

Distribution of conjugated linoleic acid and metabolites in different lipid fractions in the rat liver

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Abstract Conjugated linoleic acid (CLA) is known to provide certain health benefits in experimental animal models. The major CLA isomer in food is *c*9,*t*11-CLA. A primary objective of this study was to investigate the uptake of *c*9,*t*11-CLA and its downstream metabolites into various lipid fractions in the liver of rats fed either a high or low CLA diet (containing 0.1 or 0.8 g CLA/100 g diet, respectively). As expected, the levels of all conjugated diene (CD) fatty acids (CD 18:2 + CD 18:3 + CD 20:3 + CD 20:4) were elevated about 8-fold in the high CLA diet group. However, there was no change in the distribution of CLA and CLA metabolites into various lipid fractions due to CLA intake. Unlike linoleic acid or γ -linolenic acid, which were distributed mainly in phospholipids, CD 18:2, CD 18:3, and CD 20:3 were incorporated primarily in neutral lipid. Furthermore, the incorporation of all nonconjugated unsaturated fatty acids was not perturbed by CLA. Regardless of the level of CLA in the diet, CD 20:4 was predominantly enriched in phosphatidylserine and phosphatidylinositol. In contrast, arachidonic acid was primarily enriched in phosphatidylcholine and less so in phosphatidylethanolamine. The above findings may have potential implication regarding the role of CLA in modulating eicosanoid metabolism. —Banni, S., G. Carta, E. Angioni, E. Murru, P. Scanu, M. P. Melis, D. E. Bauman, S. M. Fischer, and C. Ip. Distribution of conjugated linoleic acid and metabolites in different lipid fractions in the rat liver. *J. Lipid Res.* 2001. 42: 1056–1061.

Supplementary key words conjugated linoleic acid • CLA metabolism • neutral lipid CLA • phospholipid CLA

There has been an explosion of interest in conjugated linoleic acid (CLA) in recent years that was ignited by the spectrum of biological activities attributed to this minor fatty acid from meat and dairy products (1). In experimental models, CLA has a number of beneficial effects including protection against cancer and atherosclerosis, stimulation of certain immune functions, reduction in body fat, and normalization of impaired glucose tolerance in diabetes. Little information is available, however, on its

mechanism of action. A number of reports have provided either direct or indirect evidence suggesting that CLA may interfere with prostaglandin production through a decrease in the supply of arachidonic acid precursor (2–5). Recent molecular assays have also indicated that CLA is a high affinity ligand of peroxisome proliferator activated receptor (PPAR), which is a family of transcription factors known to affect gene expression (6, 7).

The term CLA actually refers to a collection of positional and geometric isomers of octadecadienoic acid with conjugated double bonds. With the exception of the more recent studies in which individual CLA isomers were evaluated, most of the previous work with CLA was done with a commercial or custom-synthesized preparation containing a mixture of *c*9,*t*11- and *t*10,*c*12-isomers as free fatty acids, although CLA in food is predominantly the *c*9,*t*11-isomer present in triacylglycerols (8). We have reported the use of a natural source butter fat *c*9,*t*11-CLA in our research on CLA modulation of mammary gland development and mammary carcinogenesis in the rat (9). Regardless of which signaling pathway(s) might be involved in mediating the effect of CLA, the intracellular disposition of *c*9,*t*11-CLA and its metabolites is of primary interest because it could provide valuable clues to potential biochemical target sites.

The conversion of CLA (also designated as CD 18:2; the CD prefix indicates the presence of a conjugated diene structure in the fatty acid) to CD 18:3, CD 20:3, and CD 20:4 by desaturase and elongase enzymes has been described previously (10, 11). At the most fundamental level, there is a lack of an understanding with respect to the uptake of CLA and CLA metabolites into neutral lipid

Abbreviations: CD, conjugated diene; CLA, conjugated linoleic acid; NL, neutral lipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PPAR, peroxisome proliferator activated receptor.

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and various phospholipids. One objective of the present study was to address this gap of knowledge. A second objective was to compare the incorporation profiles of conjugated diene fatty acids (e.g., CD 18:2, CD 18:3, CD 20:3, and CD 20:4) with that of the other unsaturated fatty acids (e.g., 16:1, 18:2, 18:3, 20:3, and 20:4) in different classes of cellular lipids. The purpose was to identify any distinctive patterns that may be suggestive of potential metabolic perturbations. Our aims were to evaluate specifically whether CLA metabolites show a lipid distribution profile that is different from that of CLA itself, and whether a high CLA intake will affect the incorporation of arachidonic acid into phospholipids. The lipid analysis was carried out in liver samples collected from rats fed either a control butter fat diet (low CLA intake) or a CLA-enriched butter fat diet (high CLA intake). These were the same diets used in a previous study (9). Thus CLA was provided as a natural component of food with both the CLA isomer (predominantly *c*9,*t*11-isomer) and lipid class (triglyceride) being characteristic of the dietary supply of CLA for humans. We focused on the liver to investigate the distribution profile of CLA and CLA metabolites because of the central role this organ plays in lipid metabolism, and because of its content of neutral lipids and phospholipids.

MATERIALS AND METHODS

Female Sprague-Dawley rats (Charles River Breeding Laboratories, Raleigh, NC) were fed a diet containing 20% by weight of control butter fat or CLA-enriched butter fat for 1 month (9). The remaining ingredients in the diet consisted of 23.5% casein, 44.8% dextrose, 4.1% AIN-76 mineral mix, 1.2% AIN-76A vitamin mix, 5.9% alphacel, 0.3% methionine, and 0.2% choline bitartrate. Additional details on the diets were described in our previous publication (9). To produce the CLA-enriched butter, milk fat was obtained from cows that had been fed a low-fiber diet supplemented with sunflower oil (12). Control butter and high CLA butter contained 5.1 and 41 mg CLA/g of fatty acids, respectively, and over 90% was the *c*9,*t*11-isomer. The fatty acid composition of these two types of butter is shown in **Table 1**. Rats fed the control butter fat diet or high CLA butter fat diet received 0.1 or 0.8 g CLA per 100 g of diet, respectively (9).

After excision, liver samples were stored at -80°C until they were ready for analysis. Total lipid was extracted by the method of Folch et al. (13). Separation of major lipid classes was carried out according to the method described by Pietsch and Lorenz (14) with slight modifications. Briefly, samples containing about 5 mg of total lipid in 0.5 ml of chloroform were placed on an aminopropyl bond large reservoir capacity column (Chrompack International BV, Middleburg, The Netherlands) for solid phase extraction. Each lipid class was eluted using different solvents: 5 ml of chloroform-isopropanol (2:1, v/v) for neutral lipid (NL); 5.5 ml of acetic acid-diethyl ether (2:98, v/v) for free fatty acid (FFA); 20 ml of acetonitrile-n-propanol (2:1, v/v) for phosphatidylcholine (PC); 7 ml of methanol for phosphatidylethanolamine (PE); 7.4 ml of isopropanol-3N HCl in methanol (4:1, v/v) for phosphatidylserine (PS); and 10 ml of methanol-3N HCl in methanol (9:1, v/v) for phosphatidylinositol (PI).

Each fraction was collected and the solvent evaporated. With the exception of the FFA fraction, the other lipid fractions were mildly saponified using a procedure described by Banni et al. (15) in order to obtain FFA for HPLC analysis. Separation of CLA and its metabolites was carried out with a Hewlett-Packard

TABLE 1. Fatty acid composition of control butter or high CLA butter

Fatty acid	Control Butter	High CLA Butter
% of total fatty acids		
Butyric	4:0	4.2
Caproic	6:0	2.5
Caprylic	8:0	1.5
Capric	10:0	3.5
Lauric	12:0	4.0
Myristic	14:0	12.0
Myristoleic	14:1	1.2
Pentadecylic	15:0	1.1
Palmitic	16:0	28.6
Palmitoleic	16:1	1.4
Margaric	17:0	0.5
Stearic	18:0	9.8
Oleic	<i>c</i> 9-18:1	18.5
trans-Octadecenoic ^a	trans-18:1	3.8
Linoleic	<i>c</i> 9, <i>c</i> 12-18:2	2.8
Conjugated linoleic ^b	CD-18:2	0.5
γ -Linolenic	18:3	0.4
Others		3.7
Total	100.0	100.0

^a This represents total *trans*-18:1, of which vaccenic acid (*t*11-18:1) accounted for \sim 24.8 and 48.7% in control butter and high CLA butter, respectively.

^b CD denotes conjugated diene.

1050 HPLC system (Hewlett-Packard, Palo Alto, CA) equipped with a diode array detector 1040M. A C-18 inertsil 5 ODS-2 Chrompack column (Chrompack International BV, Middleburg, The Netherlands), 5 μm particle size, 150×4.6 mm, was used with a mobile phase of $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{CH}_3\text{COOH}$ (70:30:0.12, v/v/v) at a flow rate of 1.5 ml/min (10). Nonconjugated diene unsaturated fatty acids were detected at 200 nm and conjugated diene unsaturated fatty acids detected at 234 nm. Spectra (195–315 nm) of the eluate were obtained every 1.28 s and were electronically stored. Second-derivative UV spectra of the conjugated diene fatty acids were generated using the Phoenix 3D HP Chemstation software (Hewlett-Packard, Palo Alto, CA). These spectra were taken to confirm the identification of the HPLC peaks. Details of the methodology regarding the characterization of conjugated diene unsaturated fatty acids in both reference and biological samples have been published (10, 16).

INSTAT software (GraphPad Software, San Diego, CA) was used to calculate the mean and standard deviation of tissue fatty acid measurements. One way ANOVA was applied to evaluate statistical differences between the two groups, as described in a previous paper (5). This program is based on the Bonferroni method, which requires a higher threshold for statistical significance.

RESULTS

Liver CLA analysis was focused exclusively on the *c*9,*t*11-isomer because this was the predominant form in the butter fat-containing diet. The concentrations of both conjugated and nonconjugated unsaturated fatty acids in the liver of rats fed either the control butter fat diet or high CLA butter fat diet are shown in **Table 2**. Clearly, in terms of total amount, the nonconjugated unsaturated fatty acids (18:2 + 18:3 + 20:4) were present at much greater

TABLE 2. Unsaturated fatty acid concentrations in the liver of rats fed either the control butter fat diet or the high CLA butter fat diet

Fatty Acid ^a	Control Butter Group ^b	High CLA Butter Group ^b
	μg/mg lipid	
18:1	191 ± 26.3	152.7 ± 31.7
18:2	39.5 ± 11.5	45.3 ± 9.1
18:3	0.6 ± 0.1	0.7 ± 0.1
20:4	106.5 ± 25.8	123.4 ± 9.9
CD 18:2	2.5 ± 0.8	15.7 ± 2.6 ^c
CD 18:3	0.1 ± 0.03	0.7 ± 0.1 ^c
CD 20:3	0.4 ± 0.1	3.9 ± 0.3 ^c
CD 20:4	0.1 ± 0.03	1.0 ± 0.3 ^c

^a CD denotes conjugated diene.

^b Mean ± SD (n = 6).

^c P < 0.05 compared to the corresponding value in the control group.

levels than the conjugated diene fatty acids (CD 18:2 + CD 18:3 + CD 20:3 + CD 20:4). There was no difference in the concentrations of 18:1, 18:2, 18:3, or 20:4 in the liver between the two dietary groups. As expected, the concentrations of CD 18:2, CD 18:3, CD 20:3, and CD 20:4 were significantly elevated (P < 0.05) in rats fed the high CLA butter fat diet compared to those fed the control butter fat diet. In sum, the levels of all conjugated fatty acids (CD 18:2 + CD

18:3 + CD 20:3 + CD 20:4) were about 8-fold higher in the former group. It is interesting to note that although the high CLA butter contained similar quantities of linoleic acid and CLA (Table 1), the concentration of linoleic acid in the liver was about 3× higher than that of CLA.

In order to better understand the metabolic disposition of conjugated diene fatty acids in general, we carried out a comprehensive analysis of the incorporation profiles of CLA and CLA desaturation and elongation metabolites into different lipid fractions of the liver. The data for the control butter fat group (panels A to D) and the high CLA butter fat group (panels E to H) are presented in a pie-chart form in Fig. 1. Each pie-chart consists of a color-coded composite of NL, FFA, PC, PE, PS, and PI. The easiest way to look for concordances or deviations in the pattern is to size up the various color segments. By comparing the top four panels with the bottom four panels, it can be seen that the color profile of each pie chart was very similar between the two dietary groups. This finding suggests that a high CLA intake has little effect on the distribution of CLA and CLA metabolites into various lipid fractions. There were some very minor differences in the distribution of CD 20:3 between the two groups, as indicated by an asterisk beside the PC and PE fractions. Suffice it to note that CD 20:3 was present in very low levels in the liver (Table 2). Thus a

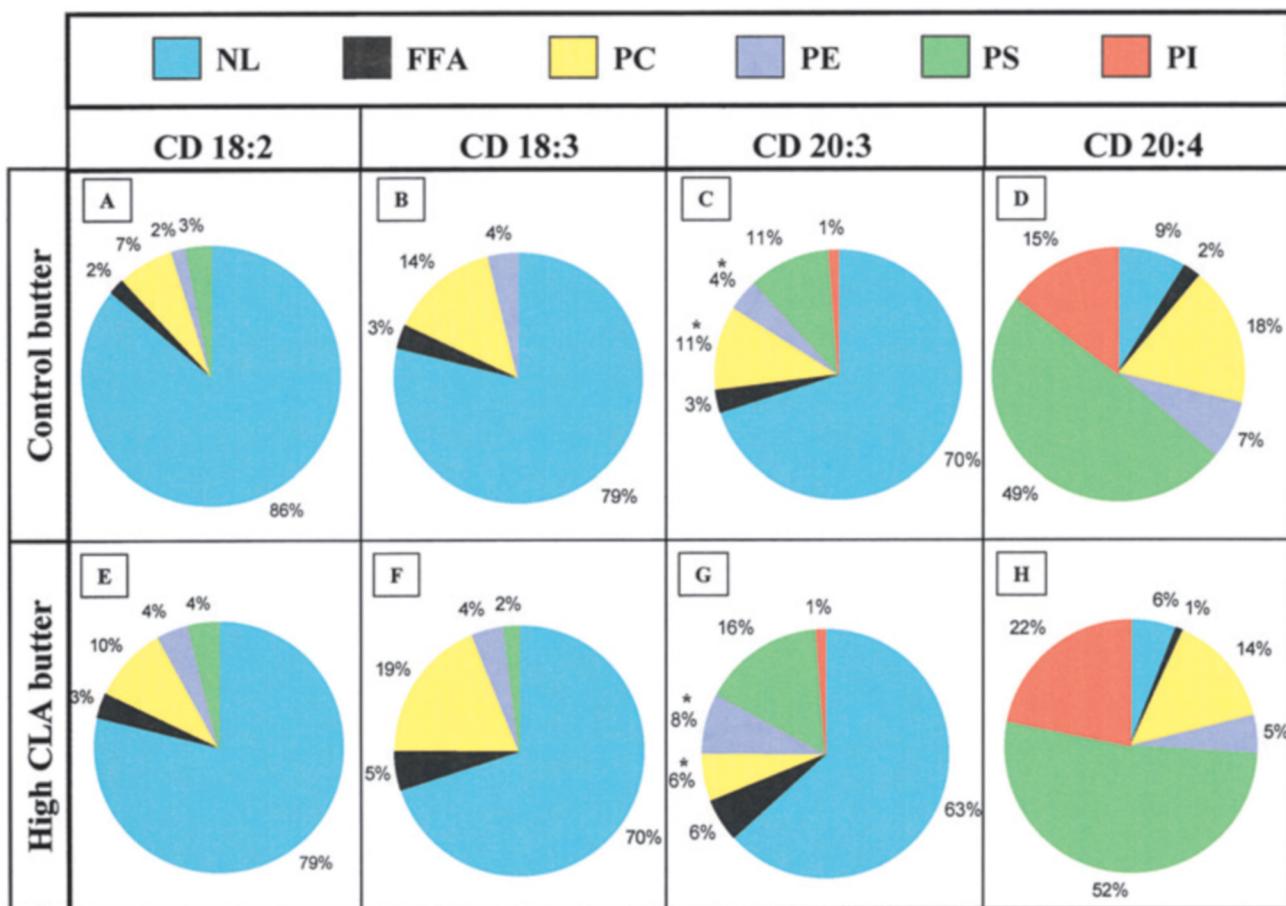


Fig. 1. Incorporation of CLA/CLA metabolites into different lipid fractions in the liver of rats fed either the control butter fat diet or high CLA butter fat diet. The pooled SD is about 20% of the mean. An asterisk indicates significant differences between the two groups (P < 0.05).

measurement of CD 20:3 in separate lipid fractions would tend to erode the precision of the analysis. These small differences are likely to be coincidental.

Even though a high CLA intake did not affect the accumulation of the other nonconjugated unsaturated fatty acids (Table 1), we also examined their incorporation into various lipid fractions in case there might be subtle changes in the distribution. Fig. 2 depicts the lipid profiles of 18:1 (oleic acid), 18:2 (linoleic acid), 18:3 (γ -linolenic acid), and 20:4 (arachidonic acid) in the control butter fat group (panels A to D) and high CLA butter fat group (panels E to H). A high CLA intake did not appear to perturb the incorporation of 18:1, 18:2, and 18:3 into either neutral lipid or phospholipids. Arachidonic acid was the only fatty acid that showed a slightly different pattern between the two groups. On a proportionate basis, less 20:4 was found in the PS fraction (green) with a high CLA intake than with a low CLA intake (5% vs. 16%, $P < 0.05$).

The data from the high CLA butter fat group are discussed in greater detail in order to unearth additional information regarding the behavior of CLA and its metabolites. Several characteristic features become immediately apparent by comparing the data shown in Fig. 1 and Fig. 2. First, with the exception of CD 20:4, the other CD-fatty acids (i.e., CD 18:2, CD 18:3, and CD 20:3) tend to be in-

corporated preferentially into NL (blue, 63% to 79%). The amount of each fatty acid present in all phospholipids constitutes a much smaller portion of the total, ranging from 18% to 31%. Second, in contrast to CD 18:2 and CD 18:3, linoleic acid (18:2) and γ -linolenic acid (18:3) have a very different pattern. Most of the latter two fatty acids are incorporated into phospholipids, a total of 81% for 18:2 and 76% for 18:3; in particular, a substantial portion is present in the PC fraction (yellow). Third, the profiles of CD 18:2 and CD 18:3 resemble closely the profile of 18:1. Thus the CD 18-carbon fatty acids behave like oleic acid and not like linoleic acid or γ -linolenic acid. Fourth, CD 20:4 and 20:4 show vastly different patterns of incorporation despite their similarity in structure. The two major phospholipid fractions containing CD 20:4 are PS (green) and PI (red), whereas 20:4 is found primarily in PC (yellow) and PE (violet). These differences are of special interest because 20:4 (arachidonic acid) serves as the precursor for the biosynthesis of eicosanoids.

DISCUSSION

By using a naturally produced CLA-enriched butter fat in our animal feeding experiment, we were able to investigate

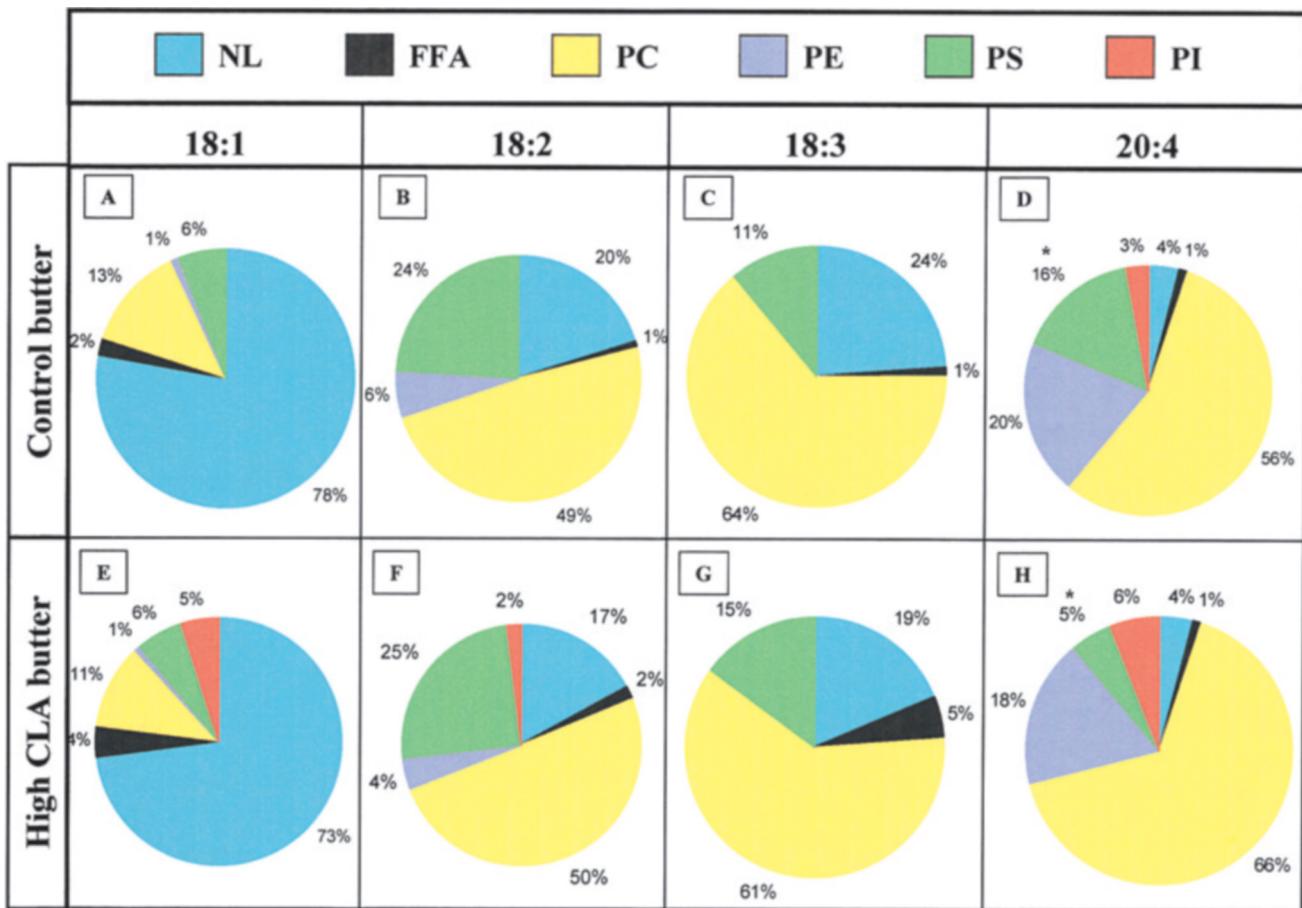


Fig. 2. Incorporation of nonconjugated unsaturated fatty acids into different lipid fractions in the liver of rats fed either the control butter fat diet or high CLA butter fat diet. An asterisk indicates significant differences between the two groups ($P < 0.05$).

CLA metabolism in a situation that mimics the intake of CLA in humans. Dairy foods are a good source of CLA which is present primarily as the *c*9,*t*11-isomer in triglyceride. This is the reason for limiting our analysis of CLA/CLA metabolite distribution to the *c*9,*t*11-CLA. The present study shows that a high CLA intake does not channel the additional CLA into a particular lipid class, nor does it perturb the incorporation of other unsaturated fatty acids in various lipid fractions. NL is the preferred lipid fraction for CLA in the rat liver. This distribution pattern is also true for two other CLA metabolites, viz CD 18:3 and CD 20:3.

Our data also demonstrate that CLA does not behave like linoleic acid. Instead it identifies more closely with oleic acid in terms of the lipid fractionation profile. The distinctive incorporation signatures associated with CLA and linoleic acid respectively may represent the outcome of one of two possibilities. Because tissue concentration of linoleic acid is higher than that of CLA, the more plentiful linoleic acid may tend to exclude CLA for incorporation into phospholipids and drive it into storage as a component of NL. Alternatively, there might be discriminating controls for linoleic acid and CLA uptake into phospholipids based on structural or biochemical determinants. It is possible that the NL pool of CLA and CLA metabolites may represent a relatively inert storage form, whereas the fast turnover phospholipid pool of CLA may be more important biochemically because of its ready accessibility as a signaling molecule. Unsaturated fatty acids in general are hydrolyzed from the sn2 position by various phospholipase A₂ enzymes in response to a spectrum of endogenous and exogenous stimuli. The incorporation of CLA into the sn2 position remains to be verified, and so does the ability of different phospholipases to release CLA from the various phospholipids (17, 18).

In contrast to CLA, over 90% of CD 20:4 is found in phospholipids, and only 6% in NL. The incorporation of CD 20:4 is thus likely to be influenced by dietary linoleic acid because CD 20:4 goes mainly into phospholipids. In fact, previously we failed to detect the presence of CD 20:4 when CLA was included in a diet rich in linoleic acid (5). Further studies will be needed to determine whether CD 20:4 is not produced under this condition, or if it is produced, if it is not being incorporated into phospholipids because it is readily metabolized to other products.

PI appears to be a major source of arachidonic acid (20:4) for eicosanoid biosynthesis (19, 20). According to the results shown in Fig. 1 and Fig. 2, about 15–22% of CD 20:4 is incorporated in PI, whereas only 3–6% of 20:4 is found in this fraction. In view of the fact that the concentration of 20:4 is more than 100× greater than that of CD 20:4 (Table 2), it is unlikely that the uptake of 20:4 into PI will be adversely affected by CD 20:4. Consequently, changes in eicosanoid production may not be due to the reduced bioavailability of phospholipid-20:4 following CLA treatment. CLA is known to modulate immune functions (21–23), atherogenesis (24), carcinogenesis (25, 26), and tumor promoter-mediated biochemical changes in keratinocytes (27). Although eicosanoids are believed to be intimately involved in all these events, addi-

tional studies will be needed to determine whether CLA is able to modulate eicosanoids in the target site of interest, and if so, the molecular mechanism by which CLA or CLA metabolite achieves these changes.^{fig}

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