Comparative Lipoprotein Metabolism of Myristate, Palmitate, and Stearate in Normolipidemic Men

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This project was designed to test the hypothesis that long-chain saturated fatty acids (myristate, palmitate, and stearate) are metabolized differently in human subjects, and that these differences may therefore account for the changes in plasma lipoprotein composition when these fatty acids are altered in the diet. Ethyl esters of each of the stable-isotope–labeled fatty acids ($^{2}H_3$- or $^{2}H_4$-myristate, $^{13}C_{16}$-palmitate, and $^{13}C_{18}$-stearate) were fed to five nonhyperlipidemic men. The concentration of each labeled fatty acid was monitored for up to 72 hours as the fatty acids were assimilated into the lipid components (phospholipid [PL], triglyceride [TG], and cholesteryl ester [CE]) of the plasma lipoproteins (TG-rich lipoproteins [TRL], intermediate-density [IDL], low-density [LDL], and high-density lipoprotein [HDL]). Over 95% of the myristate was incorporated into TG, whereas 33% and 9% of the stearate and 18% and 7% of the palmitate were incorporated into PL and CE, respectively. The mean residence times (MRTs) for myristate in TG (8.6 to 9.9 hours) and PL (6.7 to 10.9 hours) in the individual lipoprotein subfractions were significantly shorter than for either palmitate (TG, 12.7 to 15.3 hours; PL, 19.6 to 21.3 hours) or stearate (TG, 10.7 to 15.5 hours; PL, 17.8 to 19.9 hours). The MRTs for stearate were shorter than for palmitate in PL. These data indicate that TG fatty acid in general, and myristate TG in particular, is the most rapidly cleared of the saturated fatty acids. There was a rapid transfer of labeled TG and PL between the lipoproteins. We were unable to detect any significant amount of stearate desaturation or elongation. In conclusion, these data demonstrate that myristate, palmitate, and stearate are metabolized in unique ways, and that it may therefore be inappropriate to continue to regard all "saturated fatty acids" as metabolically similar in clinical studies. Rather, it is important that we elucidate more clearly the specific metabolic pathway of each fatty acid to understand the mechanisms by which it alters plasma lipoprotein concentrations and composition and influences atherogenesis.

IT IS NOW WELL ESTABLISHED that dietary ingestion of different fatty acids will have significantly different effects on lipoprotein composition. Mattson and Grundy[1] demonstrated that the unsaturated fatty acids, oleate (C18:1 ω-9) and linoleate (C18:2 ω-6), both decrease low-density, lipoprotein (LDL) cholesterol when substituted for palmitate (a saturated fatty acid, C16:0) in nonhyperlipidemic individuals. In addition, linoleate was also more likely than oleate to decrease high-density lipoprotein (HDL) cholesterol. Neither oleate nor linoleate decreased triglyceride (TG) levels in that study,[1] but others have reported a reduction of plasma TG when polyunsaturated fats were included in the diets.[2] Several recent reviews[3-5] have further suggested that the saturated fatty acids, myristate (C14:0), palmitate (C16:0), and stearate (C18:0), have different effects on LDL cholesterol, namely that myristate produced the largest increase in LDL, whereas stearate appeared to be neutral (similar to oleate). Palmitate appears to have a variable impact. Zock et al[6] and Tholstrup et al[7] demonstrated effects of palmitate on LDL similar to those of myristate, whereas Sundram et al[8] found a smaller impact of palmitate relative to myristate and Ng et al[9] reported palmitate effects to be neutral (ie, similar to oleate). Khosla and Hayes[10] may have helped to resolve some of these conflicts by demonstrating a hypercholesterolemic effect of palmitate only when subjects were hyperlipidemic or when the diet was also supplemented with cholesterol. Tholstrup et al[7] demonstrated clearly that stearate was less hypercholesterolemic than palmitate; however, Lichtenstein et al[11] showed that supplementation with stearate adversely affected lipids relative to linoleate.

Some potential mechanisms for the reduced hypercholesterolemic effect of stearate have recently been suggested. Several studies have reported a reduced intestinal absorption of stearate-containing TGs in rats,[12-14] hamsters,[15] and humans.[16] However, Emken et al[17] demonstrated that tristearin was absorbed as well as tripalmin if these lipids were maintained at a temperature above their melting points. Others have suggested that stearate acts like oleate because it is converted endogenously to oleate. Whereas some studies have supported this hypothesis by demonstrating a high conversion rate of stearate to oleate in rodent liver TG[12,18] and mouse macrophage PLs,[19] others have been unable to demonstrate this conversion in rat liver TG.[14,20] The only human study that has addressed this issue[17] used chemical ionization–mass spectroscopy to estimate that 9% of the stearate they fed to young men was desaturated to oleate. Perhaps the most consistent differential effect of the saturated fatty acids has been the preferential incorporation of stearate (relative to palmitate) into phospholipids. This effect has been seen in various mouse tissues,[18] cultured rat hepatocytes,[21] rat liver "in vivo,"[20,22,23] and humans.[17] Myristate appears to be a poor substrate in rats for hepatic esterification into PL, TG, or cholesterol esters (CE). As a result, myristate appears to be preferen-

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tially oxidized in the liver or used by peripheral tissues.[23] There have been no previous human studies that have examined myristate metabolism.

The major impact of linoleate on LDL metabolism relative to monosaturated and unsaturated fatty acids appears to be its ability to increase hepatic LDL receptor number and thus LDL clearance,[2,24] with little change in LDL production. Jones et al[25] actually reported an increase in hepatic cholesterol synthesis in human subjects fed linoleate- versus stearate/palmitate-enriched foods. However, in animal studies, stearate-enriched diets also appear to increase LDL receptors, LDL catabolism, and neutral fecal steroid excretion (while reducing hepatic cholesterol content) relative to myristate- or palmitate-enriched diets.[14,15,26]

From these studies, it is apparent that carbon-chain length and the number and location of double bonds can have important effects on fatty acid and lipoprotein metabolism. The current studies were designed to determine whether myristate, palmitate, and stearate are distributed differently into the lipid components (PL, TG, and CE) of chylomicrons (CMs), very–low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), LDL, and HDL in man. This altered dissemination could then be expected to alter the metabolism of these lipoproteins. A second aim was to determine whether palmitate and/or stearate are desaturated to potentially less hypercholesterolemic monounsaturated fatty acids.

SUBJECTS AND METHODS

Subjects

Five lean (body fat, 19%) nonhyperlipidemic (cholesterol, 168 ± 17 mg/dL; TG, 83 ± 14 mg/dL) adult (age, 36 ± 7 years) men were studied (Table 1). They were not receiving any medication and had no personal or family history of atherosclerotic disease. None of the subjects had diabetes mellitus, thyroid disease, anemia, liver disease, or renal disease. A diet history indicated that the usual dietary intake of total fat was 34% of calories, and that 39% of these calories were derived from saturated fat. Fatty acid analysis of the subjects' fasting plasma lipids on the first day of these studies showed similar distributions of saturated and unsaturated fatty acids (Table 2). This protocol was approved by the Institutional Review Board of the University of Tennessee and the Scientific Advisory Committee of the General Clinical Research Center. All subjects signed an informed-consent form.

Protocol

Body fat composition was determined by bioelectric impedance (RJL Systems, Mount Clemens, MI). Body fat distribution was estimated by the waist to hip ratio. Subjects were admitted to the

Table 1. Subject Characteristics

Characteristic	Subject No. 1	2	3	4	5	Mean ± SD	Normal Value
Body composition							
Age	42	23	42	33	39	36 ± 7	
Weight (kg)	105.0	75.0	79.3	77.5	72.0	81.8 ± 11.9	
Height (cm)	176.5	185.4	180.3	174.0	184.2	180.1 ± 4.4	
Frame	Large	Large	Large	Medium	Large		
%IBW	107	98	101	113	91	102 ± 8	
WHR	ND	0.857	0.934	0.894	0.804	0.872 ± 0.048	
Body fat (%)	23	18	29	15	10	19 ± 7	
Lean weight (kg)	81	62	56	66	66	66 ± 8	
Total body water (L)	56	43	41	48	50	48 ± 5	
Estimated body metabolism (kcal)	2,294	1,905	1,662	2,018	2,007	1,977 ± 204	
Lipoproteins (mg/dL)							
TC	146	177	196	158	164	168 ± 17	<238
TG	103	72	73	69	98	83 ± 14	<178
VLDL	13	8	13	11	16	12 ± 3	<33
IDL	11	11	14	7	14	11 ± 3	<23
LDL	78	109	107	97	84	95 ± 12	67-148
HDL	44	49	63	43	50	50 ± 7	32-63
Apo B	54	47	68	62	57	58 ± 7	49-110
Apo A-I	173	175	170	128	171	163 ± 18	103-193
Apo A-II	45	60	68	44	53	54 ± 9	26-59
Apo C-III	13	17	12	16	13	14 ± 2	4-17
Diet composition							
Total protein (%kcal)	14	13	23	16	17	17 ± 3	
Total carbohydrate (%kcal)	57	55	28	43	42	45 ± 10	
Total fat (%kcal)	29	31	35	40	36	34 ± 4	
%saturated fat	33	43	38	38	45	39 ± 4	
%monounsaturated fat	38	37	44	38	38	39 ± 3	
%polyunsaturated fat	29	20	19	24	17	22 ± 4	
Cholesterol (mg)	536	353	494	214	410	401 ± 113	
Alcohol (g)	1.6	2.6	45.3	2.4	27.2	15.8 ± 17.6	

Abbreviations: IBW, ideal body weight; WHR, waist to hip ratio; TC, total cholesterol; ND, not determined.

Table 2. Fasting Plasma Fatty Acid Composition (% total)

Parameter	Subject No. 1	2	3	4	5	Mean ± SD
TGs						
Myristate	4.8	3.6	3.7	1.3	2.0	3.1 ± 1.3
Palmitate	25.9	21.8	26.4	26.6	26.1	25.4 ± 1.8
Stearate	6.6	5.8	4.9	3.4	3.2	4.8 ± 1.3
Palmitoleate	8.1	6.9	8.2	3.8	5.4	6.5 ± 1.7
Oleate	32.7	38.6	32.8	37.8	40.9	36.6 ± 3.3
Linoleate	16.1	17.7	17.0	23.9	18.4	18.6 ± 2.7
Arachidonate	1.8	2.0	1.7	2.1	1.2	1.8 ± 0.3
Docosahexanoate	0.0	0.3	0.5	0.0	0.2	0.2 ± 0.2
PLs						
Myristate	0.9	0.6	0.7	0.3	0.7	0.6 ± 0.2
Palmitate	23.3	19.1	23.1	26.6	26.3	23.7 ± 2.7
Stearate	13.7	12.7	12.9	13.8	15.5	13.7 ± 1.0
Palmitoleate	1.1	1.9	2.7	0.9	0.7	1.5 ± 0.7
Oleate	17.5	20.0	16.7	14.7	11.4	16.1 ± 2.9
Linoleate	17.6	18.0	19.8	21.4	21.3	19.6 ± 1.6
Arachidonate	10.0	10.5	8.9	14.0	12.7	11.2 ± 1.9
Docosahexanoate	2.8	1.2	2.9	2.1	2.7	2.3 ± 0.6
CEs						
Myristate	2.2	1.4	1.9	0.7	1.2	1.5 ± 0.5
Palmitate	17.7	14.0	14.4	14.0	13.1	14.6 ± 1.6
Stearate	2.0	1.8	2.1	2.1	1.2	1.8 ± 0.3
Palmitoleate	5.9	7.1	10.8	2.5	3.9	6.0 ± 2.9
Oleate	21.4	23.7	22.7	17.8	22.1	21.5 ± 2.0
Linoleate	39.4	39.0	35.6	51.2	46.3	42.3 ± 5.6
Arachidonate	7.4	9.4	8.0	9.8	8.5	8.6 ± 0.9
Docosahexanoate	0.0	0.5	0.6	0.3	0.5	0.4 ± 0.2
Free fatty acids						
Myristate	4.6	1.6	3.2	2.8	1.4	2.7 ± 1.2
Palmitate	24.0	20.8	25.1	23.4	26.1	23.9 ± 1.8
Stearate	9.1	10.6	10.8	8.3	10.5	9.9 ± 1.0
Palmitoleate	4.7	3.0	3.6	4.1	3.7	3.8 ± 0.6
Oleate	31.3	37.4	32.3	36.2	41.9	35.8 ± 3.8
Linoleate	18.6	21.2	17.9	21.1	13.7	18.5 ± 2.7
Arachidonate	0.0	1.9	0.8	2.3	1.6	1.3 ± 0.8
Docosahexanoate	0.0	0.8	0.2	0.3	0.0	0.3 ± 0.3

Clinical Research Center at 7 AM after a 12-hour fast. To establish a stable postprandial condition, each patient received 16 hourly feedings from 7 AM until 10 PM . Each meal consisted of one sixteenth of their normal daily caloric intake, and was composed of a combination of Sustacal (a premixed standard meal containing 55% carbohydrate, 24% protein, and 21% fat; Mead Johnson, Evansville, IN), ice cream, and corn oil (to increase the total fat composition to 35%: 38% saturated, 18% monounsaturated, and 44% polyunsaturated fatty acids).

To reduce the possibility of differences in fatty acid absorption,[12-16] ethyl esters of each saturated fatty acid were prepared. The esters have lower melting points than the triacylglycerols and are therefore easier to disperse in aqueous solutions than are the triacylglycerols of the fatty acids. Approximately 8 g stable-isotope–labeled myristate ethyl ester ($^2H_3$ or $^2H_4$; Cambridge Isotope Laboratories, Andover, MA) and 4 g each of labeled palmitate ($^{13}C_{16}$) and stearate ($^{13}C_{18}$) ethyl ester (Martek, Columbia, MD) were mixed with the 10 AM meal and ingested over 5 minutes by each subject. More ^2H-myristate was utilized because the threshold for detection of this isotope was less than that of the ^{13}C isotopes (see later discussion). Lipoprotein analyses were performed at 1, 2, 3, 4, 6, 12, 24, and 48 hours after this meal. Patients remained in a fasting state after the 10 PM meal until they returned the following day at 10 AM for the 24-hour lipoprotein analysis. They were then allowed to eat their usual diet as they wished, outside of the research center, until 10 PM, when they again fasted until 10 AM the following morning, at which time the final blood sample was obtained.

Lipoprotein Isolation

Thirty milliliters of blood was drawn into EDTA tubes, and red blood cells were removed by centrifugation. Ultracentrifugation of the plasma was performed within 24 hours as described previously.[27] Nine milliliters of plasma was increased to a density of 1.27 g/mL with KBr and added to an ultracentrifuge tube. A second layer of buffer with a density of 1.20 g/mL (12 mL) was added, and then the tube was filled with buffer (d = 1.006 g/mL). The plasma was centrifuged in a Ti70 rotor in a Beckman (Fullerton, CA) L8-80 ultracentrifuge at 15°C for 3.25 hours at 70,000 rpm (361,000 × *g*). The tubes were emptied by pumping the effluent out of the top of the tube, and 1.0-mL fractions were collected. These fractions were pooled into the following lipoprotein subfractions: VLDL + CM, IDL, LDL, and HDL.

A "CM + large-VLDL" subfraction was isolated with a modified gradient. Five milliliters of plasma was increased to a density of 1.27 g/mL with KBr and added to an ultracentrifuge tube. The tube was filled by layering on a buffer with a density of 1.006 g/mL. The plasma was centrifuged for 30 minutes at 25,000 rpm (85,000 × *g*) at 15°C in a Ti70 rotor in a Beckman L8-80 ultracentrifuge. The tube was emptied as above and the fractions containing CMs and large VLDL were pooled. The major apolipoprotein (apo) B band in this subfraction was found to be apo B-100 by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. A small band of apo B-48 was also seen. This result is consistent with the finding that postprandial VLDL attains a size and density similar to that of CMs. Thus, it was not possible by these techniques to separate intestinal-derived lipoproteins from liver-derived lipoproteins.

Apolipoprotein and Lipid Analysis

An aliquot of each lipoprotein subfraction was delipidated with 5.0 mL hexane-isopropanol (3:2 vol/vol) after adding a known amount of protein internal standard (insulin) and lipid internal standards (free fatty acids, TG, PL, and CE containing 100% heptadecanoate [17:0]). A second delipidation step was performed with 4.0 mL hexane. The hexane layers from each step were pooled and dried under nitrogen for fatty acid analysis (described later). The delipidated apolipoproteins were dried and resolubilized in 3 mol/L guanidine HCl and analyzed by reversed-phase high-performance liquid chromatography using an acetonitrile-water gradient (27% to 59%) containing 0.1% trifluoroacetic acid.[27] Peaks were detected at a UV absorbance of 214 nm, and peak areas were determined using a Shimadzu integrator (Columbia, MD). The concentration of each apolipoprotein in the lipoprotein subfractions was calculated using the known response factor for each protein (apo A-I, A-II, C-III, C-II, C-I, and E) relative to insulin.

The apo B content of LDL was determined[27] by diluting an aliquot of LDL 1:5 with water and mixing 100 μL diluted LDL with 100 μL 100-mmol/L SDS in 0.2N NaOH. Lowry reagents were added, and the absorption was measured at 640 nm. Bovine serum albumin was used as a standard. Apo B concentrations in VLDL and IDL were determined by precipitation of Apo B with 50% isopropanol/water.[27] Three aliquots (100 μL) of each sample were washed twice with 1.0 mL isopropanol (IPA)/water and once with hexane/IPA (3:2 vol/vol). Apo B was precipitated by centrifugation (2,500 rpm for 20 minutes), and the supernatant containing the soluble proteins and salt was discarded each time. The pellet

was dried under vacuum and resolubilized in 100 μL 100-mmol/L SDS in 0.2N NaOH. This step required incubation overnight at 37°C. One hundred microliters of water was added, followed by the Lowry reagents as above.

Total cholesterol (reagent #352100; Sigma Diagnostics, St Louis, MO), free cholesterol (reagent #139 050; Boehringer Mannheim Diagnostics, Indianapolis, IN), TG (reagent #339-50; Sigma Diagnostics), and PL (reagent #271-54008; Wako Pure Chemical Industries, Osaka, Japan) were assayed with commercially available kits using a 96-well microtiter plate reader. Each assay used standards obtained from the College of American Pathologists, if available. Triplicates of both "normal" and "elevated" controls (provided with each kit) and a pooled-plasma control, prepared locally and stored at −80°C, were assayed with each plate. KBr present in the lipoprotein subfractions did not interfere with the results of the assays. This laboratory participates in the laboratory quality-control testing program sponsored by the Centers for Disease Control for the total cholesterol and TG assays. The Centers for Disease Control does not provide unknowns for the free cholesterol or PL assays.

Fatty Acid Analysis

PL, CE, and TG were separated by thin-layer chromatography, using petroleum ether/anhydrous ether/acetic acid (84:15:1 by volume). The fatty acids were esterified with boron trifluoride in methanol, and analyzed by gas chromatography/mass spectroscopy using a Supelcowax 10 bonded-phase column (30 m, 0.5-μm film thickness, 0.32 mm ID, fused silica; Belle Fonte, PA). The amount of each fatty acid was determined in each lipid class (TG, CE, and PL) from each lipoprotein subfraction by using the internal standard added during delipidation.

A Hewlett Packard (Atlanta, GA) mass-selective ion detector, which operates in the electron ionization mode, was used for these analyses. Two different stable-isotope–incorporated myristate labels were used for these studies. One contained three deuterium atoms on the terminal carbon (C-14) of the fatty acid, and the other contained four deuterium atoms, two on carbon 10 and two on carbon 11 of the fatty acid. The molecular weight of the unlabeled methyl myristate is 242 daltons. The principal fragment ion is $(M-31)^+$, which is the loss of OCH_3, at *m/e* 199, and it contains all of the carbons of the original fatty acid. Therefore, the corresponding stable-isotope–containing fragment ions were quantified using either *m/e* 202 or 203 (depending on the label used). No fragment ions were observed at either *m/e* 202 or 203 in the unlabeled material. The molecular ion ($M^{+\cdot}$) was also monitored, but it was not the primary ion utilized, since its abundance was only about one third that of the *m/e* 199 fragment.

Palmitate and stearate were both labeled with ^{13}C in all 16 and 18 of the original carbons, respectively. This level of incorporation allowed us to monitor any fragment that provided the most accurate quantitative data. The two most abundant fragment ions occurred at *m/e* 74 (the McLafferty rearrangement ion containing the carboxy-terminal two carbons) and *m/e* 87 (the direct cleavage ion containing the carboxy-terminal three carbons). The *m/e* 74 fragment was the most abundant ion (by ~30%), but there was a small amount of native material at *m/e* 76 that would have obscured our enrichment calculations. However, there was no native material at *m/e* 90, so the shift from *m/e* 87 to 90 (due to three labeled carbons) was the most easily detected and most accurately quantified ion. The molecular ion of each fatty acid was also monitored. However, the sensitivity of this measurement was only approximately one tenth that of the *m/e* 87 fragment ion. On the other hand, the specificity of detecting the molecular ion mass shift of 16 or 18 mass units was excellent. Therefore, if the $M^{+\cdot}$ was detected, it provided strong corroboration for the presence of the isotope in the particular compartment in question. Our threshold for detecting labeled palmitate and stearate was obviously greater than that for detecting labeled myristate (due to the greater abundance of the *m/e* 87 *v m/e* 199 fragment ion). We attempted to overcome this difference by feeding twice as much myristate as palmitate and stearate. Unfortunately, myristate labeled in the three carboxy-terminal carbons was not available when the study was initiated.

Monounsaturated fatty acids are fragmented by electron ionization much more than are saturated fatty acids. Therefore, the *m/e* 87 fragment ion was much less abundant in oleate than in stearate. However, there was still no native material at *m/e* 90; thus, the *m/e* 87 to 90 shift still provided the most accurate results.

The amount of labeled fatty acid (milligrams per deciliter) in each lipid in each lipoprotein class was calculated by (1) determining the percent enrichment of myristate, palmitate, and stearate (ie, area of the *m/e* 90 fragment ion of palmitate divided by the area of the *m/e* 87 fragment ion of palmitate); (2) multiplying the enrichment by the percentage of the total of each fatty acid (ie, 4% enrichment of palmitate multiplied by the 30% of total fatty acids that were palmitate yields a labeled fraction in this lipid subfraction of 0.012); (3) multiplying the labeled fraction by the plasma concentration of the specific lipid (ie, LDL PL level of 45 mg/dL multiplied by 0.012 indicates that the concentration of labeled palmitate in LDL PL is 0.54 mg/dL).

Data Analysis

Pharmacokinetic analysis was performed using published model-independent methods.[28] Concentration-time profiles were constructed for each lipid compartment in individual patients. Areas under the plasma concentration-time curve (AUC) and under the first-moment curve (AUMC) were calculated using the linear trapezoid rule. The magnitude of the AUC is directly proportional to the dose and to the fraction of the dose incorporated into that compartment (f) and indirectly proportional to the clearance of the fatty acid from that compartment (CL), as denoted by the equation, AUC = (f × dose)/CL. The AUC for the first 12 hours was calculated. Mean residence times (MRTs) were calculated as the AUC divided by the AUMC for each lipid compartment, and correspond to the mean time spent by a labeled fatty acid molecule in the measured compartment from its entrance into the body to its definitive exit from the measured compartment. For intravenous doses, MRT correspond to the inverse of the elimination rate constant, *k* (ie, $MRT_{iv} = 1/k$). However, MRT for extravascular administration (eg, oral administration as used in this study) includes the mean absorption time (MAT) and thus will always be larger than the MRT observed after intravenous administration ($MRT_{ex} = MRT_{iv} + MAT$).

For total plasma MRT and AUC, data were analyzed using repeated-measures ANOVAs for a complete block design without replication, with subjects as blocks and isotopes as the treatments. To examine the relative distribution and kinetics of each isotope in each of the three lipid fractions, data were analyzed using a repeated-measures two-way ANOVA for a complete block design without replication, with subjects as blocks, isotopes and lipid fractions as main effects, and the various interaction effects; each main effect was tested with the appropriate error term (ie, the interaction of subjects with isotopes or subjects with fractions or the residual). Planned nonorthogonal contrasts were made between various pairs of means using the residual mean square error as the denominator in the F tests.[29] The specific hypotheses tested were the following: (1) there are no differences among the isotopes for the same lipid fraction, and (2) there are no differences among the lipid fractions for the same isotope. To test these hypotheses, we subdivided the isotope-lipid fraction sum of squares (type III)

and the degrees of freedom into separate contrasts and constructed F tests. Data for MRT and AUC of various lipids contained in the lipoprotein fractions were characterized by heteroscedasticity; in addition, data for AUCs were log-normally distributed. Therefore, data for each lipid fraction were transformed, if appropriate, and analyzed separately using repeated-measures one-way ANOVA with planned nonorthogonal contrasts, as already described. Because no myristate appeared in the PL of two subjects, MRTs in PL of myristate (n = 3), palmitate (n = 5), and stearate (n = 5) in these lipoprotein subfractions were compared by paired *t* tests. The specific hypothesis tested was that there are no differences among the isotopes for a particular lipid contained in a specific lipoprotein fraction. The level of significance for all statistical tests was .05. For balanced situations, results are reported as the mean ± SE; for unbalanced situations, results are expressed as the least-square mean ± SE.[30] All calculations and statistical analyses were performed using the SAS program (SAS Institute, Cary, NC).

RESULTS

Total Plasma

Approximately twice as much labeled myristate was given to each patient as the other two stable-isotope–labeled fatty acids. The peak concentration of myristate in total plasma lipids (TG + PL + CE) was approximately twice that of palmitate and stearate (2.8 *v* 1.4 and 1.2 mg/dL, respectively), and all peaks occurred at a similar time (between 2 and 6 hours) (Fig 1). These data suggest that the three fatty acids had similar absorption parameters. The total plasma MRT observed for myristate (9.6 ± 1.1 hour) was significantly shorter ($P = .003$) than for palmitate (16.7 ± 1.1 hour) or stearate (16.7 ± 1.1 hour) (Table 3). Despite the higher dose of myristate, the total plasma AUC of myristate (35.1 ± 2.8 mg · h/dL) was not significantly different from those of palmitate (27.2 ± 2.8 mg · h/dL, $P = .07$) or stearate (30.7 ± 2.8 mg · h/dL, $P > .10$). These data indicate that the almost twofold faster plasma clearance of myristate offset its twofold higher dosage, resulting in similar AUCs.

This hypothesis is corroborated by examining the relative distribution and kinetics of each isotope in each of the three lipid components. Incorporation of the labeled fatty acids into the lipid components was dependent on their chain length. During the first 12 hours (when influx of lipid tends to overshadow clearance), the labeled isotopes partitioned as follows (AUC): myristate, 96.7% TG, 2.3% PL, and 0.9% CE; palmitate, 75.5% TG, 17.9% PL, and 6.6% CE; and stearate, 57.9% TG, 33.2% PL, and 8.9% CE. These data suggest that compared with palmitate or stearate, myristate is preferentially incorporated into TG. This preference appears to contribute to the threefold higher peak myristate concentration in TG despite its only twofold higher initial dosage (Fig 1). However, the myristate AUC in TG (30.0 ± 2.2 mg · h/dL) for the total 72 hours was only twice the AUCs observed for palmitate (15.7 ± 2.2 mg · h/dL, $P = .003$) and stearate (12.3 ± 2.2 mg · h/dL, $P = .0001$). This lower relative AUC was again the result of a shorter myristate MRT in TG, compared with palmitate and stearate (Table 3).

In PL, the stearate AUC (16.6 ± 2.2 mg · h/dL) was greater ($P = .002$) than the palmitate AUC (8.79 ± 2.23 mg · h/dL), which was greater ($P = .0001$) than the myristate AUC (0.65 ± 2.23 mg · h/dL) (Fig 1). The higher concentration of stearate relative to palmitate was clearly due to differences in incorporation, since the stearate MRT tended to be slightly shorter than the palmitate MRT (Table 3). In contrast, the small AUC of myristate in PL

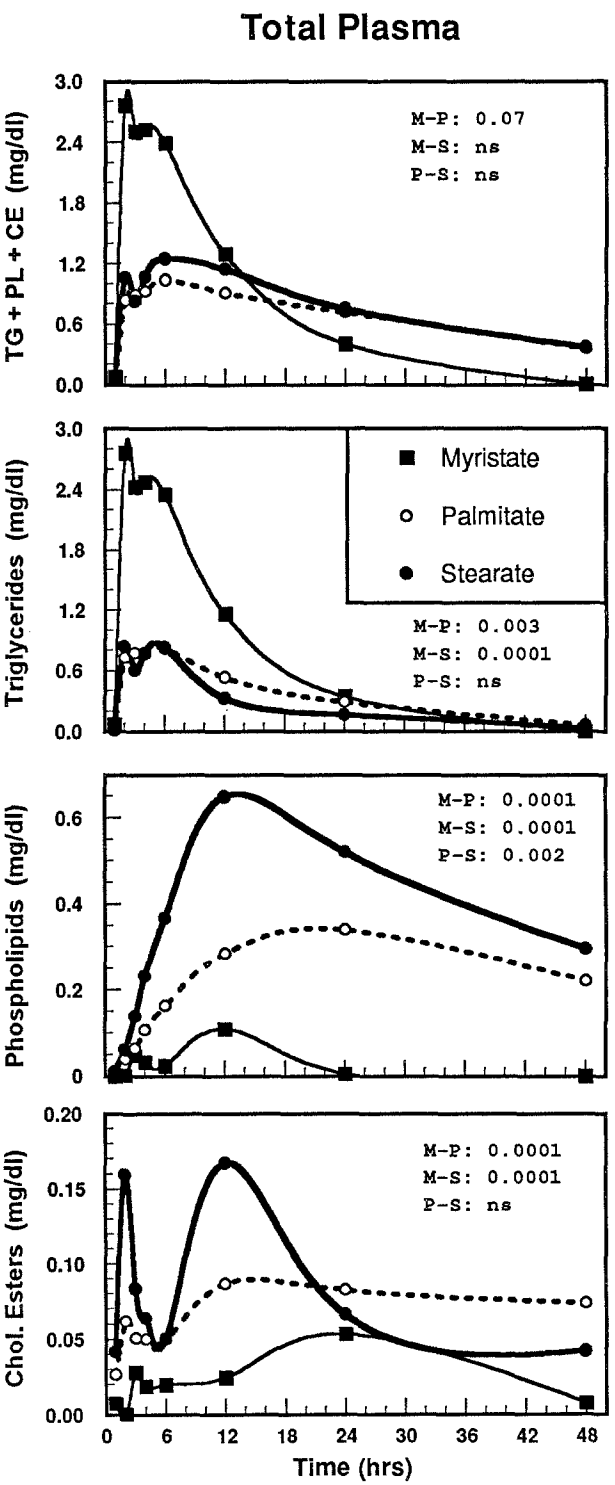

Fig 1. Stable-isotope–incorporated fatty acid concentrations in total esterified fatty acids, TGs, PLs, and CEs in total plasma after feeding the isotopes. *P* values for differences in AUCs are listed: M-P, myristate *v* palmitate; M-S, myristate *v* stearate; P-S, palmitate *v* stearate.

Table 3. Kinetics in Total Plasma: MRT (h)

	TG	PL	CE	Total Plasma
Myristate	8.5 ± 1.0	10.3 ± 1.3 (n = 4)	21.3 ± 1.3§	9.6 ± 1.1
Palmitate	12.6 ± 1.0*	21.8 ± 1.0†‡	18.5 ± 1.3‡	16.7 ± 1.1†
Stearate	11.6 ± 1.0*	19.8 ± 1.0†§	16.4 ± 1.3*§	16.7 ± 1.1†

NOTE. Results are the mean ± SEM; n = 5 unless otherwise noted.
*P < .05 *v* myristate.
†P < .005 *v* myristate.
‡P < .05 *v* TG.
§P < .005 *v* TG.

was the result of a low level of incorporation in combination with a substantially shorter MRT relative to both palmitate and stearate. A similar trend was observed for CE AUCs (stearate, palmitate, and myristate: 5.34 ± 2.23, 3.97 ± 2.23, 0.88 ± 2.23 and mg · h/dL, respectively). However, unlike TG and PL, myristate CE MRT was significantly longer than stearate (P = .009) and slightly longer than palmitate (Table 3). Therefore, the low concentration of myristate in CE was entirely due to low incorporation, because once myristate was incorporated into CE, it was removed more slowly than the other two fatty acids. In fact, myristate CE was retained in plasma longer than any of the fatty acids except palmitate in PL.

In summary, myristate was predominately incorporated into TG, but was more rapidly cleared from both TG and PL than palmitate and stearate. Thus, the relatively small total myristate AUC was due to its predominant incorporation into the most rapidly cleared compartment (TG) and its more rapid removal from both the TG and PL compartments. Conversely, when myristate was incorporated into CE, it was retained for a relatively long duration. Unequivocal confirmation of this hypothesis will only be obtained in studies using intravenous administration of the labeled fatty acids.

TGs

When TGs were fractionated into the various lipoproteins, it became apparent that the largest portion of each label was found in the VLDL + CM fraction (total TG-rich lipoproteins [TRLs]; Fig 2). As observed in the total plasma TG analysis, the AUC for each fatty acid was dependent on the dose administered. Although the myristate dose was only twofold greater than the palmitate and stearate doses, the myristate AUC in VLDL + CM (26.1 ± 2.0 mg · h/dL) was approximately threefold higher than both the palmitate (10.6 ± 2.0 mg · h/dL, P = .0002), and stearate (8.25 ± 2.00 mg · h/dL, P = .0001) AUCs. As seen with the total plasma TG, the myristate MRT in VLDL + CM TG (9.05 ± 0.69 hours) was shorter than the palmitate MRT (12.7 ± 0.7 hours, P = .005) or stearate MRT (10.7 ± 0.7 hours, P = nS), indicating that myristate was cleared up to 30% faster from this compartment than the other two fatty acids. Thus, the larger myristate AUC in VLDL + CM TG was the result of the higher dose administered and a greater incorporation of myristate into the TG compartment. The palmitate and stearate peak concentrations in VLDL + CM TGs were almost identical. However, the palmitate AUC was larger than the stearate AUC (P = .04; Fig 2) because of a longer palmitate MRT (P = .05) in this compartment (Fig 3).

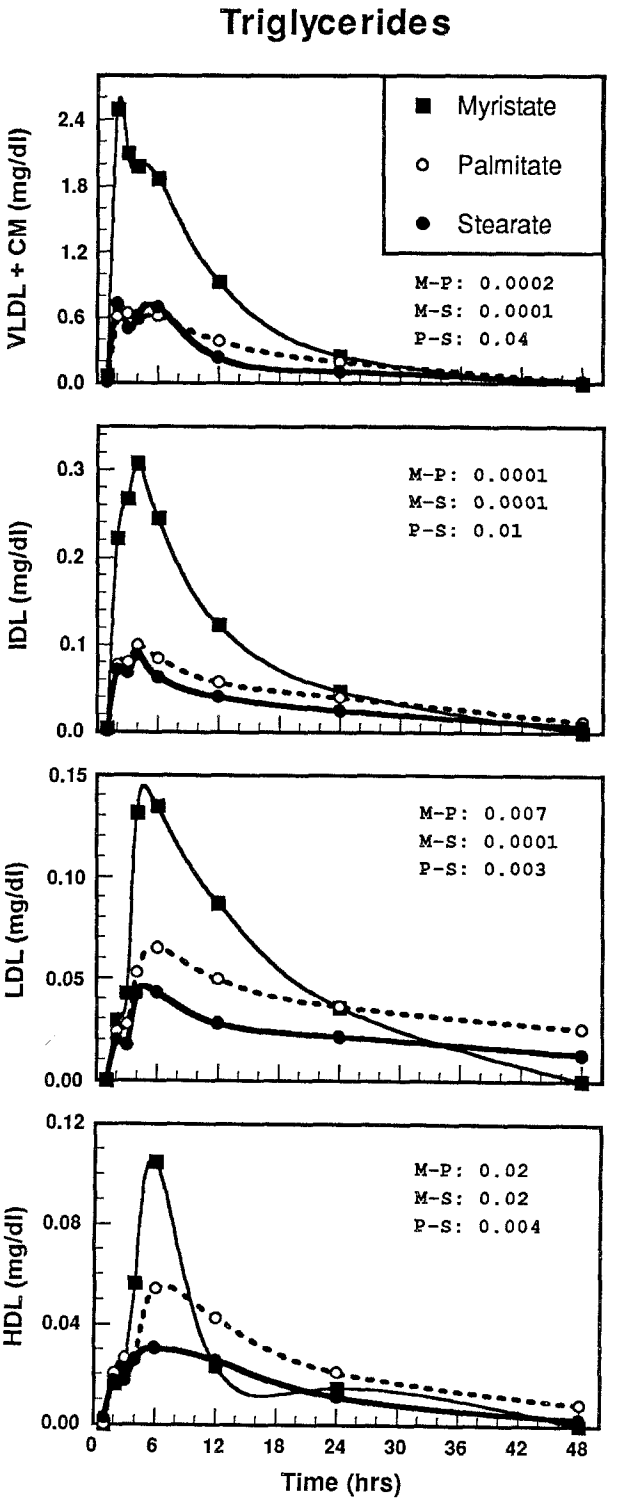

Fig 2. Stable-isotope–incorporated fatty acid concentrations in VLDL + CM, IDL, LDL, and HDL TG after feeding the isotopes. *P* values for differences in AUCs are listed: M-P, myristate *v* palmitate; M-S, myristate *v* stearate; P-S, palmitate *v* stearate.

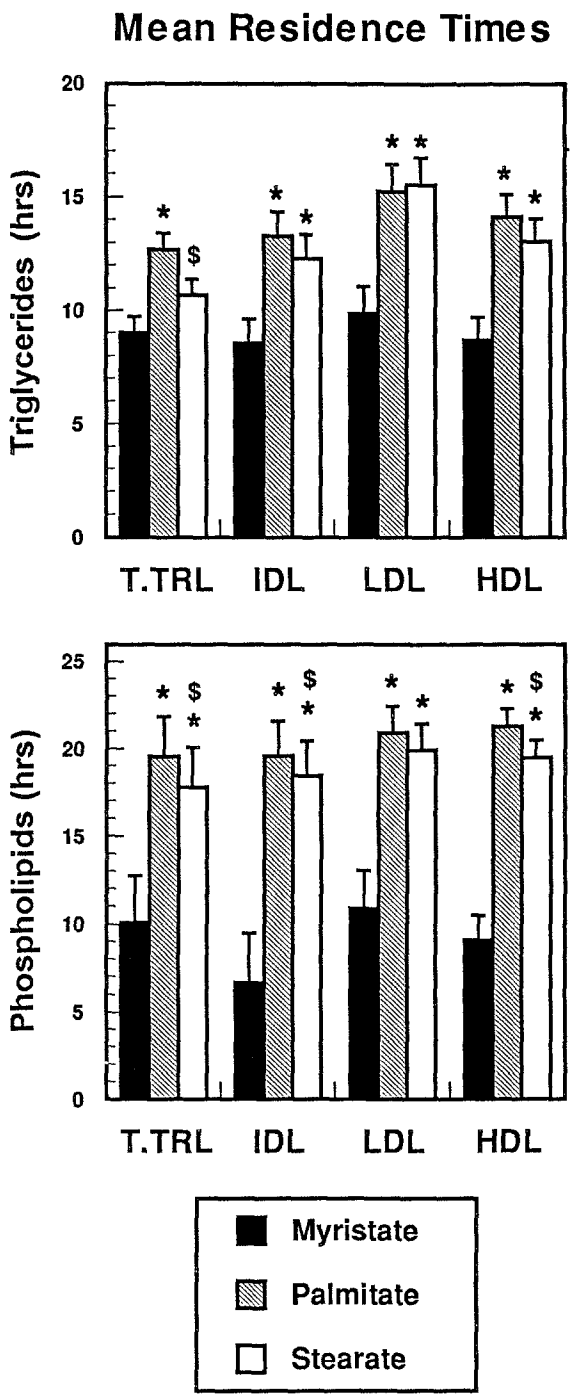

Fig 3. MRTs (mean ± SEM) for each stable-isotope–incorporated fatty acid in TG and PL of total TRL (T.TRL), IDL, LDL, and HDL. MRT for myristate in PL was calculated from only 3 individuals, because the other 2 subjects had no detectable stable-isotope–incorporated myristate in PL. *$P < .05$ *v* myristate; \$$P < .05$ *v* palmitate.

In the other lipoproteins, the peak TG concentrations of myristate and stearate decreased progressively (IDL to LDL to HDL) relative to those of palmitate (Fig 2). Similarly, the AUC of myristate became progressively smaller relative to palmitate until HDL actually had a larger palmitate AUC (0.94 ± 0.10 *v* 1.06 ± 0.10 mg · h/dL, $P = .02$). The substantially shorter MRT of myristate relative to palmitate in all three lipoproteins (Fig 3) was clearly a major factor in this reversal of AUCs, because even though the relative influxes of myristate became progressively smaller, the myristate peaks remained higher than the palmitate peaks in all lipoproteins, even HDL. The progressive separation of palmitate and stearate AUCs in IDL, LDL, and HDL was primarily caused by progressively reduced influxes of stearate relative to palmitate, because the MRTs of palmitate and stearate were similar in all three lipoproteins (Fig 3).

The enrichment of each stable-isotope–incorporated fatty acid in total TRL (VLDL + CM) and IDL TG was similar at all times, and was maximal at 6 hours (Fig 4). However, the enrichment of palmitate (and possibly stearate) in large TRL was greater than either the total TRL or IDL. LDL enrichment of each stable-isotope–incorporated fatty acid was also maximal at 6 hours, and equilibrated with VLDL + CM and IDL by 12 hours. However, HDL palmitate and stearate enrichment reached their peaks later than the other lipoproteins, and their peak enrichments were similar to VLDL + CM. HDL myristate, on the other hand, peaked at 6 hours and then decreased, so it never equilibrated with the other lipoproteins.

PLs

The isotope-labeled fatty acid concentrations in lipoprotein PLs followed patterns similar to the total plasma PLs (Fig 5). The highest concentrations were seen in HDL and the lowest in IDL. Stearate AUCs were significantly greater than the palmitate AUC in IDL ($P = .009$), LDL ($P = .023$), and HDL ($P = .073$), despite shorter MRTs (Fig 3), indicating that stearate was preferentially incorporated into the phospholipids of these lipoproteins. Myristate AUCs were smaller than the palmitate AUC in IDL ($P = .0005$), LDL ($P = .0001$), and HDL ($P = .0001$), because of both reduced influx and shorter MRTs (Fig 3). These data support the hypothesis that incorporation of fatty acids into PLs is chain-length–dependent. On the other hand, myristate may be incorporated so readily into TGs that little is left over for PL synthesis.

Peak enrichments occurred between 12 and 24 hours (Fig 4), with equilibration of palmitate and stearate occurring by 24 hours. Large TRL PLs were highly enriched very early (1 hour) with all three stable-isotope–incorporated fatty acids. There were secondary peaks at 3 (myristate), 6 (palmitate), and 12 (stearate) hours in this subfraction. Similar to the TGs, the relative enrichment of each stable-isotope–incorporated fatty acid was as follows: large TRL > total TRL, IDL > LDL, HDL.

CEs

The stable-isotope–incorporated fatty acid metabolism in CEs was much more difficult to analyze because of their rapid transit through these compartments. Individual patients would have relatively large amounts of one or more

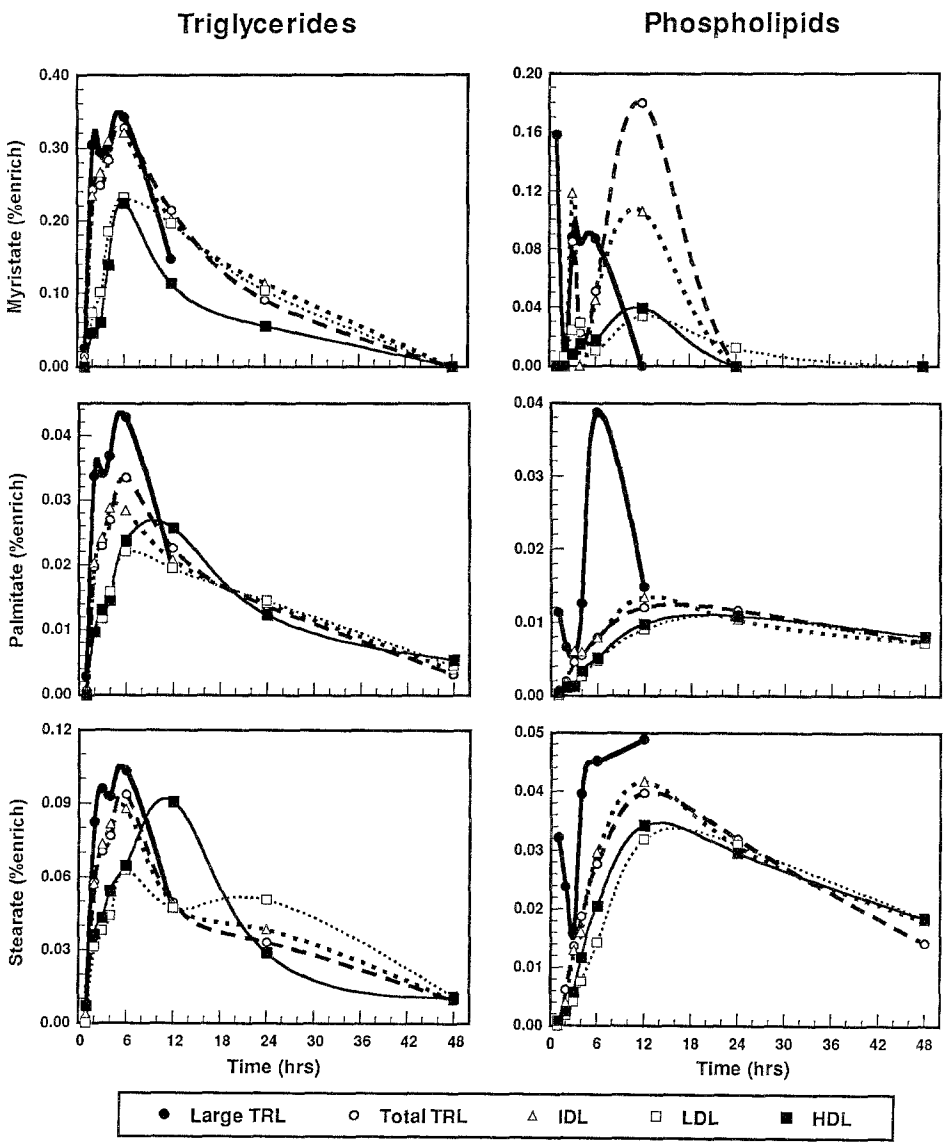

Fig 4. Stable-isotope–incorporated fatty acid enrichments (isotope concentration divided by the total fatty acid concentration in the specific lipoprotein lipid) in TG and PL of large TRL (VLDL + CM), total TRL (T.TRL), IDL, LDL, and HDL.

stable-isotope–incorporated fatty acids at one time point and nondetectable levels within 1 to 2 hours. This pattern was not believed to be an artifact, because all of the subjects demonstrated similar patterns. There was substantial incorporation of all three isotope-labeled fatty acids into all lipoproteins within 3 hours of feeding, with relative concentrations of stearate greater than palmitate greater than myristate (Fig 6). This CE labeling was seen before any significant PL labeling, except in large TRL. There were secondary peaks of CE labeling of each lipoprotein between 12 and 48 hours. The AUCs for myristate in all subfractions were less than those for palmitate and stearate ($P < .05$), except for VLDL + CM palmitate.

Elongation and Desaturation

In only one patient were we able to identify oleate derived from labeled stearate. This labeled oleate was seen in VLDL TG in the third hour. We found no evidence of elongated labeled fatty acids in the lipoprotein lipids, even though our analytical technique would have been able to detect these fatty acids if there had been a sufficient quantity present.

DISCUSSION

These studies demonstrated substantial differences in the way saturated fatty acids were metabolized in these normolipidemic men. Initially, myristate was incorporated primarily into the TGs of VLDL + CM and IDL, which equilibrated rapidly with LDL and HDL (Fig 4). This myristate TG was cleared quickly (relative to the other fatty acids) from all lipoproteins (Figs 2 and 3). There was only sporadic and minimal assimilation of stable-isotope–incorporated myristate into either PLs or CEs. These data are consistent with previous observations in rats that hepatic esterification of myristate is limited.[23] It is of interest that when myristate was identified in PL it had a relatively short MRT similar to its MRT in TG, but it had a

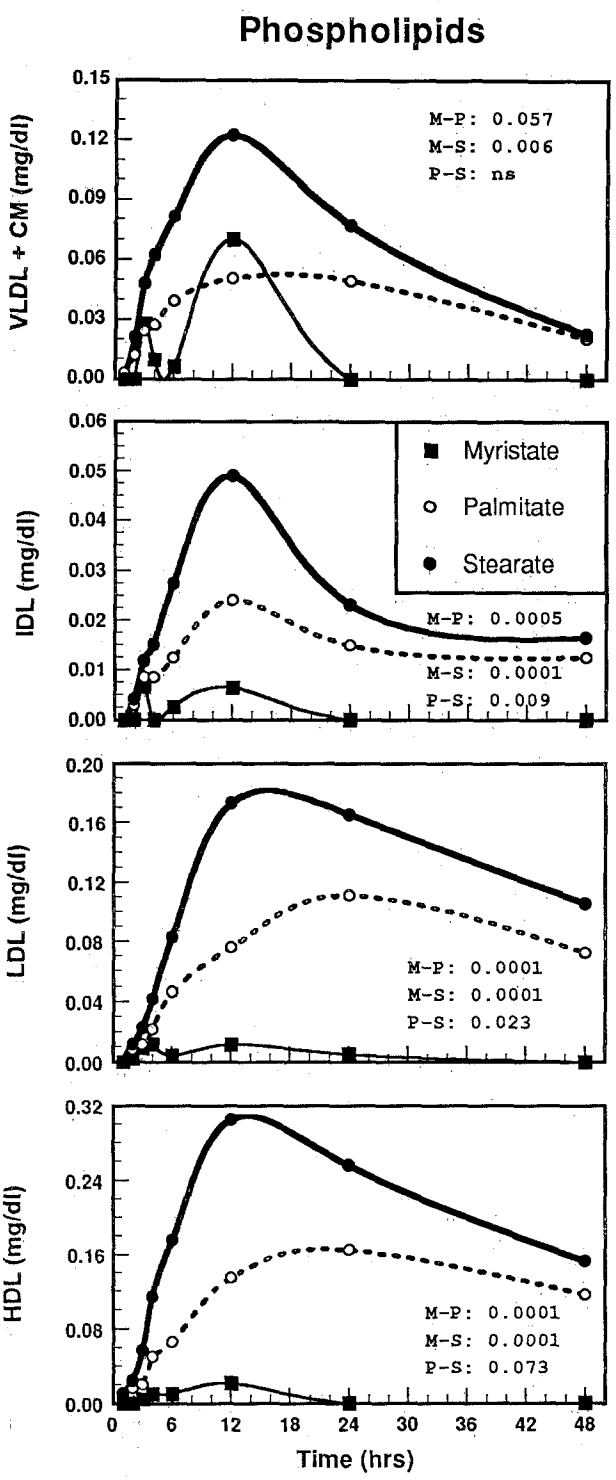

Fig 5. Stable-isotope–incorporated fatty acid concentrations in VLDL + CM, IDL, LDL, and HDL PL after feeding the isotopes. *P* values for differences in AUCs are listed: M-P, myristate *v* palmitate; M-S, myristate *v* stearate; P-S, palmitate *v* stearate.

relatively long MRT in CE. Myristate is known to be one of the most hypercholesterolemic of the saturated fatty acids.[3,4,6,8] Because we found little myristate in LDL and because myristate was so rapidly cleared from the plasma, it would seem likely that myristate exerts its effects by altering VLDL and LDL metabolism in the liver. Several potential mechanisms for its hypercholesterolemic effect are apparent. First, myristate may directly alter LDL binding to its receptor by altering the plasma membrane composition. Second, myristate incorporation into secreted VLDL may result in an increased cholesterol to TG ratio in the particle. Finally, myristate may alter the free cholesterol–CE balance (by influencing ACAT activity) toward increasing the regulatory pool of free cholesterol. This modification would lead to a downregulation of LDL receptors[31] and an increase in plasma LDL concentrations. Further studies will be required to elucidate the actual mechanisms responsible for these events.

Stearate, compared with myristate, appears to represent an opposite extreme of saturated fatty acid metabolism. A substantial portion of stearate was incorporated into PLs (Fig 5), and it had the highest percentage incorporation into CEs (Fig 6). The differences we observed between stearate and palmitate were similar to those reported by Emken et al.[17] However, they did not study myristate, nor did they separate the various lipoproteins. Stearate concentrations in all lipoproteins were higher than palmitate concentrations despite stearate's shorter MRT (Fig 5). The enrichment of large VLDL + CM with stearate was biphasic, with a high enrichment only 1 hour after the isotope load, followed by a subsequent decrease and a secondary slightly higher peak at 6 hours (Fig 4). These findings are consistent with the model in which PLs derived from the gastrointestinal tract are labeled rapidly, cleared from the plasma, and then replaced by a second influx of PLs in large TRLs secreted from the liver.

Stable-isotope–incorporated stearate was also seen early in the CEs of all the lipoproteins, well before it was observed in HDL PLs (Fig 6). It is therefore likely that these CEs were derived from cholesterol esterification in the intestine (ACAT) rather than from the activity of plasma lecithin-cholesterol acyltransferase. Stable-isotope–incorporated palmitate was also seen at the same times, but always at lower concentrations, suggesting a transferase preference for stearate. CE metabolism of all the fatty acids was difficult to characterize, because individual patients would have highly labeled fractions 1 hour and undetectable label the next. Clearly, these fatty acids were moving rapidly through these compartments, and more frequent sampling will be required to adequately analyze these pathways.

We were able to detect stable-isotope–incorporated oleate in only one patient at one time point. This result is not surprising, because Emken et al[17] observed that less than 10% of stearate is desaturated in normolipidemic men. They used a mass spectroscopic technique (chemical ionization) that could detect much lower concentrations of labeled unsaturated fatty acids. Conversion of stearate to oleate may contribute to the neutral effect of stearate on LDL metabolism, but it is unlikely that this pathway is the major mechanism, because greater than 90% of the stearate is apparently not desaturated.

The influx of palmitate into PL (Fig 5) and CE (Fig 6) appeared to be intermediate between that of myristate and stearate. However, the palmitate enrichment of large

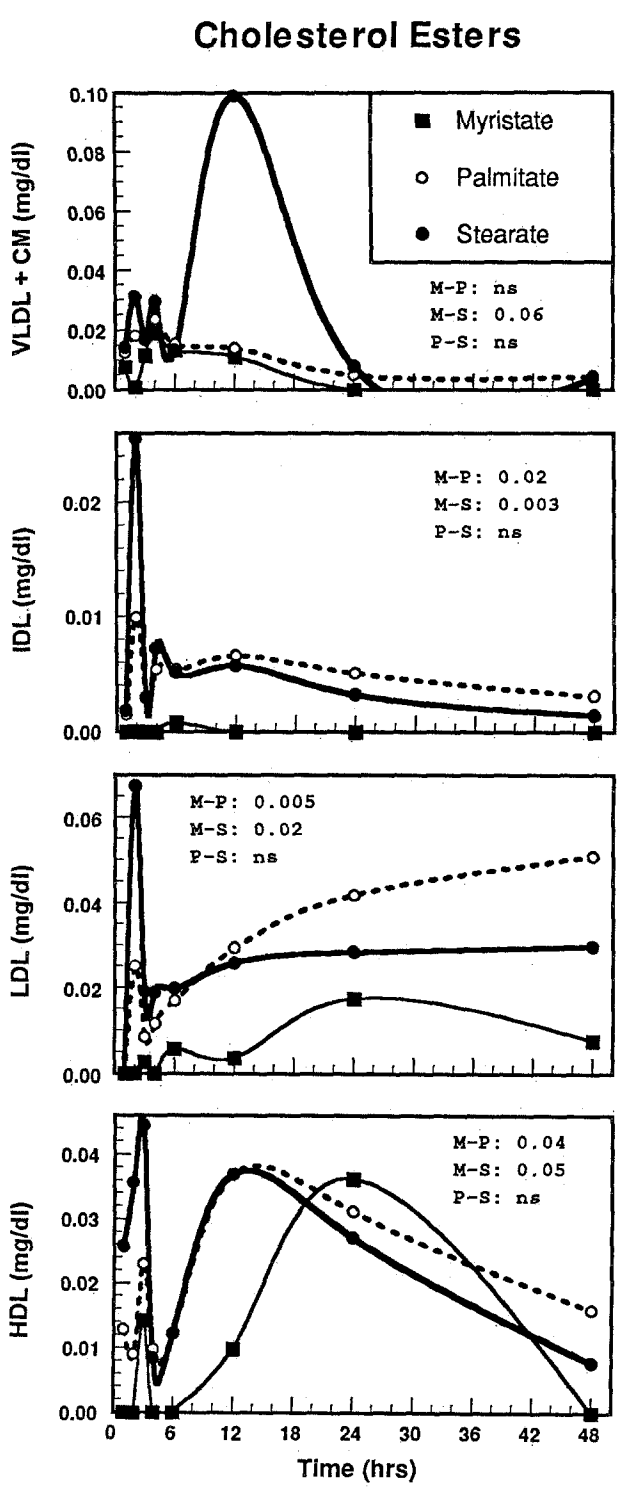

Fig 6. Stable-isotope–incorporated fatty acid concentrations in VLDL + CM, IDL, LDL, and HDL CE after feeding the isotopes. *P* values for differences in AUCs are listed: M-P, myristate *v* palmitate; M-S, myristate *v* stearate; P-S, palmitate *v* stearate.

VLDL + CM PLs during the secondary peak (between 4 and 12 hours) was much higher than the enrichment seen in the other lipoproteins (Fig 4). This relatively high enrichment of large VLDL + CM PL was not seen with either myristate or stearate, and suggests a preferential incorporation of palmitate into the PLs of large hepatic particles with subsequent direct clearance from this compartment.

Palmitate generally had the longest MRT in both TGs and PLs in most of the lipoproteins, and even though this difference was not large compared with stearate, it was significant in many instances (Fig 3). Three possible explanations for this observation are (1) the other fatty acids were the preferred substrates for lipoprotein lipase and hepatic lipase, (2) palmitate was incorporated into a relatively protected position in TGs and PLs, or (3) particles enriched in palmitate were cleared more slowly from the plasma. Palmitate-containing TG also appeared to be the preferred moiety for transfer from VLDL + CM to HDL, because the peak concentration and AUC for palmitate in HDL were greater than those for stearate (Fig 2). These results were observed despite similar concentrations of palmitate and stearate in VLDL + CM TGs. Similarly, variances in the peak concentrations of myristate and palmitate were substantially smaller in HDL than in VLDL + CM, suggesting a more rapid transfer of palmitate than myristate.

A significant technical problem with our studies was that we used a different isotope for myristate (deuterium) than for palmitate and stearate ($^{13}C_{16}$ and $^{13}C_{18}$, respectively). This difference dictated that we monitor a different, less abundant fragment ion for the myristate (*m/e* 202 or 203). To compensate for the lower detection threshold for this fragment ion, we fed larger amounts of myristate. Since these studies were completed, we have obtained $^{13}C_3$-myristate, and have studied several subjects after feeding similar amounts of the three isotope-incorporated fatty acids and monitoring the same fragment ions of all three fatty acids. The results from these more recent subjects are similar to the data reported here.

The tedious process of formally modeling these data is under way. This analysis should further our understanding of the metabolism of these specific saturated fatty acids. More study is required in human and animal models to elucidate the exact mechanisms by which these fatty acids impact lipoprotein metabolism, especially in the intestine and liver. However, it is already clear that myristate, palmitate, and stearate are metabolized in unique ways. Therefore, it does not seem appropriate to continue to regard all saturated fatty acids as metabolically similar in clinical studies. Rather, it is important that we more clearly define the specific metabolic pathways of each fatty acid to understand the mechanisms by which they alter plasma lipoprotein concentration and composition and influence atherogenesis.

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