Incorporation of deuterium-labeled fatty acids into human milk, plasma, and lipoprotein phospholipids and cholesteryl esters


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Abstract Fatty acid metabolism and the contribution of dietary fatty acids to milk cholesteryl ester (CE) and phospholipid (PL) were investigated in normal lactating mothers. The approach used was to feed mixtures of triglycerides containing deuterium-labeled palmitic acid (16:0-2H₂), oleic acid (18:1-2H₄), and linoleic acid (18:2-2H₄). Milk and plasma samples were collected for 72 hr. Triglyceride (TG), CE, and PL fractions from milk, plasma, and lipoprotein were isolated and analyzed by gas-liquid chromatography and mass spectrometry. Data for the ratios of the deuterated fatty acids incorporated into the milk CE and PL samples, their incorporation times and isotopic enrichment, data, it appears that these fatty acids are supplied mainly by the TG derived from chylomicrons and very low density lipoproteins. Plasma and lipoprotein CE data showed a progressive increase in 18:2-2H₄ content, with 16:0-2H₂ and 18:1-2H₄ remaining relatively constant over the collection period. Plasma and lipoprotein PL data showed a higher rate for incorporation of 18:2-2H₄ than 16:0-2H₂ and 18:1-2H₄ over the course of the sampling period. Comparison to previous data from adult males indicates lactation does not have a major effect on the general metabolism of these fatty acids. An increase with time in the isotopic enrichment of 18:2-2H₄ in the plasma and lipoprotein CE and PL samples was observed which is consistent with in vitro selectivities reported for lecithin:cholesterol acyltransferase and phosphatidylcholine acyltransferase. Compared to 18:2, dietary 16:0 and 18:1 were preferentially incorporated into CE by both intestinal and mammary cells. These data suggest that acyl coenzyme A:cholesterol acyltransferase in primarily responsible for synthesis of CE in the intestinal cells and in the mammary gland. The results show that the influence on fatty acid composition of milk TG, PL, and CE occurs 8-10 hr after dietary fat is consumed. The same fatty acid pool is apparently used for synthesis of milk TG, PL, and CE. — Emken, E. A., R. O. Adlof, D. L. Hachey, C. Garza, M. R. Thomas, and L. Brown-Booth. Incorporation of deuterium-labeled fatty acids into human milk, plasma, and lipoprotein phospholipids and cholesteryl esters. J. Lipid Res. 1989. 30: 395-402.

Supplementary key words lactation • triglycerides

Various studies have shown that dietary fatty acids contribute a substantial portion of the fat present in human milk lipids and influence the composition of milk triglycerides (1-5). Recent results for lactating women fed triglycerides containing deuterium-labeled palmitic, oleic, and linoleic acids showed that about 10% of the labeled fatty acids were nonselectively incorporated into breast milk TG. A lag time of about 6 hr was observed between the time of maximum isotopic enrichment in the plasma TG and maximum enrichment of the labeled fatty acids in milk TG (6). From these and other data, dietary fat is estimated to contribute 25 to 30% of the fatty acids present in human milk lipids. The effects of dietary fats on human milk TG composition, metabolism of lipids by the mammary gland, and transfer of dietary fats to human milk TG have been studied and are well summarized in recent reviews (7-9).

In contrast to the information available on the synthesis and origin of milk TG, less is known about the origin of milk PL and CE in human subjects. As summarized by Patton and Jensen (7), glycerol-3-phosphate is synthesized de novo by the mammary gland, and then acylated to yield phosphatidic acid which is converted to various phospholipid subclasses. These phospholipids are concentrated...
trated in the membrane structure that surrounds the milk droplet as it is pinched off during secretion. The origin of milk CE is less clear. Cholesterol may enter the mammary cells from both the diet and serum where it is then acylated. The possibility has been suggested that a portion of the milk CE may be derived as an intact molecule from chylomicrons and very low density lipoprotein particles.

Some aspects of lipid metabolism in healthy women appear to be similar to those of men. The dietary effects of high and low ratios of polyunsaturated-to-saturated fatty acids on serum cholesterol levels in women (10) were similar to results reported for men. Earlier kinetic data indicated that women clear very low density lipoprotein TG more efficiently than men (11), but overall plasma TG turnover rates were similar for men and women (12). Differences in hormone levels may be responsible for some of the differences in lipoprotein lipid metabolism of men and women. The higher levels of high density lipoprotein (HDL) observed in premenstrual women compared to men is believed due to differences in estrogen level (13). In a comparison of lactating and nonlactating women, it was found that lactating women had higher levels of HDL cholesterol and phospholipid (14). It is reasonable that this difference is caused by a higher rate of catabolism of TG-rich lipoproteins due to prolactin activation of mammary gland lipoprotein lipase.

The purpose of this research was to measure and compare the differences in incorporation of specific dietary fatty acids into milk, plasma, and lipoprotein PL and CE samples from lactating mothers. The approach used was to feed mixtures of TG containing deuterium-labeled fatty acids into milk and plasma lipids. These data provide additional insight into the transport and metabolism of dietary fatty acids in lactating mothers. Data for the distribution of these deuterium-labeled fatty acids in milk, plasma and lipoprotein triglycerides have been reported previously (6).

MATERIALS AND METHODS

Materials

Deuterium-labeled triglycerides containing [9,10-3H2]hexadecanoic acid (16:0-2H2), [14,14,15,15,17,18-3H6]cis-9-octadecenoic acid (18:1-3H6), and [16,16,17,17-3H4]cis-9, cis-12-octadecadienoic acid (18:2-3H4) were synthesized (15-17) and blended at 85°C with Sustacal liquid (Mead Johnson, Evansville, IN) as previously described (6). The fatty acid composition of the test diet and isotopic enrichment of the deuterated fatty acids in the final mixture are summarized in Table 1.

Subjects and study protocol

Details concerning the three lactating mothers, diets, milk composition, and the protocol followed have been described in a previous publication (6). A brief summary of the general experimental design follows.

Three lactating mothers were admitted to the Clinical Research Center study unit and their usual diet was replaced on an equal caloric basis with Sustacal (Mead Johnson and Co.). The Sustacal diet was continued during the 4-day experimental period. The subjects were allowed to supplement their diet (up to 15% of total caloric intake) with fruits, vegetables, and cereals. The subjects were admitted the evening before the mixture of deuterated fats blended with Sustacal was fed in place of their usual breakfast. Milk and blood samples were collected at 0, 1, 2, 4, 6, 8, 10, 12, 16, 20, 24, 36, 48, and 72 hr after

<table>
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<th>Fatty Acid</th>
<th>Total Mixture</th>
<th>Susetacal</th>
<th>Isotopic Enrichment</th>
<th>Weight</th>
<th>3H</th>
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<td>g</td>
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<tr>
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<td>3.00</td>
<td>38.4</td>
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</table>

*Three g of unlabeled 16:0 was fed to subject 2 in place of 16:0-3H2.
feeding the test diet. Blood samples for lipoprotein lipid analysis were collected at 4, 6, 8, 12, and 16 hr. Lipoprotein fractions, chylomicron (CHYLO), very low density (VLDL), low density (LDL), and high density (HDL), were obtained by preparative ultracentrifugation according to standard procedures (18).

The protocol was approved by the Institutional Review Boards for Human Research of Baylor College of Medicine and Texas Children's Hospital and informed consent was obtained prior to feeding the deuterated fats.

**Milk, plasma, and lipoprotein extractions and separations**

Total lipids from milk, plasma, and lipoprotein samples were extracted with chloroform–methanol following appropriate procedures as described earlier (6). Milk and plasma lipids were isolated by separation with disposable aminopropyl solid phase extraction columns (Bond Elut, Analyticchem International, Harbor City, CA) by the procedure described previously (6). Lipoprotein lipid classes were separated by preparative thin-layer chromatography by following procedures described elsewhere (19, 20).

Valid data for milk CE and PL samples from subject 1 were not obtained because substantial deterioration occurred before the samples were analyzed. Methyl esters of the isolated lipid classes were prepared by saponification of the samples with KOH (21), and treatment of the extracted free fatty acids with either methanol–dimethoxypropane-HCl (22), or methanol-HCl (21).

**Gas-liquid chromatographic and mass spectrometric analyses**

The fatty acid methyl esters were analyzed with a Hewlett-Packard gas chromatograph (Model 5840) equipped with either a 25 m × 0.5 mm Silar-10c glass capillary column or a 30 m × 0.32 mm DB-225 fused silica capillary column. Authentic methyl ester standards (Supelco, Inc., Bellefonte, PA) were used for identification of the fatty acids in the samples by comparison of retention times.

A MAT 212 GLC-mass spectrometer with an SS-200 data system (Finnigan MAT, San Jose, CA) and interfaced with the Hewlett-Packard gas chromatograph was used to determine isotopic enrichments of 16:0-2H2, 18:0-2H4, and 18:2-2H4 in the palmitate, oleate, and linoleate peaks.

The percentages of deuterium-labeled 16:0, 18:1, and 18:2 in the total fatty acids from the various lipid classes were calculated by multiplying the percent isotopic enrichment by the percent of 16:0, 18:1, and 18:2 in the total sample. These data were then adjusted for isotope dilution by the unlabeled fatty acids in the Sustacal diet. These calculations adjusted the data to a common basis, which facilitated comparison of data from each subject.

**RESULTS**

**Total plasma and milk cholesteryl ester and phospholipid data**

The incorporation and turnover of deuterium-labeled 16:0, 18:1, and 18:2 in plasma CE fractions from three lactating mothers are plotted in Fig. 1. The data indicate an average incorporation of ca. 16 times more 18:2-'H4 than 16:0-'H2 and ca. 7 times more 18:2-'H4 than 18:1-'H6.

The maximum enrichment of 18:2-'H4 in the total fatty acids of the CE samples was variable (range 1.5 to 5.7%) but the ratios for 18:2-'H4 incorporation relative to deuterated 16:0 and 18:1 were consistent.

Data for plasma PL samples from two subjects (2 and 3) are plotted in Fig. 2. Data for plasma PL from subject 1 are not plotted because this group of samples showed
evidence of deterioration. The data in Fig. 2 indicate a three- to fivefold preferential incorporation of 18:2-$^3$H$_4$ relative to 16:0-$^2$H$_2$ and 18:1-$^2$H$_6$. Maximum levels of deuterated fatty acid incorporation into the plasma phospholipids were consistent for these two subjects.

Milk CE and PL data from subjects 2 and 3 are compared in Fig. 3. Milk CE and PL data indicate a substantial discrimination against incorporation of 18:2-$^2$H$_4$ relative to 18:1-$^2$H$_6$ and 16:0-$^2$H$_2$. This discrimination against 18:2-$^2$H$_4$ in these milk lipids is in marked contrast to the preferential incorporation of 18:2-$^2$H$_4$, relative to 18:1-$^2$H$_6$ and 16:0-$^2$H$_2$, observed for the plasma CE and PL samples.

**Lipoprotein CE and PL data**

Data for comparison of the incorporation of 16:0-$^2$H$_2$, 18:1-$^2$H$_6$, and 18:2-$^2$H$_4$ into milk and lipoprotein CE fractions from subject 3 are plotted in Fig. 4 for the 4-hr and 16-hr lipoprotein CE samples and the 8-hr milk CE sample. Similar data for the PL fractions are shown in Fig. 5 for the 4- and 12-hr lipoprotein PL samples and the 10-hr milk PL sample. Only data for subject 3 are shown in these figures in order to facilitate comparisons. The trends shown in Figs. 4 and 5 for subject 3 were similar to the deuterated fatty acid patterns for milk and lipoprotein samples from subjects 1 and 2. Milk CE and PL data were not available for subject 1, but data for subjects 2 and 3 were nearly identical. The data for the 12-hr and 16-hr lipoprotein samples from all subjects were also similar. The only difference was in the isotopic enrichment in the lipoprotein samples which is also reflected in the plasma CE data in Fig. 1 and, of course, the data for subject 2 did not contain information for 16:0-$^2$H$_2$. The most variability was found in the data for the 4-hr lipoprotein samples from the different subjects. This is expected because: 1) the low isotopic enrichments in these samples increase the analytical error; and 2) the sampling times are not frequent enough to ensure that samples represent the same point on the absorption curve for each subject. From the chylomicron and VLDL TG data, we know that the absorption times varied by 1 to 2 hr for these subjects.

For all subjects, incorporation of 18:2-$^2$H$_4$ into the lipoprotein CE samples progressively increased from relatively low values in the 4-hr samples to relatively high values in the 16-hr lipoprotein samples. The percentages of 16:0-$^2$H$_2$ and 18:1-$^2$H$_6$ in the lipoprotein fractions remained fairly constant over this time period, which is similar to the plasma CE data (Fig. 1). The 4-hr lipoprotein CE and PL samples from subject 3 contained 2 times or more 16:0-$^2$H$_2$ than 18:2-$^2$H$_4$. In contrast, the 16-hr lipoprotein CE samples and 12-hr LDL and HDL PL samples contained a much higher proportion of 18:2-$^2$H$_4$ relative to 16:0-$^2$H$_2$ and 18:1-$^2$H$_6$ (Figs. 4 and 5).

The overall fatty acid composition of the plasma and lipoprotein CE and PL samples was consistent with reported composition data (23, 24) despite the fact that the polyunsaturate-to-saturate (P/S) ratio (2.57) of the
Fig. 4. Comparison of the distribution of 16:0-3H2, 18:1-3H6, and 18:2-3H4 in chylomicron (CHYLO), very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) CE samples to 8-hr milk CE data from subject 3; (A) 4-hr lipoprotein and (B) 16-hr lipoprotein samples.

Sustacal diet was 5 to 6 times higher than in typical U.S. diets (25).

DISCUSSION

Overall, the milk CE and PL data (Fig. 3) showed a definite lower incorporation of 18:2-3H4 relative to 16:0-3H2 and 18:1-3H6 than did plasma CE and PL data (Figs. 1 and 2). However, the pattern for the deuterated fatty acids in the 4-hr lipoprotein CE samples more closely reflected the pattern in milk CE than did the data for the 16-hr lipoprotein CE samples (Fig. 4). These data indicate that the enzymes involved with CE synthesis in the mammary tissue and the intestinal cells have similar specificities.

The reason the ratios for the deuterated fatty acids in the 4-hr lipoprotein CE samples differ substantially from the ratios in the 16-hr samples (Fig. 4) is probably due to the difference in the selectivities of the acyl coenzyme A:cholesterol acyltransferase (ACAT) and lecithin:cholesterol acyltransferase (LCAT). ACAT is selective for 18:1 (26, 27) and is apparently involved in synthesis of CE during absorption and incorporation into chylomicrons and VLDL by the intestinal cells. Cholesterol esterase, which is relatively nonselective for 18-carbon acids, could also be involved (27). Once these lipoprotein particles enter the circulation system, the 18:2-3H4 content of the CE progressively increases because of the high selectivity of LCAT for n-6 polyunsaturated fatty acids (28, 29). In humans, ACAT is primarily located in tissue (26) and its likely presence in mammary tissue is probably responsible for the higher content of 18:1-3H6 and 16:0-3H2 in milk CE.

The higher ratios of 16:0-3H2 and 18:1-3H6 relative to 18:2-3H4 in the milk PL samples compared to the 10-hr plasma (Fig. 2), and 12-hr lipoprotein PL samples (Fig. 5) are consistent with the concept that the mammary tissue utilizes the deuterated fatty acids from chylomicrons and VLDL TG to synthesize PL. The 4-hr lipoprotein TG data are compared to milk CE and milk PL data in

Fig. 5. Comparison of the distribution of 16:0-3H2, 18:1-3H6, and 18:2-3H4 in chylomicron (CHYLO), very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) PL samples to 10-hr milk PL data from subject 3; (A) 4-hr lipoprotein and (B) 12-hr lipoprotein samples.
Lipoprotein TG fractions were the primary source of the total isotopic enrichment was high enough to provide the isotopic enrichment observed in the 10-hr milk PL sample. These data suggest that fatty acids from the 4-hr lipoprotein TG fractions were the primary source of the deuterated fat found in the 8-hr milk CE and 10-hr milk PL fractions and that milk CE and PL synthesis in the mammary glands utilizes enzymatic pathways similar to PL fractions and that milk CE and PL synthesis in the mammary glands utilizes enzymatic pathways similar to CE and PL synthesis in the intestinal cells.

The reason for the higher percentage of 16:0-2H2 and 18:1-2H6 relative to 18:2-2H4 in the 4-hr lipoprotein PL samples compared to the 12-hr PL samples is less clear. One hypothesis is that PL synthesis by the intestinal cells involves a phospholipid acyltransferase that discriminates against 18:2 or is selective for 16:0. Alternatively, formation of PL during absorption of dietary fatty acids may utilize the cytidine pathway to convert a 1,2-diglyceride enriched with 16:0-2H2 to phosphatidylcholine (30). Regardless of the mechanism, the data are consistent with the observation that 16:0 percentages in bile phosphatidylcholine are considerably higher than in plasma phosphatidylcholine (31). Once the PL associated with lipoprotein enters the blood system, the fatty acid composition is apparently modified by selective exchange for 18:2 and/or additional PL, rich in 18:2, is synthesized by phosphatidylcholine acyltransferase (32).

Based on data from studies with goats and rats in which 32P-labeled lipoproteins were used (33), circulating plasma phospholipids do not directly contribute to the milk phospholipid pool. The backbone of the milk phospholipids is synthesized de novo in the mammary cell.

These data are consistent with the differences between the lipoprotein and milk phospholipid data in Fig. 5. The maximum deuterium isotopic enrichment in the milk TG samples was approximately 24% which is much higher than the 6% total enrichment in milk PL, but the time for maximal enrichment is the same (8-10 hr) for both TG and PL. The similarity in time of maximal enrichment suggest that TG and PL are formed from the same fatty acid pool. The lower isotopic enrichment for PL compared to TG indicates that the pool size of unlabeled PL is much larger in proportion to the amount of newly synthesized PL than the pool size of unlabeled TG relative to the amount of newly synthesized TG. The reason for this difference in isotope enrichment is because the amount of new PL needed for the membrane surrounding the milk TG droplet is much smaller than the amount of new TG required to form the milk lipid droplet. The times for maximal enrichment (8-10 hr) and the total isotopic enrichment (ca. 3%) for CE are similar to those for PL. These data for milk CE also suggest that the pool size of unlabeled CE is larger in proportion to the amount of newly synthesized CE than the pool size of unlabeled TG relative to the amount of newly synthesized TG.

Since stearoyl-CoA desaturase (acyl-CoA desaturase) in mammary tissue from mice (34) and cows (35) has been shown to also desaturate 16:0 at appreciable rates, it was anticipated that deuterated 16:1 might be present in the milk samples. However, no deuterated 16:1 could be detected in any of the milk samples, which indicates that acyl-CoA desaturase activity in the human mammary tissue is low. Also, no deuterated 18:0 was detected, which indicates that the rates for elongation of 16:0-2H2 to 18:0-2H2 and the conversion of 18:1-2H6 to 18:0-2H6 are very low. In addition, conversion of 18:2-2H4 to 20:4-2H4 was not detected.

Overall, the results suggest that the chylomicron TG and the VLDL TG particles transport the dietary fatty acids to the mammary gland where the epithelial cells synthesize TG, CE, and PL from the same fatty acid pool. The TG, CE, and PL containing these dietary fatty acids are then secreted 6 to 8 hr later as part of the total milk lipids. This 6-8 hr time period for synthesis of the milk globule in humans agrees well with the 4-6 hr time period estimated from goat data (7, 33). The metabolism of the dietary fatty acids not immediately removed from the chylomicron particles by the mammary gland is consistent with data reported for males (36) and nonlactating females (37). The deuterium-labeled fatty acids transferred to LDL and HDL do not enter the milk lipids unless they are recycled back into chylomicron and VLDL particles formed during absorption of fatty acids from subsequent meals. The results indicate that the fatty acid composition of each meal influences the fatty acid composition of milk TG, CE, and PL but the influence on the fatty acid composition of milk TG is greater than for milk CE and PL. Apparently this is because the enzymes involved in PL.
and CE synthesis are designed to selectively use fatty acids that impart physical properties required by the membrane surrounding the secreted TG droplet. Milk lipid fatty acids are also supplied by adipose tissue TG which reflect the long term average of the fatty acids in the diet. In this way, adipose tissue serves to moderate the fluctuations in dietary fatty acid patterns which in turn reduces the variation in the fatty acid composition of milk. 

REFERENCES


