Long-chain conversion of [^{13}C]linoleic acid and α-linolenic acid in response to marked changes in their dietary intake in men

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Abstract **We studied the long-chain conversion of [U-^{13}C]α-linolenic acid (ALA) and linoleic acid (LA) and responses of erythrocyte phospholipid composition to variation in the dietary ratios of 18:3n-3 (ALA) and 18:2n-6 (LA) for 12 weeks in 38 moderately hyperlipidemic men. Diets were enriched with either flaxseed oil (FXO; 17 g/day ALA, n = 21) or sunflower oil (SO; 17 g/day LA, n = 17). The FXO diet induced increases in phospholipid ALA (>3-fold), 20:5n-3 [eicosapentaenoic acid (EPA), >2-fold], and 22:5n-3 [docosapentaenoic acid (DPA), 50%] but no change in 22:6n-3 [docosahexanoic acid (DHA)], LA, or 20:4n-6 [arachidonic acid (AA)]. The increases in EPA and DPA but not DHA were similar to those in subjects given the SO diet enriched with 3 g of EPA plus DHA from fish oil (n = 19). The SO diet induced a small increase in LA but no change in AA. Long-chain conversion of [U-^{13}C]ALA and [U-^{13}C]LA, calculated from peak plasma ^{13}C concentrations after simple modeling for tracer dilution in subsets from the FXO (n = 6) and SO (n = 5) diets, was similar but low for the two tracers (i.e., AA, 0.2%; EPA, 0.3%; and DPA, 0.02%) and varied directly with precursor concentrations and inversely with concentrations of fatty acids of the alternative series. [^{13}C]DHA formation was very low (<0.01%) with no dietary influences.**—Hussein, N., E. Ah-Sing, P. Wilkinson, C. Leach, B. A. Griffin, and D. J. Millward. **Long-chain conversion of [^{13}C]linoleic acid and α-linolenic acid in response to marked changes in their dietary intake in men.** ***J. Lipid Res.*** **2005.** 46: **269–280.**

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The potential of dietary α-linolenic acid (ALA) to exert the favorable effects on cardiovascular disease associated with its longer chain relatives in fish oil (1, 2) will depend to a large extent on the rate and efficiency of its long-chain conversion. An improved understanding of how diet can regulate this conversion may help to explain why there is currently a lack of evidence to support a cardioprotective role for dietary ALA (3–9).

Experimental studies on the characteristics of the polyunsaturated fatty acid desaturase enzymes suggest that long-chain conversion of PUFA precursors is probably rate limited by the Δ6-desaturase (10–14), with competition between the n-3, n-6, and n-9 substrates for the enzyme and relative affinities varying as n-3 > n-6 > n-9. This suggests that in practice, the main influences on the elongation of ALA come from competition from linoleic acid (LA) and possibly *trans* fatty acids. Given that levels of the latter in human diets are decreasing markedly with the newer *trans*-free spreads and that LA intake continues to increase, the current issue of most practical importance is likely to be the dietary ratio of LA to ALA.

Supplementation studies have generally indicated ALA conversion to eicosapentaenoic acid (EPA), as indicated by increased phospholipid EPA levels, with most (but not all) studies showing no influence on phospholipid docosahexanoic acid (DHA) levels. However, stable isotope studies of ALA long-chain conversion using either deuterium-labeled ALA in infants (15–17) or adults (18–23) or [^{13}C]ALA in infants (24) or adults (25–29) have all reported conversion to both EPA and DHA, although DHA synthesis is generally low. Although an increased intake of dietary ALA might be expected to upregulate ALA conversion, this has either not been found (27) or ALA conversion has been downregulated (29), a finding also observed in response to increased intake of DHA in some (20) but not all studies (23). Taken together, these findings suggest that ALA can be converted to EPA and therefore can increase phospholipid EPA levels, but the main

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; APE, atom percent excess; AUC, area under the curve; BMI, body mass index; DGLA, dihomo-χ-linolenate; DHA, docosahexanoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester; FXO, flaxseed oil; LA, linoleic acid; LCP, very long-chain polyunsaturated fatty acid; SO, sunflower oil; SOF, fish oil supplementation.

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dietary regulation of ALA conversion to EPA and especially DHA is by no means clear, possibly because the tracer kinetics of fatty acid long-chain formation in vivo represents a complex problem with variable approaches to the interpretations of the studies reported to date.

The present study was designed to examine the long-chain conversion of both ALA and LA in subjects for whom long-chain conversion of ALA may be of particular importance: middle-aged men with an atherogenic lipoprotein phenotype (30). We sought to evaluate the influence of a marked variation in the dietary ALA/LA ratios on their long-chain conversion in terms of *a*) their accumulation in phospholipids, with a comparison with preformed EPA and DHA from dietary fish oils, and *b*) the relative conversion rates of [U-^{13}C]ALA and [U-^{13}C]LA to their respective long-chain products. In the latter studies, we also took into account differential isotope dilution attributable to between-diet and between-series differences in ALA and LA pool sizes. The influence of these dietary changes on cardiovascular risk factors known to be responsive to fish oil supplements has been described elsewhere (31).

MATERIALS AND METHODS

Subjects

Normal, healthy, free-living male volunteers aged between 35 and 60 years were recruited for the study, which was approved by the University of Surrey Ethics Committee. The main selection criterion was an atherogenic lipoprotein phenotype [i.e., a moderately increased plasma triacylglycerol (>1.5 mmol/l), low HDL cholesterol (<1.1 mmol/l), and the predominance of small, dense LDLs (>40%)] (30). Subjects expressing an atherogenic lipoprotein phenotype were invited by letter to take part in the dietary intervention. Exclusion criteria were hypertension, smoking, excessive alcohol consumption (>30 units/week), therapy known to affect lipid metabolism, and any biochemical evidence of abnormal liver, kidney, or endocrine function or abnormal hematology. In total, 57 subjects completed the study, with a mean baseline body mass index (BMI) of 28.5 kg/m^2. The 12 subjects randomly selected for the isotope substudy at the end of the intervention had a similar mean BMI of 29.4 kg/m^2 and did not differ from the main group in terms of the main selection criteria (i.e., triacylglycerol mean value of 1.7 mmol/l, HDL cholesterol mean value of 1.0 mmol/l, and small, dense LDL mean value of 45%).

Diets and study design

The study was of a randomized, single-blind design that aimed to compare single-step diets, fed over 12 weeks in parallel, that differed markedly in their ALA and LA contents. The main study compared a high-ALA/low-LA flaxseed oil (FXO) diet with a low-ALA/high-LA sunflower oil (SO) diet. A third diet was included, low-ALA/high-LA enriched with fish oil (SOF), to serve as a positive control for the enrichment of phospholipids with EPA and DHA and for influences on cardiovascular risk factors (not discussed here; see ref. 31) with which the FXO diet could be compared. The stable isotope study was conducted on a subset of subjects on the FXO and SO diets during the last 2 weeks of the intervention to address the specific question of the impact of the dietary LA/ALA ratio on the long-chain conversion of ALA and LA. The diets involved substituting 45 g of the usual fat intake with either FXO (56% ALA) and other high-ALA, low-LA oils and spreads (FXO diet) or SO (69% LA) and other high-LA, low-ALA spreads (SO diet). The FXO and SO were supplied to subjects in laminated foil sachets (17 g of oil per sachet), the oils having been packaged under nitrogen and stored at −40°C before use. Further enrichments were achieved through the provision of cooking oils (rapeseed oil for the FXO diet, SO for the SO and SOF diets) and specially formulated spreads containing either high (FXO diet) or low (SO diet) amounts of rapeseed oil. The SOF diet group received six 1 g fish oil capsules (Pikasol; Pronova Biocare), of which 50% was EPA and DHA (i.e., 370 mg of EPA and 230 mg of DHA per capsule, 3 g of EPA+DHA per day). The target n-6:n-3 dietary ratios of <1 (FXO diet) or >25 (SO diet) were calculated to require an increased intake of ALA or LA of 18 g above background intake. The feasibility of achieving this level of intake had been previously established in a pilot study in normal volunteers using FXO as the principal dietary source of ALA (32). Periodic measurement of lipid peroxide levels showed the stored oils to be stable over the experimental period. To avoid the potentially adverse effects of direct heating, the oils were introduced into the diet by incorporation into cooked foods, including pasta sauces, salad dressings, and milkshakes. Subjects were also provided with cooking oils [i.e., rapeseed oil (8% ALA, low n-6:n-3 ratio, FXO diet); SO (SO and SOF diets)] and spreads, either rapeseed enriched for the FXO diet or a standard formulation (Mono; St. Ivel) for the SO and SOF diets. The foil sachets, cooking oils, and spreads were identified by the letters X, Y, and Z (FXO, SO, and SOF, respectively). The habitual diet of the subjects was assessed by 7 day food diaries (33). Before each dietary intervention, subjects received dietetic counseling on an individual basis. This consisted of giving advice to subjects in the FXO group to avoid foods containing high levels of n-6 PUFAs and for subjects in both the FXO and SO groups to abstain from consuming any source of long-chain n-3 PUFAs, chiefly oily fish. Subjects attended the Human Investigation Unit at the University of Surrey on three occasions, always after an overnight fast (12–14 h): at baseline (pre-diet) and after 6 and 12 weeks (post-diet). Height, total body weight, an estimate of percentage body fat, and blood pressure were measured at each visit. Blood samples were taken by venipuncture for the analysis of plasma lipids, lipoproteins, and hemostatic variables. Dietary compliance was assessed midway through the intervention (6 weeks) by 3 day food diaries and at the end of the intervention (12 weeks) by a 7 day food diary.

In view of the relatively large dietary load of PUFAs, vitamin E intake and status were monitored in terms of both α- and χ-tocopherol levels in the oils and plasma by liquid chromatography time-of-flight mass spectrometry (34).

Analyses

Erythrocyte membrane phospholipid fatty acid profiles were measured at baseline and at 6 and 12 weeks. Blood samples were collected into containers with EDTA and sodium citrate. Blood cells were separated by low-speed centrifugation at 1,100 *g* for 15 min at 2°C and washed three times with sodium chloride (0.9% NaCl), followed by centrifugation at 3,100 *g* for 15 min at 2°C. The washed cells were transferred to a glass tube with 250 μl of butylated hydroxytoluene in propan-2-ol (0.1%) added as an antioxidant, mixed, and stored at −80°C until analyzed.

Lipids were extracted from the erythrocyte membranes with a mixture of 6 ml of chloroform-methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene as an antioxidant (35). Fatty acid methyl esters (FAMEs) were prepared with 1 ml of sodium methoxide (0.5 M solution in methanol), incubated at 60°C for 15 min, acidified with 50 μl of glacial acetic acid, and finally extracted twice with 3 ml of hexane (36).

The resulting FAMEs were analyzed by gas chromatography using a Varian 3400 gas chromatograph with a PAG capillary column (30 m × 0.25 mm × 0.25 μm film thickness; Supelco, Inc.) and a flame ionization detector (250°C), with helium as the carrier gas. Temperature was programmed at 4°C/min from 150°C to 220°C. The fatty acid peaks were identified against standard fatty acid mixtures of known composition run on the same column under identical conditions (Supelco 37 Component FAME Mix; Sigma Chemical Co.). Individual standards [docosapentaenoic acid (DPA, n-3), docosatetraenoic acid (DTA)] were also used because these were not included in the standard mixture. The injection volume was 2 μl and the run time was 45 min. Results are expressed as percentages of the sum of all identified peaks.

Stable isotope studies

[U-^{13}C]ALA and [U-^{13}C]LA (free fatty acid, >98 atom%) were purchased from Martek Biosciences Corp. (Columbia, MD). This preparation contained 97.1% ALA and 98% LA, the remainder being short- and medium-chain fatty acids as indicated by GC analysis. Solvents were from Fisher Chemicals Ltd. (Loughborough, Leicestershire, UK). Fatty acid standards and all other reagents were obtained from Sigma (Poole, Dorset, UK). The high cost of the stable isotope limited the studies to a subset of subjects. Because the objective was to examine any marked difference in the dietary ratio of LA and ALA on very long-chain polyunsaturated fatty acid (LCP) formation rates of the n-6 and n-3 precursors (LA and ALA), a subset of 12 subjects were randomly selected from the FXO diet (n = 6) and SO diet (n = 6) and studied during week 12 of the interventions; however, samples from 1 SO subject were lost, so the data presented here for the SO group is n = 5.

The subjects of the substudy were given a single oral dose of 400 mg of [U-^{13}C]ALA and [U-^{13}C]LA in a milkshake at breakfast after an overnight fast. The milkshake was similar to that described by Burdge, Jones, and Wootton (25), an emulsion made from double cream (22 g), casein (12 g), beet sugar (4.5 g), glucose (9 g), chocolate Nesquik (10 g), and the ^{13}C tracers (0.8 g) in water (160 g). This was followed by a standard test breakfast comprising whole milk (150 ml), orange juice (200 ml), white bread toast (72 g), margarine (14 g), marmalade (20 g), and cereal (50 g).

Blood samples (10 ml) were collected from a forearm vein by venipuncture at 1, 2, 3, 7, 10, and 14 days. Total lipids were prepared from plasma (1 ml) by extraction with 6 ml of chloroform-methanol (2:1, v/v) (34) containing butylated hydroxytoluene (50 mg/ml). Heptadecanoic acid (C17:0) was added as an internal recovery standard. Purified lipids were dissolved in toluene and converted to their corresponding FAMEs by the addition of 2 ml of methanol containing 2% (v/v) H_2SO_4 and incubation at 50°C for 18 h. The reaction mixture was neutralized with 2 ml of 0.25 M potassium bicarbonate ($KHCO_3$) and 0.5 M potassium carbonate (K_2CO_3), and the FAMEs were extracted with 2 ml of hexane (37). Samples were dried under nitrogen and redissolved in 200 μl of hexane; 2 μl aliquots of this solution were used for analysis of isotopic enrichment by gas chromatography-combustion-isotope ratio mass spectrometry via an HP6890 gas chromatograph (Hewlett-Packard, Wokingham, Berkshire, UK), with FAMEs converted to CO_2 using a Thermofinnegan gas chromatograph combustion III isotope ratio mass spectrometer interface and with $^{13}CO_2/^{12}CO_2$ ratio determined by a Thermofinnegan Delta Plus X stable isotope analyzer. At suitable time points, pulses of CO_2 with known isotopic ratios [calibrated against the international standard Pee Dee Belemate limestone (38)] were introduced as reference standards (δ −32.84‰).

All samples from an individual were analyzed in one series to eliminate interassay variation in the results, with each sample run in duplicate. Concentrations of individual fatty acids were calculated from peak area compared with the internal standard.

Quantification of ^{13}C-labeled fatty acids involved comparing ^{13}C enrichment indicated by the 45:44 CO_2 signal isotope ratios with the fatty acid concentration assessed from integrating the signal of mass 44 CO_2 produced from the individual fatty acids and relating it to the signal resulting from the C-17:0 heptadecanoic acid methyl ester standard. This enables ^{13}C tracer concentration in each fatty acid to be expressed as micrograms of ^{13}C per milliliter of plasma, calculated by multiplying the concentration (micrograms) of the fatty acid by the ^{13}C increase over basal ^{13}C (at percent excess).

Stable isotope calculations

When ^{13}C incorporation into conversion products was very low, a limit of detectability for the enrichment for each fatty acid was calculated as 2 SD above the mean baseline value (all subjects) for each fatty acid. Values greater than this were considered to be enriched. The experimental design focused on the extent of tracer incorporation into LCPs, especially DHA, rather than the early changes in ALA or LA, using approximate measures of ALA or LA conversion. This is justified given the lack of access to actual tissue metabolite pools, which is required for multicompartmental modeling. We calculated two measures of the relative conversion rates on the two diets. The first was the absolute conversion as the fraction of the dose appearing in plasma rather than being oxidized or deposited in intracellular pools, as indicated by the maximum plasma ^{13}C concentration of each LCP. This is expressed as the percentage of the dose appearing in LCP in the plasma pool, assuming that plasma represents 4.5% of body weight. Second, we compared area under the curve (AUC) for the 14 day time course (AUC_{14d}) of ^{13}C tracer in each LCP (micrograms of ^{13}C fatty acid per milliliter of plasma), as reported by others (18–20, 39).

Modeling considerations

Because the transfer of isotope from LA and ALA into LCPs reflects both the rate of conversion and enrichment [atom percent excess (APE)] of precursor, comparisons of LCP AUC values between the two diets and the two tracer series (n-3 and n-6) had to take into account differences in the relative tracee enrichments (APE) attributable to variations in tracee pool sizes. The problem is shown schematically in **Fig. 1**. Variation in ALA and LA pool size means that the observed relative rates of ^{13}C-labeled LCP formation between diets and between series will not reflect the true relative conversion rates unless both tracer doses and tracee pool sizes are the same. This effect will be most apparent for n-3 and n-6 comparisons, as shown in Fig. 1, but will also be a problem for within-series, between-diet comparisons when there are dietary effects on pool sizes, as in the present study. Furthermore, these considerations apply to each step in LCP formation. With adequate data, compartmental analysis can take into account both pool sizes and precursor-product tracer/tracee ratios. However, with the present limited data set, a simple algorithm was devised to adjust AUC values to take account of between-diet and between-series variations in tracee pool size and consequent variations in tracer/tracee ratios.

First, variation in the initial enrichments between diets and subsequent precursor-product variation was accounted for by normalizing each ^{13}C isotope concentration throughout the time course. This was done by dividing the value by the ratio of the AUC ^{13}C APE of the presumed precursor up to that time point measured in the subject by the mean value of the same measure obtained for all subjects on both diets. This reduced ^{13}C concen-

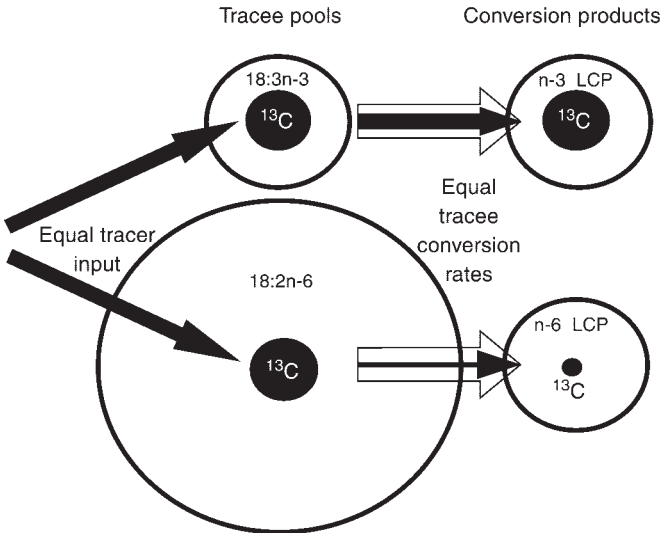

Fig. 1. Scheme showing the effect of tracee pool size on relative rates of tracer appearance in long-chain conversion products. With a 7-fold greater pool size of linoleic acid (LA) compared with α-linolenic acid (ALA), the same tracer doses of [^{13}C]LA and [^{13}C]ALA result in a 7-fold difference in relative enrichment (atom percent excess) of LA and ALA pools. This means that with similar absolute very long-chain polyunsaturated fatty acid (LCP) conversion rates, the n-3 LCPs will contain 7-fold more total ^{13}C than the n-6 LCPs. Without taking this into account, LCP formation from ALA will appear much greater than that from LA. The algorithm described in the text adjusted the ^{13}C content of each LCP to what would have been observed if the tracer/tracee ratio of the precursor had been the mean value of all subjects (n = 11).

trations when the presumed precursor APE was higher than average and vice versa and resulted in more accurate relative, between-diet rates of LCP formation within each series. Second, the diet-adjusted n-6 series LCP conversion to DGLAand arachidonic acid (AA) was then normalized to comparable values observed for the n-3 series by increasing the AUC isotope concentration values according to the ratio of ALA-to-LA enrichments (^{13}C APE AUC_{14d}), mean value, both diets. The result of these adjustments is that both between-diet and between-series variation in n-6 and n-3 LCP conversion will better reflect actual differences that would have been observed if the series precursor ^{13}C APE had been the same for LA and ALA on each diet. The detailed calculations were as follows.

a) Between-diet comparisons. For LCP conversion in the series $FA_1 \rightarrow FA_2 \rightarrow FA_3 \rightarrow FA_n$, values for ^{13}C concentration at time points t_0–t_{14} were adjusted according to the following expressions:

For FA_2 to FA_n at each time =

(Eq. 1)

$$\text{FA (diet adjusted)}^{13}\text{C}((\mu\text{g})/\text{ml}_{t=t}) =$$

$$\text{FA}_n(\text{mg}/\text{ml})_{t=t} \times \text{FA}_n{}^{13}\text{C APE}_{t=t} \times \frac{\text{mean}(n=11)\text{FA}_{n-1}\text{AUC}(t_0-t_t)^{13}\text{C APE}}{\text{FA}_{n-1}\text{AUC}(t_0-t_t)^{13}\text{C APE}} \times 10$$

These diet-adjusted values were than used to calculate the AUC_{14d} (diet-adjusted) ^{13}C values for FA_2 to FA_n

b) Between-series comparisons.

For dihomo-χ-linolenate (DGLA) and AA at each time = t

(Eq. 2)

$$\text{FA (diet and series-adjusted)}^{13}\text{C}(\mu\text{g}/\text{ml}_{t=t}) =$$

$$\text{FA (diet-adjusted)}^{13}\text{C}(\mu\text{g}/\text{ml}_{t=t}) \times \frac{\text{mean (n = 11) ALA AUC}_{14d}{}^{13}\text{C APE}}{\text{mean (n = 11) LA AUC}_{14d}{}^{13}\text{C APE}}$$

These diet- and series-adjusted values were than used to calculate the AUC_{14d} (diet-adjusted) ^{13}C values for DGLA and AA.

Adjustment 1 resulted in values for ^{13}C and consequent AUC values that would have occurred if the precursor enrichment up to that time point had been the same as the average value for that series. The factor 10 ensures values are as micrograms per milliliter.

Adjustment 2 resulted in AUC values for dihomo-χ-linolenate (DGLA) and AA that would have been observed if the LA APE had been the same as that for ALA, enabling more accurate n-6 and n-3 comparisons. The AUC_{14d} APE value was used rather than the 24 h enrichment because this takes into account both the initial enrichment and the persistence of enrichment with time, which differed between LA and ALA because of the different pool sizes and relative turnover rates. The value for the ratio of AUC_{14d} ^{13}C APE for ALA to LA used in equation 2 was 4.98, whereas the ratio of the ^{13}C APE_{24h} values was 9.7.

Statistical methods and calculations

For the red cell fatty acid profiles, the statistical model was an analysis of the dietary influences on the changes from baseline at 6 and 12 weeks. The significance of any change from baseline was examined by paired *t*-tests. Where significant changes occurred, any dietary influences on the extent of the changes were examined by ANOVA with post hoc comparisons by the Tukey HSD test. Homogeneity of variances was tested by the Brown and Forsythe modification of Levene's test, and normality was examined by the Shapiro-Wilk test on the original or log-transformed data after trimming for outliers (>±3 SD). Where the ANOVA assumptions were violated, nonparametric tests were used (i.e., Kruskal-Wallis ANOVA by ranks with post hoc testing by Mann-Whitney *U* test).

For the ^{13}C measurements, a two-tailed, unpaired *t*-test was used to test for significant differences between AUC values from subjects fed the SO and FXO diets. Overall conversion of tracer to AA, EPA, DPA, and DHA in the plasma pool was calculated from the maximum observed plasma ^{13}C concentration (micrograms per milliliter) as a proportion of the dose, assuming that the plasma volume was 4.5% of body weight. This will be less than overall whole body tracer to the extent that ^{13}C-labeled LCPs are incorporated into tissue lipid pools. Fractional conversion rates were calculated for DGLA→AA, EPA→DPA, and DPA→DHA from ratios of AUC values. Values for LA→DGLA and ALA→EPA were not calculated since the AUC values for ALA and LA were underestimates of the actual values because peak enrichment was likely to have occurred before the first sampling time at 24 h.

RESULTS

Dietary compliance and intakes

The dietary oils were well tolerated, and the FXO and SO were successfully incorporated into foods, as we had shown to be feasible in a preliminary pilot study of the incorporation of FXO into various foods and meals (32). Dietary

compliance was excellent (**Table 1**), meeting the planned intakes of ALA (18.6 g/day in the FXO diet, 0.9 g/day in the SO and SOF diets compared with 0.8 g/day at baseline), LA (24.8 g/day in the SO diet, 8.4 g/day in the FXO and SOF diets compared with 5.8 g/day at baseline), and longer chain n-3 (2.2 g/day EPA and 1.4 g/day DHA in the SOF diet, 0.8 g/day EPA+DHA in the FA and SO diets compared with 0.7 g/day EPA+DHA at baseline). Thus, the three diets were accompanied by significant changes in the ratio of dietary n-6 to n-3 PUFAs, from 8.4 to 0.5 (FXO; $P < 0.001$), 9.5 to 27.9 (SO; $P < 0.05$), and 9.2 to 5.2 (SOF; $P < 0.05$). Subjects recorded when they incorporated the sachets of additional oils (FXO, SO, and SOF) into foods and meals, and from this information compliance with the diets seemed satisfactory, indicating the anticipated increased dietary intake of dietary ALA. All three diets were associated with a small increase in energy derived from dietary fat at the expense of dietary carbohydrate, which was significant for the SO group (+14%; $P < 0.01$). Nevertheless, there were no increases in mean body weight, percentage body fat, or BMI over the intervention period.

The dietary oils provided vitamin E mainly as χ-tocopherol in the FXO diet and α-tocopherol in the SO diet, so that plasma levels of χ-tocopherols tended to be higher in the FXO subjects at 6 and 12 weeks. However, for the major vitamin E species, α-tocopherol, there were no differences between subjects on the three diets at 6 or 12 weeks in concentration or in α-tocopherol/cholesterol ratios (data not shown).

Fatty acid composition of erythrocytes

There were no significant differences in any fatty acid between the three diet groups at baseline. The ratios of n-6:n-3 and AA/EPA were 4.2 ± 2.5 and 12.0 ± 8.6, respectively. Any influence of either ALA or LA levels in the background diets on very long-chain n-3 PUFAs was examined by correlation analysis. ALA concentrations were unrelated to those of its long-chain conversion products (EPA, DPA, or DHA), and LA was unrelated to AA or C22n-6, although it was positively correlated with GLA and DGLA. In fact, the main relationship was an inverse correlation between PUFAs (whether n-3 or n-6) and palmitic acid (PA), stearic acid (SA), and oleic acid (OA).

With few exceptions, the responses to the dietary interventions, shown in **Fig. 2A**, **B** as changes from baseline at 6 and 12 weeks, were effectively complete by 6 weeks. All three diets induced an exchange of palmitic acid and oleic acid for PUFAs, with no change in stearic acid, increasing the polyunsaturated/saturated (P/S) ratio on average from 1.17 to 1.42, with no differences between the diets.

With the SO diet, LA increased from 14.1 ± 3.1% to 16.0 ± 2.3% ($P < 0.05$) at 12 weeks, with no changes in the n-6:n-3 or AA/EPA ratio.

With the SOF diet, all n-3 LCPs increased significantly, mainly in exchange for palmitic and oleic acids. Thus, EPA doubled and DPA and DHA increased by 50%, so that the n-6:n-3 ratio decreased from 3.6 to 2.2 ($P < 0.02$) and the AA/EPA ratio declined from 8.4 to 3.2 ($P < 0.01$).

With the FXO diet, ALA increased >3-fold (from 0.36 ± 0.3% to 1.33 ± 0.8%; $P < 0.001$), EPA increased >2-fold (from 1.47% to 3.85%; $P < 0.001$), and DPA increased by 50% (from 2.72% to 3.44%; $P = 0.02$), with no change in DHA. Because there were only small changes in LA (declining from 13.7 to 12.5; $P = 0.1$) and no change in AA concentrations, the decrease in AA/EPA and n6:n3 ratios, from 13.9 to 5.5 and 4.3 to 3.0, respectively ($P < 0.001$ in each case), reflected only the increase in the n-3 LCPs. Thus, EPA and DPA formation from ALA meant that the increases in these two fatty acids did not differ from those in the SOF group (Mann-Whitney *U* tests: $P = 0.24$ and $P = 0.16$, respectively) (Fig. 2B). The similar changes in EPA concentrations with the FXO and SOF diets after intakes of 18 g/day ALA and 3 g/day preformed EPA+DHA containing 1.7 g of EPA indicates a dietary equivalence of a maximum of 10:1.

ALA long-chain conversion is thought to reflect competition between n-6 and n-3 substrates, and although the relationship between the LA/ALA ratio and EPA concentration was weak within the FXO group ($r^2 = 15\%$, $P = 0.08$ for EPA at 12 weeks) and not evident in the SO group, the changes in membrane EPA from baseline did reflect ALA levels. Increases in EPA from baseline in the FXO group correlated with increases in ALA at 6 weeks ($r^2 = 18\%$) and at 12 weeks ($r^2 = 19\%$).

Stable isotope analysis

The fatty acid profile of erythrocytes (**Fig. 3**) of the subgroups was similar to that of the main cohort (i.e., lower

TABLE 1. Dietary composition: pre- and postintervention dietary intakes

Diet	Total Energy	Fat	Carbohydrate	Protein	n-3 PUFA	n-6 PUFA	n-6:n-3
	kcal/day		*% energy*		*g/day (% energy)*		*ratio*
FXO (n = 21)							
Pre-diet	2,413 ± 443	37 ± 7	42 ± 7	17 ± 3	1.2 ± 2.2[a] (0.6)	5.8 ± 5.4[a] (2.4)	8.4 ± 5.5[a]
Post-diet	2,603 ± 636	41 ± 6	39 ± 7	15 ± 3	19 ± 1.3 (6.9)	8.7 ± 3.1 (3.1)	0.5 ± 0.16
SO (n = 17)							
Pre-diet	2,391 ± 274	36 ± 6	43 ± 7	15 ± 2	0.8 ± 0.4[a] (0.3)	7.6 ± 6.3[a] (3.0)	9.5 ± 4.2[a]
Post-diet	2,426 ± 494	41 ± 6[a]	41 ± 7	15 ± 3	1.5 ± 0.8 (0.5)	25.2 ± 5.8 (9.3)	27.9 ± 29
SO + SOF (n = 19)							
Pre-diet	2,655 ± 430	37 ± 6	40 ± 6	16 ± 2	0.6 ± 0.4[a] (0.2)	5.7 ± 3.9[a] (1.9)	9.2 ± 4.9[a]
Post-diet	2,570 ± 374	41 ± 7.6	38 ± 7	14 ± 2	4.7 ± 0.9 (1.6)	24.0 ± 3.1 (8.4)	5.2 ± 0.81

FXO, flaxseed oil; SO, sunflower oil; SOF, fish oil supplementation. Values are means ± SD.
[a] $P < 0.05$ (pre-diet versus post-diet).

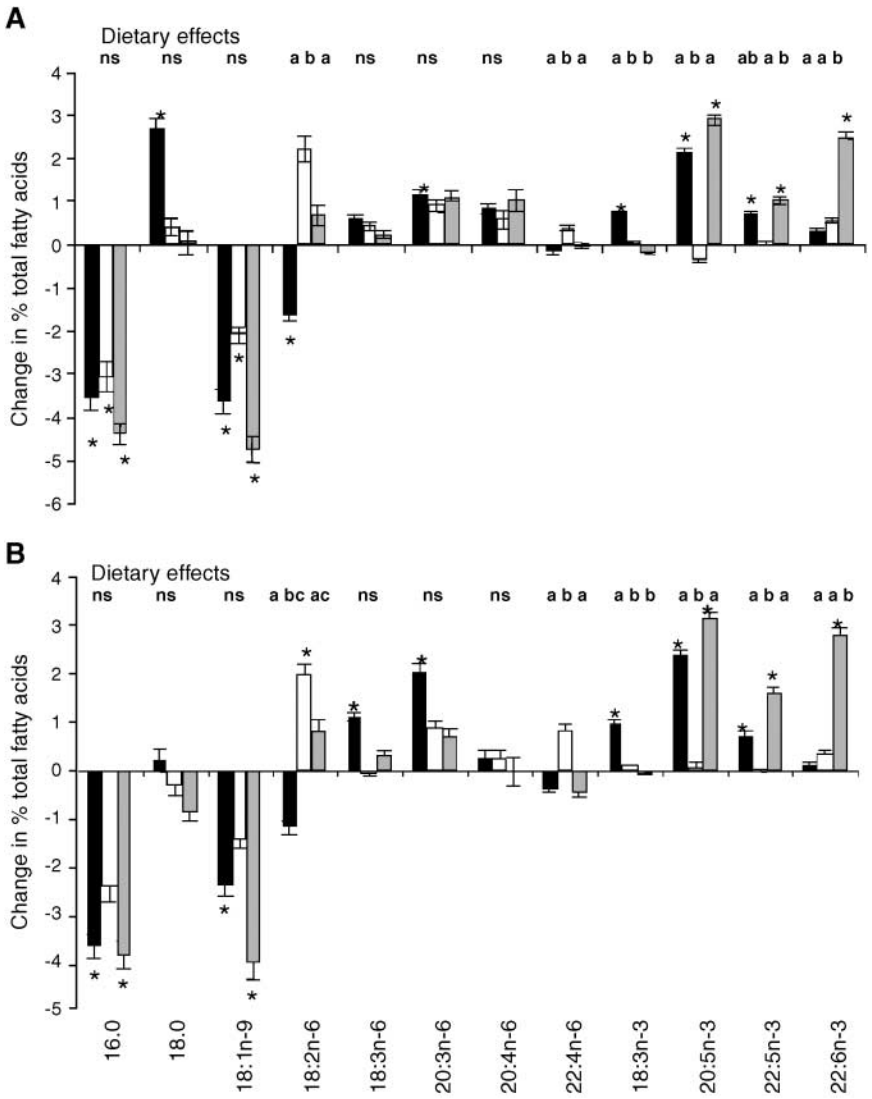

Fig. 2. Responses of erythrocyte phospholipid fatty acid profiles to interventions. A: Six weeks. B: Twelve weeks. Black bars, flaxseed oil (FXO) diet (n = 21); white bars, sunflower oil (SO) diet (n = 17); gray bars, fish oil supplementation (SOF) diet (n = 19). Values are means ± SD. Asterisks indicate significant differences from baseline ($P < 0.05$). Different letters indicate significant differences between diets ($P < 0.05$).

levels of LA and significantly higher values for the n-3 fatty acids apart from DHA in the FXO diet compared with the SO diet). For the sampled plasma lipid pool (Fig. 3), the dietary influences were broadly similar, with significantly higher values for all n-3 fatty acids apart from DHA in the FXO diet compared with the SO diet and with lower n-6 levels (but not significantly so) in the FXO diet compared with the SO diet.

^{13}C enrichment was observed in LCPs of both n-6 (DGLA and AA) and n-3 (EPA, DPA, and DHA) series. Differences in tracee pool sizes resulted in differential dilutions of tracer both between PUFA series and between diets within each series. Thus, there was a much lower initial ^{13}C enrichment (APE) of LA at 24 h after the dose compared with ALA (i.e., the ALA/LA APE_{24h} ratio was 9.7 ± 4.50 overall) and higher values in the n-6 (13.1 ± 4.8) compared with the FXO diet (7.4 ± 2.7; $P < 0.05$). However, the slower turnover of LA compared with ALA (half-life = 2.2 and 1.1 days for LA and ALA, respectively), no doubt reflecting the much larger LA pool, meant that the differences in LA and ALA enrichment over the 14 day study was not as marked as at 24 h (^{13}C APE AUC_{14d} ALA/LA ratio = 5.0 ± 3.1; n = 11). Fourteen day ALA/LA enrichment ratios were again higher in subjects fed the n-6 (8.2 ± 2.4) compared with the FXO diet (2.9 ± 0.9; $P < 0.05$).

^{13}C concentrations (micrograms per milliliter of plasma) at 24 h were similar for each tracer and each dietary group (ratio for both diets = 0.89 ± 0.31), suggesting similar initial tracer partition between oxidation, tissue storage, and entry into the sampled plasma pool. As with enrichment, the slower turnover of LA meant that the AUC_{14d} ^{13}C concentration was higher for LA than for ALA (ALA/LA ratio = 0.45; n = 11).

Overall conversion rates of LA and ALA, calculated from peak ^{13}C LCP concentrations adjusted for dietary in-

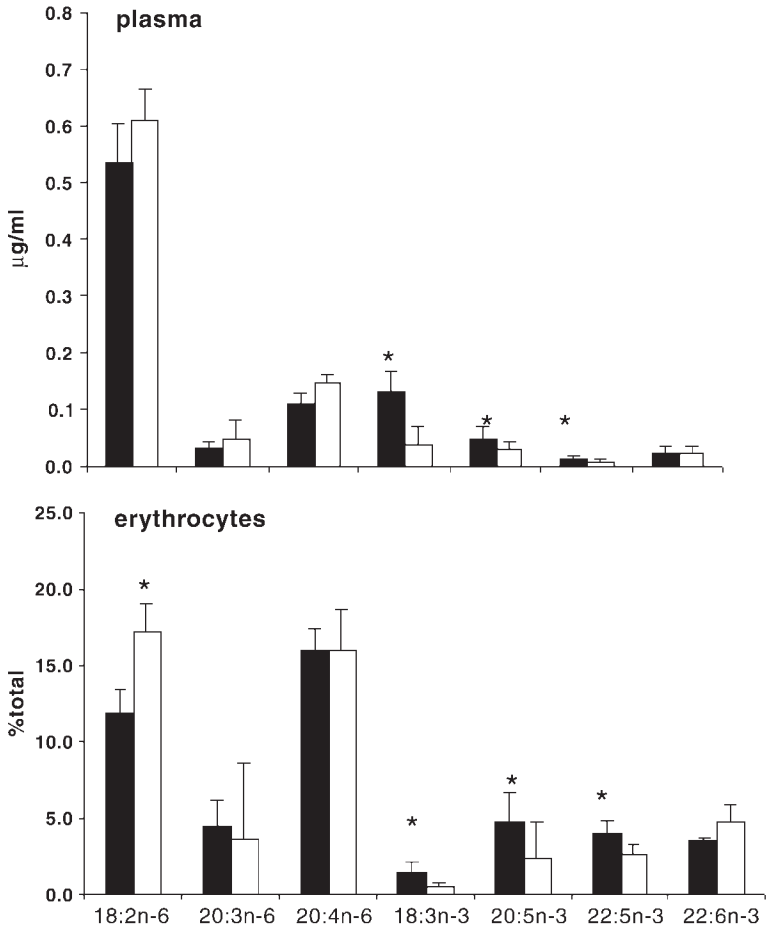

Fig. 3. Fatty acid composition of the sampled plasma pool and erythrocyte membrane during the ^{13}C tracer study. Black bars, FXO diet (n = 6); white bars, SO diet (n = 5). Values are means ± SD. * $P < 0.05$.

fluences on pool sizes of LA and ALA, were low and of similar magnitude overall for AA and EPA (0.18% and 0.26%; **Table 2**). LA→DGLA and AA formation was significantly lower on the FXO diet in each case, with ALA→EPA and DPA formation on average higher on the FXO diet, although the differences were not significant. Conversion of tracers to DHA was much less. Thus, whereas all six SO subjects demonstrated ^{13}C enrichment in DHA by day 7, only three of six FXO subjects were enriched by day 7, with one subject enriched at 1 and 3 days only and two subjects showing no enrichment in either of duplicate measurements at any time.

The time course of the ^{13}C concentration values in LA, ALA, and LCPs (micrograms per milliliter of plasma) is shown in **Fig. 4** as measured values for LA and ALA and the adjusted values for the LCPs. Labeling was declining for both LA and ALA after 24 h. However, tracer was still increasing in conversion products at 24 h, peaking at 48 h for DGLA, after this for AA, by 48 h for EPA, at 72 h for DPA, and some time after this for DHA; the level of labeling of DHA was too low to identify its time course with any certainty. As reported in all previous studies, there was considerable variability between subjects in the extent of ^{13}C labeling.

A comparison of the AUC values adjusted for dietary influences on pool sizes of LA and ALA (**Table 3**) showed that LCP formation from LA was reduced by the FXO diet, with lower DGLA ($P < 0.05$) and AA ^{13}C AUC values ($P < 0.01$), approximately half the values observed on the SO diet. As might be expected, because the tracer appearing in AA was similar to or slightly greater than that in DGLA, fractional conversion of DGLA→AA was close to

TABLE 2. Overall tracer conversion to LCPs

Diet	DGLA	AA	EPA	DPA	DHA
FXO (n = 6)	0.20 ± 0.06	0.12 ± 0.05	0.29 ± 0.19	0.05 ± 0.03	<0.01 ± 0.01
SO (n = 5)	0.29 ± 0.05	0.26 ± 0.07	0.19 ± 0.06	0.02 ± 0.01	<0.01 ± 0.00
Both diets (n = 11)	0.23 ± 0.07	0.18 ± 0.09	0.26 ± 0.15	0.04 ± 0.02	<0.01 ± 0.01
P	<0.05	<0.05	NS	NS	NS

AA, arachidonic acid; DGLA, dihomo-χ-linolenate; DHA, docosahexanoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; LCP, very long-chain polyunsaturated fatty acid. Values are means ± SD. Overall conversion is indicated as maximum plasma ^{13}C content as a percentage of dose of either [^{13}C]linoleic acid (LA) or [^{13}C]α-linolenic acid (ALA). Values are adjusted for variable precursor enrichments (see text).

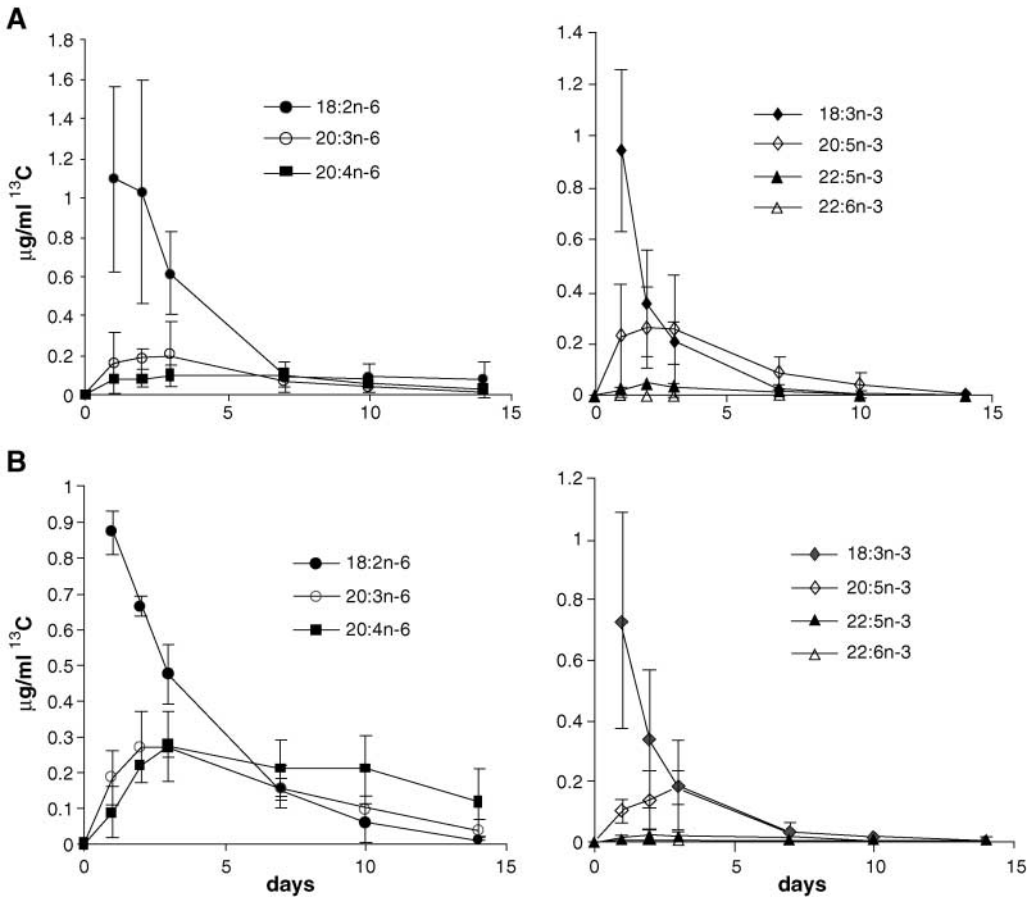

Fig. 4. Time course of ^{13}C labeling of n-3 and n-6 long-chain PUFAs in the plasma total lipid fraction. Values shown are ^{13}C concentrations as measured for ALA and LA and adjusted values for all LCPs, where the adjustment takes into account variation in precursor pool sizes and enrichments (see text). A: FXO diet (n = 6). B: SO diet (n = 5). Values are means ± SD.

unity on each diet (0.87 ± 0.30 and 1.31 ± 0.48 in the SO and FXO diets, respectively). EPA formation was on average increased with the FXO diet, although not significantly so, because of the wide variation between the FXO subjects (attributable to one subject on the FXO diet exhibiting much less ALA conversion than the other subjects).

Relative n-6:n-3 LCP formation, as judged by the ratio of AA/EPA formation, was only approximately one-third of the value on the FXO diet compared with the SO diet ($P < 0.05$). Post-EPA conversion was ~20% for EPA→DPA, 20–40% for DPA→DHA, and 7–11% for EPA→DHA, with no obvious dietary influences, although the level of enrichment in DHA was low.

Correlation analysis between tracer in LCPs and fatty acid concentrations in the sampled plasma pool (**Table 4**) showed that in general LCP formation of both n-6 and n-3 series reflected precursor concentrations with inverse correlations (in many cases) with fatty acids of the alternative series. There was little evidence of product inhibition. Thus, ALA concentrations correlated directly with [^{13}C]EPA formation ($r = 0.61$, $P < 0.05$) and inversely with [^{13}C]DGLA and [^{13}C]AA formation ($r = -0.61$, $P < 0.05$), whereas LA concentration correlated directly with [^{13}C]

TABLE 3. Adjusted isotopic enrichment (AUC_{14d}) of long-chain conversion products

Diet	AUC_{14d} Value					
	DGLA	AA	EPA	DPA	DHA	AA/EPA
			μg/ml/h			*ratio*
FXO (n = 6)	31.3 ± 14.9	24.3 ± 7.2	38.7 ± 28.6	5.7 ± 2.6	1.0 ± 0.9	1.16 ± 1.20
SO (n = 5)	50.2 ± 3.8	64.4 ± 19.8	21.4 ± 4.2	3.9 ± 1.0	1.4 ± 0.2	2.94 ± 1.13
Both diets (n = 11)	38.9 ± 17.5	40.3 ± 26.4	31.8 ± 23.8	5.0 ± 2.6	1.2 ± 0.8	1.9 ± 1.2
P	<0.05	<0.05	NS	NS	NS	<0.05

AUC_{14d}, area under the curve for the 14-day time course. Values are means ± SD. Values are adjusted for variable precursor enrichments (see text).

TABLE 4. Correlations between LA, ALA, and LCP concentrations and LCP conversion (^{13}C AUC values)

	Concentrations in Plasma Lipids						
^{13}C AUC Values	LA	DGLA	AA	ALA	EPA	DPA	DHA
μg/ml/h				*mg/ml*			
[^{13}C]DGLA	0.77	0.57	0.56	−0.34	−0.37	−0.18	−0.63
	$P < 0.01$	$P < 0.05$	$P < 0.05$	NS	NS	NS	NS
[^{13}C]AA	0.51	0.80	0.83	−0.61	−0.32	−0.47	−0.21
	$P < 0.1$	$P < 0.01$	$P < 0.01$	$P < 0.05$	NS	NS	NS
[^{13}C]EPA	0.02	−0.35	0.09	0.61	0.26	0.21	−0.28
	NS	NS	NS	$P < 0.05$	NS	NS	NS
[^{13}C]DPA	−0.54	0.38	−0.08	−0.13	0.83	0.68	0.80
	$P < 0.1$	NS	NS	NS	$P < 0.01$	$P < 0.05$	$P < 0.01$
[^{13}C]DHA	0.11	−0.18	−0.09	0.05	0.12	−0.14	−0.05
	NS	NS	NS	NS	NS	NS	NS

Pearson correlation coefficients and their significance values are shown.

DGLA formation ($r = 0.77$, $P < 0.01$). LA concentration also correlated weakly with [^{13}C]AA formation ($r = 0.51$, $P < 0.1$) and inversely with [^{13}C]DPA formation ($r = 0.54$, $P < 0.1$). Similarly EPA concentration was correlated with [^{13}C]DPA formation ($r = 0.83$, $P < 0.01$), with a weak inverse correlation with LA concentration ($r = -0.5$, $P < 0.1$), whereas DGLA concentration was correlated with [^{13}C]AA formation ($r = 0.80$, $P < 0.01$). [^{13}C]DHA formation was unrelated to the concentration of any fatty acid.

DISCUSSION

Our hypothesis in the work described here was that a marked change in the dietary LA/ALA ratio would change the relative rates of formation of n-6:n-3 LCPs from their precursor fatty acids as measured by both ^{13}C tracer studies and through compositional changes in erythrocyte fatty acid profiles.

The three dietary groups were well matched at baseline with respect to erythrocyte fatty acid profiles, with initial levels of EPA and DHA similar to those in previous studies (40), although the high levels of LA (14%) and AA (15%) suggest a high background intake of LA. The increased concentrations of ALA, EPA, and DPA on the FXO diet were at the expense of palmitic and oleic acids, but with only modest decreases in LA and no changes in AA at any time. Indeed, there was no evidence of any inverse relationship between EPA and AA concentrations in either the FXO group ($r^2 = 0.02$) or the SOF group ($r^2 = 0.07$), suggesting that EPA did not replace AA. Others have noted that reductions in dietary intakes of LA are generally ineffective at changing concentrations of LA (41) or AA in relatively short time studies (42, 43). Because [^{13}C]LA conversion to AA did reflect relative concentrations of LA and ALA, we must assume that substantial LA, and possibly AA, storage in adipose tissue (44, 45) resulted in an additional supply of LA and AA independent of acute dietary intake (42, 46). A 12 month intervention providing 2.5% dietary energy as ALA in a high-LA diet (47) did attenuate the increase in AA in serum cholesterol esters compared with a similar LA intake with less ALA, suggesting that increased ALA can modify LA-to-LCP conversion.

Our finding of a substantial increase in EPA but no change in DHA in membrane phospholipids is consistent with most (11, 14, 48, 49) but not all (50, 51) previous reports of ALA supplementation in adults. The highest levels of enrichment of EPA are usually achieved at the lower intakes of FXO (48), suggesting that high levels of ALA inhibit its conversion to EPA. Indeed, the inverse relationship between dietary ALA and the DHA content of membrane phospholipids (42) suggests that increased ALA and/or EPA may displace DHA. In a recent 6 month study with 9.5 g of ALA per day, although EPA increased in peripheral blood mononuclear cells, DHA concentration decreased (49). The ALA/LA ratio rather than the absolute amount of ALA has been suggested to regulate the conversion to EPA (52), consistent with the report that doubling the intake of ALA at a constant dietary ALA/LA value had no additional influence on platelet EPA content (43).

The ^{13}C results show that LCP formation from both dietary LA or ALA does vary with its relative supply and inversely in many cases with concentrations of fatty acids of the alternative series. This is in accord with conventional thinking from in vitro studies that LA and ALA compete for Δ6-desaturation, the first step in the biosynthesis of long-chain PUFAs (53, 54). It is important because the apparent resistance of AA concentrations to dietary change has led some authors to question conventional ideas about the regulation of LCP formation (55). Thus, formation of both [^{13}C]DGLA and [^{13}C]AA [i.e., ^{13}C (mg/ml) AUC_{14d}] varied directly with plasma LA concentration and inversely with ALA concentration, whereas [^{13}C]EPA formation varied directly with ALA concentration. This meant that the marked reduction in the n-6:n-3 ratio by the FXO diet resulted in a halving of [^{13}C]LA conversion to [^{13}C]AA and a doubling in [^{13}C]ALA conversion to [^{13}C]EPA compared with the high n-6:n-3 ratio SO diet. The same pattern of dietary effects was indicated by the comparisons of absolute rates of LCP formation from peak plasma concentrations of [^{13}C]AA and [^{13}C]EPA.

However, although our findings for the reduction in n-6 LCP formation in response to the decreased dietary n-6:n-3 ratio are robust, the increases in n-3 LCP formation were less so. Significant changes were limited to the correla-

tion between ALA concentration and [^{13}C]EPA formation. These findings need to be considered in the context of the low power of the study to detect minor dietary influences because of the high cost of U-^{13}C tracers, low levels of enrichment, and marked between-subject variability in tracer handling, as observed in all such studies reported to date (11, 14, 22, 23). Tracer studies of fatty acid metabolism in vivo represent a particularly difficult problem given the lack of access to the tissue compartments where LCP formation takes place and an inability to measure exit rates of individual LCPs in the pathway as oxidation/carbon recycling or tissue deposition. Few studies have attempted more than relatively crude estimates of isotope transfer from tracer into the various tracee pools (22, 23), and it is recognized that AUC values will overestimate true conversion rates and provide only approximate relative rates of transfer (39). The two measures of conversion calculated here, peak plasma concentration and AUC, were proportional and highly correlated (r^2 = 0.79) but involved different assumptions and gave slightly different comparative results (Tables 3, 4). Thus, the slower decay of enrichment in the n-6 series fatty acids reflecting the larger pool sizes meant that the ratio of ^{13}C AUC/peak concentration was significantly higher for this series than for the n-3 series.

Our adjustment for variation in tracee pool sizes and consequent ^{13}C enrichments between diets and between fatty acid series attempts to deal with a serious issue recognized in some (21) but not other studies (18, 29). In a tracer study of n-3 and n-6 LCP formation in adults, Salem et al. (21) interpreted the 4-fold greater isotopic enrichment in EPA compared with AA as reflecting the greater initial enrichment of ALA compared with LA, with true relative rates of EPA synthesis likely to be lower than AA rates. Others (29) interpret a 5-fold greater ^{13}C enrichment in ALA and its LCPs after a decline in dietary ALA as indicating that dietary ALA reduces ALA long-chain conversion, without commenting on the between-diet tracer dilution problem. Indeed, to our knowledge, an upregulation of ALA long-chain conversion by increased dietary ALA or a reduced LA/ALA ratio has not been demonstrated even when increased concentrations of LCPs occurred; this reflects, in part, lack of adjustment for the lower ^{13}C enrichment in ALA after a high-ALA diet (29). Others report a 5- to 10-fold higher conversion rate of ALA compared with LA as a real, between-series difference and not an artifact attributable to differential initial isotope dilution, arguing that this would not influence the weight or concentration of trace-labeled metabolites (18), an assertion that is difficult to support. Our ^{13}C adjustment algorithm, normalizing for assumed precursor enrichments, assumes that the adjustment was not markedly influenced by the lack of time points before 24 h in the AUC ^{13}C APE values. In fact, lack of early time points at peak labeling for LA and ALA ^{13}C enrichments (<24 h), with a consequent underestimate of enrichment values over time, would have underestimated the errors. This is because the ALA enrichment AUC would have been more underestimated than that of LA and more underestimated on the low-ALA diet compared with the high-ALA diet. In turn, this means that we underestimated both the upward adjustment of n-6 compared with n-3 LCPs and n-3 LCPs on the high-ALA diet compared with the low-ALA diet. This strengthens rather than weakens the findings reported here. Clearly, there is an urgent need to develop more precise and standardized analytical models for tracer studies of fatty acid metabolism.

The low long-chain conversion of LA reported here is consistent with results from other human studies (18, 39, 56, 57) and with animal data (51, 58, 59) and reflects the low initial conversion of [^{13}C]LA to [^{13}C]DGLA. Similarly, previous [^{13}C]ALA studies have shown oxidation to be its major fate (17), with conversion to EPA varying from ~8% (18, 25) to much lower values (0.2%; 22, 23), consistent with our results. With the Δ6-desaturase rate limiting LCP formation, once formed, [^{13}C]DGLA is converted almost quantitatively to [^{13}C]AA and 20–30% of EPA is converted to DPA, as reported by others (25–27, 29). Reports of the conversion of ALA to DHA range from 4% (18) to no significant DHA synthesis (25) or <0.05% (27). Our finding of an overall conversion of <0.01% of the administered [^{13}C]ALA tracer to DHA is consistent with the latter reports.

If the Δ6-desaturase is the rate-limiting step in n-3 and n-6 LCP formation, the higher tissue concentrations of LA compared with ALA might be expected to result in higher rates of LA conversion compared with ALA, even taking into account the 1.5- to 3.0-fold higher Δ6-desaturase conversion rates for ALA compared with LA indicated by animal studies (60, 61). However, product inhibition may be an important regulator of C18 conversion to LCPs in vivo. Without such a mechanism, the high intake of LA in many human diets would result in the accumulation of high levels of n-6 LCPs in tissue lipid stores. Tang et al. (62) have reported that product inhibition occurs via suppression of the human Δ6-desaturase enzyme by LCPs mediated through its activation by peroxisome proliferator-activated receptors. It must be assumed that product inhibition by AA will affect LCP conversion of both LA and ALA.

In conclusion, when variation in ^{13}C enrichment attributable to variable tracee pool sizes is taken into account, reducing the dietary LA/ALA ratio downregulates LA conversion to AA and most likely upregulates ALA conversion to EPA. This suggests that the relative concentrations of the n-6 and n-3 LCP eicosanoid precursors do reflect relative dietary intakes of LA and ALA, but the relatively high background ratio of dietary n-6:n-3 tends to minimize changes in membrane AA in short-term studies. Even the Canadian Inuit, with very high intakes of n-3 LCPs, have an AA/EPA ratio of 2 (63). The regulation of DHA levels in tissues remains an enigma. The fact that DHA can be formed from ALA, albeit at a very low rate, but cannot be increased by increased dietary ALA suggests that DHA concentrations, at least in circulating phospholipid pools, are regulated to satisfy a relatively low metabolic demand that can be satisfied by the relatively low levels observed in vegans with no dietary DHA intake and with erythrocyte DHA levels lower than EPA (64). From

this perspective, it could be argued that any cardioprotection associated with increased dietary DHA intake and increased tissue levels represents a pharmacological response to an increased risk associated with an imbalanced n:6:n3 ratio.

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