE species formed at baseline and after 6 weeks of DHA supplementation is shown in **Fig. 5**. Only the synthesis of 22:6 CE (+112%) and 16:0 CE

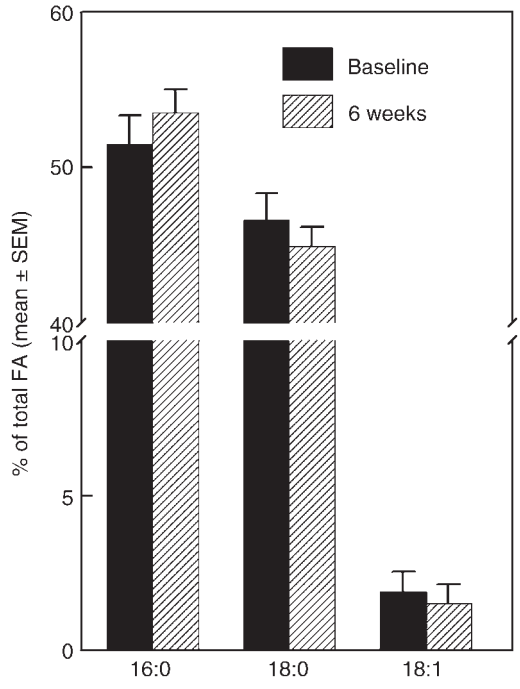

Fig. 4. Acyl composition of the *sn*-1 position of plasma PC. Plasma PC was isolated by TLC and then treated with snake venom phospholipase A_2. The fatty acid composition of the lyso-PC generated was determined by gas chromatography as described in the text. None of the changes was statistically significant by paired *t*-tests.

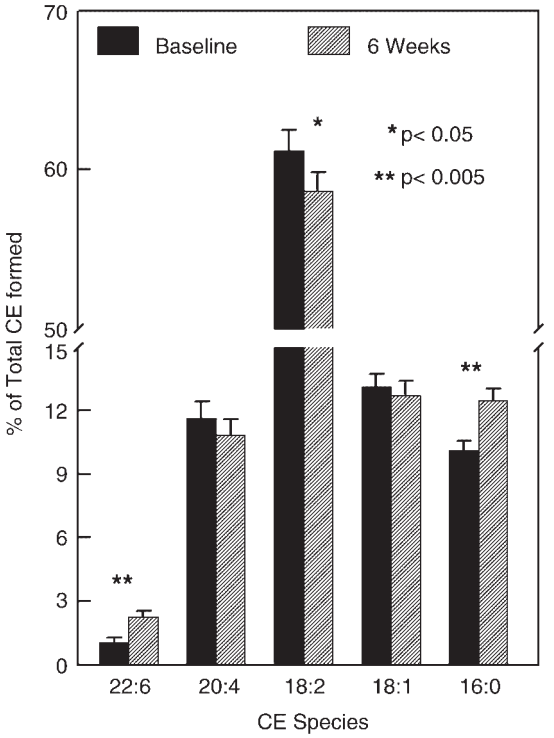

Fig. 5. Ex vivo synthesis of CE species before and after DHA supplementation. Plasma samples were prelabeled with [4-^{14}C]cholesterol and incubated with mercaptoethanol at 37°C for 3 h, to allow the LCAT reaction to take place. The labeled CEs formed by the LCAT reaction were then separated by HPLC on a C18 column, and their radioactivity was determined using an online radioactivity detector. * $P < 0.05$ and ** $P < 0.005$ by paired *t*-tests (n = 13). Values shown are mean ± SM.

(+24%) increased significantly after DHA supplementation, whereas the synthesis of 18:2 CE decreased (−4%) significantly. These results are similar to the results with the mass analysis of CE species in whole plasma. The synthesis of 20:4 CE also decreased after DHA supplementation, although this decrease was not statistically significant. These results show that the composition of CE in plasma reflects predominantly the activity of LCAT and that the contribution by tissue ACAT is minimal, as reported earlier (1).

Correlations

The novel finding of this study is an increase in the synthesis of 16:0 CE after dietary supplementation with DHA. To determine whether this 16:0 is derived only from the *sn*-2 of PC or from the *sn*-1 position of PC species that contain DHA, we investigated the correlation of changes in *sn*-2 acyl composition with the increased synthesis of 16:0 CE. If 16:0 for CE synthesis is exclusively derived from the *sn*-2 position of PC, the increase in 16:0 synthesis should correlate positively with an increase in *sn*-2-16:0. If, on the other hand, excess 16:0 is derived from the *sn*-1 position of PC species containing 22:6 at *sn*-2, it should correlate positively with the increase in *sn*-2-22:6. As shown in **Fig. 6A**, there was a positive correlation between the increase in *sn*-2-22:6 in PC and the increase in the synthesis of 16:0 CE. There was, however, no such correlation between the synthesis of 16:0 CE and the increase in 16:0 at the *sn*-2 position (Fig. 6B). Instead, there was a slight negative correlation between these two parameters (NS). These results suggest that the increased synthesis of 16:0 CE after DHA supplementation is attributable to an alteration in the positional specificity of LCAT, rather than to an increased availability of 16:0 at *sn*-2 of PC. Interestingly, the increase in 22:6 CE synthesis did not correlate positively with the increase in *sn*-2-22:6 ($r = -0.29$; NS) (results not shown).

DISCUSSION

It is well established that the LCAT reaction is responsible for the synthesis of most of the CE present in human plasma (1). Although it is widely accepted that the enzyme specifically transfers the *sn*-2 acyl group from plasma PC to free cholesterol, the CE species composition differs significantly from that of the *sn*-2 acyl groups of PC. Characteristically, the percentage of saturated CE is four to five times higher than expected from the fatty acid composition at *sn*-2 of PC (1–4). Furthermore, the CE species generated during ex vivo incubation of plasma also show this discrepancy, showing that the saturated CEs are derived from the LCAT reaction, not contributed by the ACAT reaction in liver or intestine (23). On the basis of the consumption of PC species during in vitro incubation of whole plasma, we proposed that the reason for the formation of higher than expected amounts of saturated CE by human LCAT is the utilization of the *sn*-1 acyl group from certain PC species that contain 20:4 or 22:6 at the *sn*-2 position (24). Although we provided evidence for this hypothesis using synthetic PC substrates and isolated LCAT (7, 8), it is not known whether the altered positional specificity occurs in vivo. The present study was aimed at providing direct evidence for its occurrence in vivo. The rationale for

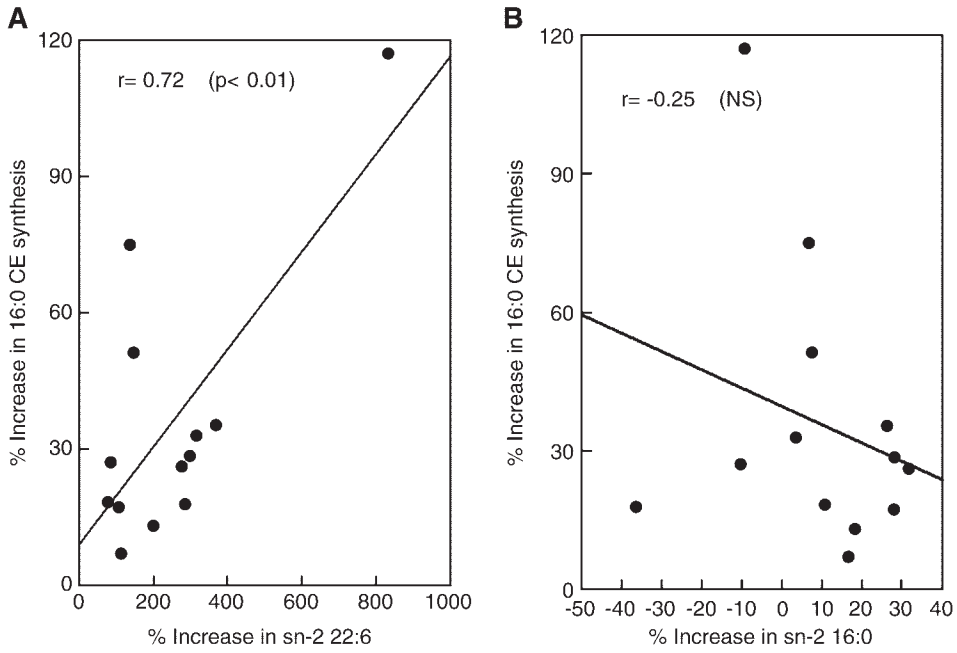

Fig. 6. Correlation of increase in the in vitro synthesis of 16:0 CE with the changes in *sn*-2 acyl composition. The percentage increase in labeled 16:0 CE synthesis after DHA supplementation was plotted against the increase in *sn*-2 22:6 (A) or *sn*-2 16:0 (B) of plasma PC. Correlation coefficients (Pearson) and significance were calculated by the SPSS statistics program (n = 13).

this study is that when the *sn*-2 position of PC is enriched with 22:6 through dietary supplementation, it will increase the synthesis of 16:0 CE as a result of the utilization of 16:0 from the increased levels of 16:0-22:6 PC. The results presented here show that after dietary supplementation with DHA-rich oil, the *sn*-2-22:6 PC increased by 3.5-fold. The 16:0 CE content was also increased significantly (+12%; $P < 0.005$), although there was no significant change in *sn*-2-16:0 PC. Furthermore, the in vitro synthesis of CE from labeled free cholesterol showed an increased formation of labeled 16:0 CE in the DHA-enriched plasma (+24%) compared with baseline levels. The increase in 16:0 CE synthesis (from baseline) correlated positively with the increase in 22:6 (but not 16:0) at the *sn*-2 position. It should be pointed out that, in addition to 16:0-22:6 PC, the presence of 16:0-20:4 PC also results in altered positional specificity of human LCAT (7, 8). The concentration of *sn*-2-20:4 PC actually decreased (−14%) after DHA supplementation (Fig. 3). Therefore, the increase in 16:0 CE would have been even higher if there were no decrease in *sn*-2-20:4 PC.

The possibility that the increase of 16:0 CE in lipoproteins is caused by its increased secretion from liver or intestine should also be considered. Although we cannot exclude this possibility completely, we believe that most of the increase in 16:0 CE is from the LCAT reaction, and not from the ACAT-2 reaction in intestine or liver, for the following reasons: *a*) quantitatively, ACAT-2 contributes only a minor percentage of the total CE present in circulating lipoproteins; *b*) the composition of CE species synthesized ex vivo (exclusively by LCAT) (Fig. 5) closely resembles that of plasma lipoproteins (Fig. 2); *c*) recent in vitro studies show that ACAT-2 is relatively nonspecific for various fatty acids (13, 14), and it is therefore unlikely that the specific increase in 16:0 CE observed after DHA feeding can be explained by an increased contribution of this enzyme; *d*) our previous in vitro studies showed that human LCAT synthesizes predominantly 16:0 CE when 16:0-22:6 PC is used as the substrate (7, 8); and *e*) there is a specific enrichment of plasma with 16:0-22:6 PC after DHA feeding, and this increase correlates positively with the increase in 16:0 CE synthesis both in vivo and in vitro.

Previous studies on the effect of feeding fish oil to humans and nonhuman primates also provide indirect evidence for the altered positional specificity of LCAT in vivo, although these studies were not aimed at addressing this issue. Thus, Holub, Bakker, and Skeaff (10) and Rapp et al. (25) reported a significant increase of saturated CE in plasma of human subjects after feeding fish oil, without any change in the 16:0 of PC. Similarly, in nonhuman primates, a significant increase in 16:0 CE was reported without a change in the 16:0 content of PC or TG (12, 22, 26). We postulate that in all of these studies, the specific increase in 16:0 CE was the result of an increase in 16:0-22:6 PC, which in turn resulted in an increase in the utilization of 16:0 from the *sn*-1 position for CE synthesis. Our earlier studies indeed showed an increase in 16:0-22:6 PC species after fish oil feeding in humans (20). Although ω-3 fatty acid feeding is known to affect the particle size distribution of HDL (27), it is unlikely that this causes increased synthesis of 16:0 CE by LCAT, because changes in the physical properties of the substrate particle do not alter the composition of CE species synthesized (7).

The physiological effects of the altered positional specificity of LCAT are not clear. One consequence of the alteration in positional specificity is the formation of 20:4 lyso-PC or 22:6 lyso-PC in the lipoproteins. The studies of Thies et al. (28, 29) showed that 22:6 or 20:4 is taken up by the brain much more efficiently if present in lyso-PC form rather than in free fatty acid form. Thus, the formation of *sn*-2-22:6 lyso-PC by the LCAT reaction may be an effective form of delivery of this essential fatty acid to the brain, where it is known to play a critical role (30). Another consequence of the altered positional specificity of LCAT is the formation of saturated CE. Previous studies reported that the saturated CEs are more atherogenic than unsaturated CEs (31). Our earlier studies showed that the alteration in positional specificity of LCAT is seen mainly in animal species known to be susceptible to atherosclerosis (e.g., rabbit, human, pig, guinea-pig), whereas LCATs from the resistant species such as rat and mouse do not show this alteration (3, 4). It remains to be seen whether the paradoxical increase in saturated CE after feeding of polyunsaturated DHA has any impact on the well-accepted beneficial effects of this n-3 fatty acid.

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