Eicosapentaenoic Acid, but Not Oleic Acid, Stimulates β-Oxidation in Adipocytes

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ABSTRACT: The beneficial roles of dietary fish oil in lowering serum TAG levels in animals and humans have been attributed in part to the high content of two n-3 polyunsaturated very long-chain FA, EPA, and DHA. Recent studies show that EPA induces mitochondrial β-oxidation in hepatocytes, which might contribute to the systemic lipid-lowering effect. Whether EPA affects FA storage or oxidation in adipocytes is not clear. To investigate this possibility, 3T3-L1 adipocytes incubated with EPA (100 μM) for 24 h were assayed for β-oxidation, carnitine palmitoyl transferase 1 (CPT-1) activity, protein, and mRNA expression of CPT-1. For comparison, cells treated with oleic acid, octanoic acid, and clofibrate, a synthetic ligand for peroxisome proliferator-activated receptor α were also analyzed. Mitochondria were isolated by differential centrifugation, and the mitochondrial membrane acyl chain composition was measured by GLC. EPA increased the oxidation of endogenous FA but did not inhibit lipogenesis. Oleic acid and clofibrate did not affect FA oxidation or lipogenesis, whereas octanoic acid suppressed the oxidation of endogenous FA and inhibited lipogenesis. Increased β-oxidation by EPA was associated with increased CPT-1 activity but without changes in its mRNA and protein expression. EPA treatment increased the percentage of this FA in the mitochondrial membrane lipids. We suggest that EPA increased the activity of CPT-1 and β-oxidation in adipocytes by altering the structure or dynamics of the mitochondrial membranes.

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The beneficial roles of dietary fish oil in the human diet have been attributed to the high content of two n-3 polyunsaturated very long-chain FA, EPA, and DHA (1–4). Animal studies as well as *in vitro* cell studies have provided some insights into possible mechanisms for these effects (5–10). Both EPA and DHA activate peroxisome proliferator-activated receptor α (PPARα), the nuclear transcription factor that regulates the expression of key enzymes in the control of FA β-oxidation in mitochondria and peroxisomes (6). EPA has been shown to induce mitochondrial FA oxidation and contribute to lipid-lowering effects (7), whereas the effects of DHA are still debatable (7,11). In hepatocytes, EPA increases the expression of carnitine palmitoyl transferase 1 (CPT-1), which increases the flux of FA toward mitochondrial β-oxidation and reduces the substrates for lipid synthesis and lipoprotein secretion. In hepatocytes EPA directly reduces TAG synthesis by direct inhibition of DAG acyltransferase activity (6). EPA also inhibits apolipoprotein secretion in both enterocytes and hepatocytes (12,13). Any one or a combination of these mechanisms could contribute to the systemic lipid-lowering effects of fish oil or EPA dietary supplements.

Several previous reports have shown that fish oil reduces adipocyte cell size and fat pad mass, and that it increases the cellular response to insulin and β-agonist-stimulated lipolysis (1,5,8,14,15). It is not clear whether these effects are caused by n-3 FA acting on adipocytes or are secondary to the changes in hepatic or systemic metabolism. In this work, we studied the effects of EPA on FA oxidation and lipid synthesis in 3T3-L1 adipocytes. We found that EPA significantly increased the β-oxidation of endogenous FA with a concomitant increase in CPT-1 enzyme activity, consistent with the prior studies on hepatocytes. In contrast, treatment with oleic acid or octanoic acid did not affect CPT-1 activity in adipocytes. On the other hand, EPA did not appear to inhibit either *de novo* FA synthesis or TAG synthesis in adipocytes, implying that the mechanisms by which EPA modulated lipid metabolism in hepatocytes (6) might not be directly extrapolated to adipocytes, although both cell types share many of their lipid metabolic pathways.

MATERIALS AND METHODS

Cell culture and FA treatment. 3T3-L1 adipocytes were prepared as described previously (16). For each experiment, cells were incubated with serum-free DMEM for 14 h before the test reagents were added. EPA (100 μM in a complex with 40 μM BSA), oleic acid (100 μM in a complex with 40 μM BSA), octanoic acid (1.0 mM, with 40 μM BSA), and clofibrate (300 μM, with 40 μM BSA) were added to the cells maintained in DMEM. The higher concentration of octanoic acid was required because of the much lower partitioning of this medium-chain FA into the plasma membrane (17). Control cells were maintained in DMEM with 40 μM BSA. All concentrations are given as final concentrations in the incubation medium.

FA oxidation. Adipocytes grown in six-well plates were preincubated with [9,10-^{3}H] oleic acid (0.5 μCi/mL) for 4 d in DMEM with 10% FBS. To enhance the enrichment of isotope in the endogenous pool, fresh isotope tracer was added every day. The absolute amount of oleic acid added by this procedure was very low (<10 nM), and its perturbation of cellular metabolism

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Abbreviations: CPT, carnitine palmitoyl transferase; PPARα, peroxisome proliferator-activated receptor α.

was considered to be negligible. Cells were then washed three times with warm PBS and added to 2 mL of phenol red-free DMEM containing the test reagents described above. After 24 h, 1.2 mL of the medium from each incubation was removed into three small plastic vials (0.4 mL/vial), which in turn were placed in one large screw-capped glass vial surrounded with 1.2 mL of unlabeled water and sealed with paraffin. This closed system was placed in an incubator at 58°C for 24 h to allow equilibrium to be reached between the labeled and unlabeled water phases. The small vial containing the original medium was then removed, and the radioactivity absorbed into the bulk water phase through vapor exchange was used for quantification by scintillation counting as described previously (18).

Incorporation of ^3H_2O and [U-^{14}C]glucose into cellular lipids. Adipocytes treated in parallel under the above conditions (except without preincubation of the isotope-labeled FA) were incubated with ^3H_2O (100 μCi/mL) or [U-^{14}C]glucose (0.5 μCi/mL). After 24 h, the cellular lipids were harvested and hydrolyzed as described previously (16). The amount of tritium accumulated in the FA fraction was quantified by β-counting and was used to calculate the amount of FA synthesized *de novo*. The amount of ^{14}C recovered from the glycerol fraction was quantified by β-counting and was used to calculate the amount of TAG synthesized in this period of time.

Mitochondrial isolation and CPT-1 activity. Mitochondria were isolated using a standard protocol (19) with slight modifications. Cultured adipocytes were harvested into 5 mL of homogenization buffer (250 mM sucrose, 10 mM Tris-HCl, 1.0 mM EDTA, 1 mM PMSF, pH 7.0). Cells were homogenized using 10 strokes of a Potter–Elvehjem tissue grinder at the maximum power setting. The homogenate was centrifuged at $600 \times g$ for 10 min. The supernatant was then centrifuged at $16{,}000 \times g$ for 10 min at 4°C. The pellet was resuspended in 10 mL of sucrose cushion (3 g sucrose dissolved in the same homogenization buffer) and centrifuged at $25{,}000 \times g$ for 10 min at 4°C. The final mitochondrial pellet was resuspended in CPT assay buffer (150 mM KCl, 5 mM Tris-HCl, 4 mM $MgCl_2$, pH 7.2). CPT activity was measured using a published protocol (20).

The assay mix (100 μL per reaction) was prepared with the assay buffer added with l-[methyl-^{3}H]carnitine (0.5 mM, 10 μCi/mL), 100 μM palmitoyl-CoA, 4 mM ATP, 0.25 mM glutathione, 2 mM KCN, 40 mg/L rotenone, and 0.5% FA-free BSA. The reaction was started by adding 20 μg of mitochondrial protein, and the assay mix was incubated at room temperature for 6 min. The reaction was stopped by adding 100 μL of 1 N HCl, followed by the extraction of palmitoyl-[^{3}H]carnitine using 1-butanol. For each measurement, the CPT activity was measured in the absence (CPT-1 and partial CPT-2) and in the presence of 100 μM malonyl-CoA (CPT-1 inhibited). The CPT-1 activity was calculated as the difference between these two measurements.

Western blot analysis. A cell lysate was prepared using a mammalian cell lysis kit according to the manufacturer's instructions. The proteins were separated by SDS-PAGE with protein loading of 15 μg/lane. The primary antibody for CPT-1 (rabbit-anti-mouse) was from Alpha Diagnostic Inc. (San Antonio, TX). The secondary antibody (goat-anti-rabbit) was from Pierce (Rockford, IL).

Mitochondrial phospholipid FA composition analysis. After measuring the CPT-1 activity, the residual mitochondrial fraction was extracted by the method of Folch *et al*. (21). The lipid mixture was separated by TLC (hexane/ether/acetic acid, 80:20:1). The phospholipid fraction was eluted from the silica gel with $CHCl_3/CH_3OH/NH_4OH$ (1:1:0.07), dried under a stream of N_2, and methylated using a BF_3/CH_3OH kit from Supelco (St. Louis, MO) according to the manufacturer's instructions. The acyl chain composition was analyzed using a GLC method as described previously (16).

RNA analysis. Total RNA was prepared and analyzed as described previously (22). The primer sequence used for CPT-1 was from the literature (23).

Other biochemical assays. Concentrations of cellular TAG, DNA, protein, and glycerol release were measured using commercial kits from Sigma as described previously (16).

Materials. Unless otherwise indicated, all regular cell culture supplies were from Fisher (Agawa, MA); all radioactive isotope-labeled chemicals were from NEN (Boston, MA); and all other reagents and organic solvents were from Sigma.

Statistics. Data are expressed as means ± SE. Comparisons between two groups of data were made using Student's *t*-test. For others, the results were analyzed using one-way ANOVA and Duncan's multiple comparison tests. Differences were considered statistically significant when $P < 0.05$.

RESULTS

EPA increased FA oxidation in adipocytes. To provide a pool of endogenous FA that could be monitored during the experiments with EPA and other additives, cells were prelabeled with [9,10-^{3}H]oleic acid (>95% of the labeled oleic acid was found in TAG after lipid fractionation analysis, data not shown). β-Oxidation was measured by quantifying the release of ^3H_2O from the endogenous esterified [9,10-^{3}H]oleic acid. As shown in Figure 1A, EPA increased β-oxidation of endogenous oleic acid in adipocytes by about 40% ($P < 0.05$, $n = 5$). Incubation with octanoic acid decreased the oxidation of [9,10-^{3}H]oleic acid, probably caused by substrate competition between octanoic acid and oleic acid, which favors octanoic acid because it can be β-oxidized independently of CPT-1 (24). Incubation with oleic acid and clofibrate slightly increased the oxidation of endogenous oleic acid, but the difference was not statistically significant ($P = 0.5$ and 0.7, respectively). The amount of glycerol released from cells treated under these five conditions was similar in all cases (250 ± 10 nmol/24 h/10^6cells), implying that the release of endogenous FA did not account for the difference in β-oxidation.

EPA increases in vitro CPT-1 activity of adipocytes. Since CPT-1 is the only known enzyme that transports activated long-chain FA into the mitochondria for β-oxidation (19), we then determined whether the observed changes in β-oxidation were associated with corresponding changes in CPT-1 activity. To

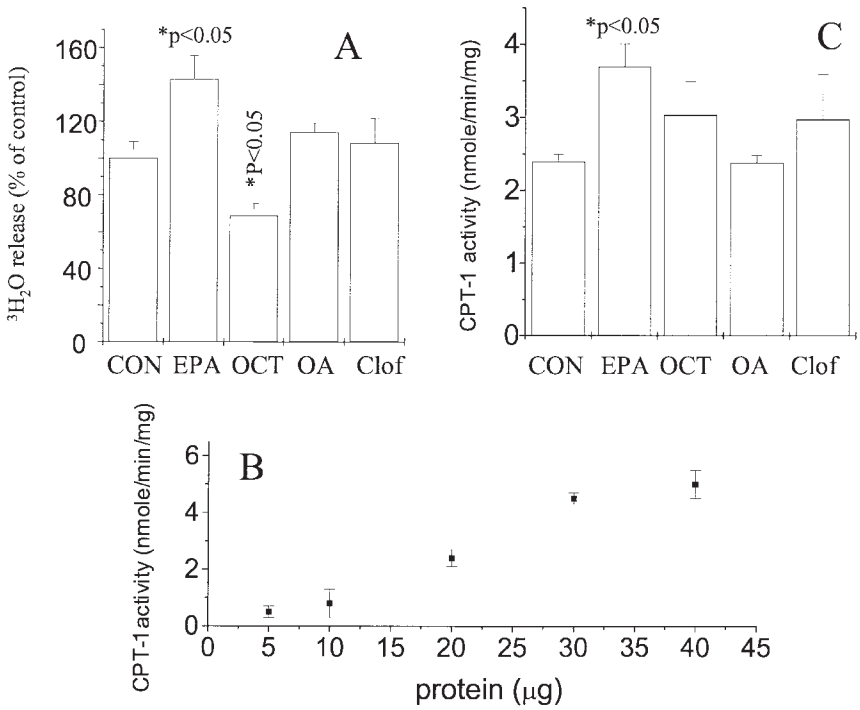

FIG. 1. Effects of EPA on β-oxidation and carnitine palmitoyl transferase 1 (CPT-1) activity. (A) Adipocytes were preincubated with [9,10-^{3}H]oleic acid for 4 d, and then treated with EPA (100 μM), oleic acid (OA, 100 μM), octanoic acid (OCT, 1 mM), or clofibrate (Clof, 300 μM) in serum-free DMEM with 0.5% BSA for 24 h. The amount of ^3H_2O liberated in this period was measured as the index for β-oxidation of endogenous FA. Data are means ± SE, n = 4; *t-test vs. control (CON). (B) CPT-1 activity was measured with different amounts of mitochondrial crude proteins from the control cells to determine the linear range of measurement. Data are means ± SE, n = 3, for each data point. (C) Cells were treated with EPA and other additives for 24 h, the same as those shown above. The mitochondrial fraction was isolated by sequential centrifugation, and the CPT-1 activity was assayed as described in the Materials and Methods section. Data are means ± SE, n = 5. *P < 0.05, vs. control.

determine the optimal mitochondrial protein concentration for the enzyme activity assay, we first measured the CPT-1 activity using different amounts of proteins prepared from the control cells, which showed a linear range from 5 to 30 μg (Fig. 1B). The final enzyme activity was measured using 20 μg of mitochondrial crude proteins. As shown in Figure 1C, CPT-1 activity in the isolated mitochondria was increased by ~40% after cells were treated with EPA. Under otherwise identical conditions, octanoic acid, oleic acid, and clofibrate did not significantly affect CPT-1 activity (P = 0.2, 0.5, 0.8, respectively). These findings show that the CPT-1 activity assayed from isolated mitochondria was proportional to the rate of β-oxidation in intact cells.

EPA did not change the expression level of CPT-1. To determine whether the changes in CPT-1 activity were associated with increased expression levels of this enzyme, the relative abundance of mRNA and protein of CPT-1 were analyzed by semiquantitative reverse-transcription (RT)-PCR. As shown in Figure 2A, EPA, as well as octanoic acid and oleic acid, did cause detectable effects on CPT-1 mRNA. There was a trend of increased CPT-1 expression in cells treated with clofibrate (Fig. 2A, 2B), but the difference did not reach statistical significance (P = 0.2 and 0.5 for mRNA and protein, respectively, n = 3 for each). The results of these measurements were validated by PCR measurements using different cycle conditions and by Western blot analysis with different protein loadings to ensure that the results were within the linear range of comparison (data not shown). As an independent verification of our methods, we also measured the mRNA in human hepatoma cells (HepG2) treated in parallel. We found that EPA, oleic acid, and clofibrate increased the expression of CPT-1 by 50, 38, and 72%, respectively (n = 3, P < 0.05 compared with control). whereas octanoic acid had no effect, consistent with previous reports (6).

Alteration in the fatty acyl composition of mitochondrial membrane lipids. Since EPA has been shown to displace proteins from membrane rafts by altering the membrane lipid composition (25), we next investigated whether the changes in CPT-1 enzyme activity were associated with a change in the mitochondrial membrane lipid composition. As shown in Figure 3, EPA typically constitutes a very small fraction of the FA in mitochondrial phospholipids. Incubation with exogenous EPA increased the percentage of EPA in the mitochondrial membrane lipids by about fivefold. This was accompanied by a reduction of 16:1 in the mitochondrial membrane lipids as compared with the control cells. The net change caused an increase in the degree of unsaturation. Incubation with either octanoic acid or clofibrate did not cause detectable changes in the mitochondrial membrane lipid composition (Fig. 3). Adding

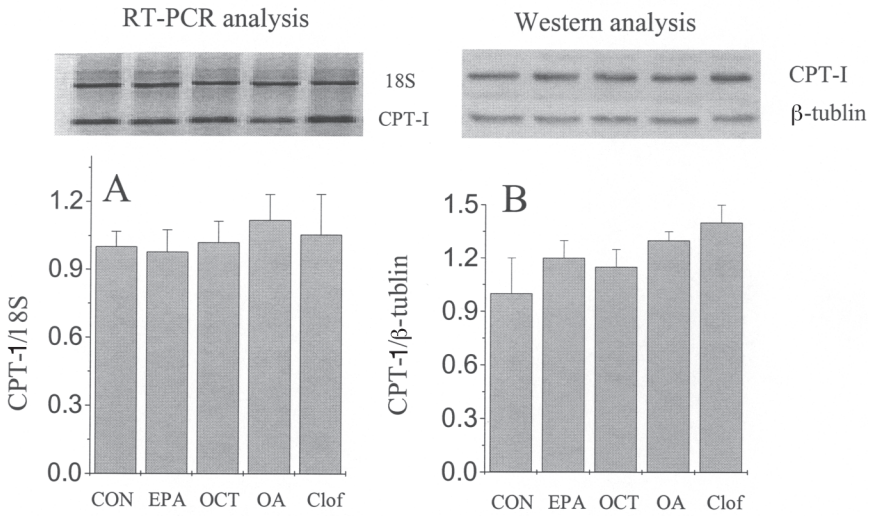

FIG. 2. Effects of EPA on mRNA (A) and protein (B) expression of CPT-1. Cells were prepared the same as those in Figure 1. RNA and protein were isolated after 24 h of incubation and analyzed as described in the Materials and Methods section (mean ± SE, $n = 3$). RT-PCR, reverse-transcription polymerase chain reaction; for other abbreviations see Figure 1.

oleic acid to the exogenous medium slightly increased the percentage of 18:1 in the membrane lipids, accompanied by a similar decrease of 16:1. Therefore, the overall acyl chain unsaturation remained unchanged.

Lipogenesis in adipocytes treated with EPA and other additives. Since lipid synthesis is one of the major metabolic events that directly affects the storage of fat in adipocytes, we investigated how treatment with EPA would affect this process, in comparison with cells treated with other additives. We found that EPA treatment did not affect *de novo* FA synthesis but increased TAG synthesis, as measured by the incorporation of glucose into the glycerol backbone (Table 1). Treatment with oleic acid caused a similar result, suggesting this might be a common effect of adding exogenous long-chain FA to cell cultures. Octanoic acid inhibited *de novo* FA synthesis but did not affect TAG synthesis, as reported previously (26). Clofibrate affected neither *de novo* FA synthesis nor TAG synthesis.

Despite the difference in β-oxidation and lipid synthesis, total TAG stores did not differ significantly in adipocytes treated as described in the Materials and Methods section, possibly because most of the stored fat was acquired before the treatments. These results also indicate that it was easier to detect the changes in a smaller pool of FA undergoing β-oxidation than in the much larger pool of FA stored in TAG.

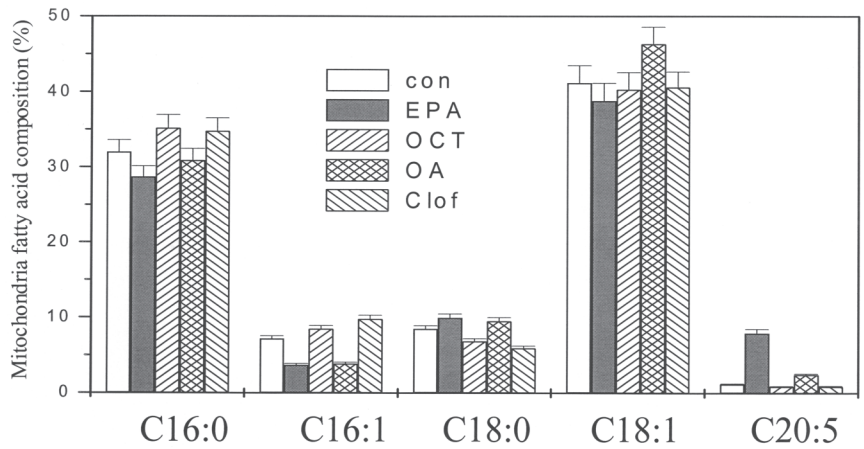

FIG. 3. Effects of EPA on mitochondrial membrane lipid FA composition. Cells were prepared the same as those in Figure 1. Mitochondrial phospholipids were isolated as described in the Materials and Methods section. The fatty acyl methyl esters were analyzed by GLC ($n = 3$). The assignment was done by reference to known fatty acyl methyl ester standards. Small amounts of other acyl chains were also detected (<3%, not shown). For abbreviations see Figures 1 and 2.

TABLE 1
The Effects of EPA on *de novo* FA Synthesis from ^3H_2O[a], TAG-Glycerol Synthesis[b], and Total Cellular TAG Content[c]

Additives	Control	EPA	Octanoic acid	Oleic acid	Clofibrate
De novo synthesized FA	5.9 ± 0.5	7.0 ± 0.8	1.9 ± 0.2*	5.0 ± 1.0	5.8 ± 0.6
Newly synthesized TAG-[U-^{14}C]glycerol	15.9 ± 0.5	22.8 ± 1.1**	16.1 ± 0.4	25.5 ± 0.7**	16.1 ± 1.1
Cellular TAG	465 ± 31	421 ± 15	441 ± 15	476 ± 21	443 ± 25

[a]Measured as pmol/h/10^6 cells.
[b]Measured as pmol/h/10^6 cells, as TAG-derived [U-^{14}C]glycerol.
[c]Measured as mg/10^6 cells, as total TAG-derived glycerol. Cells were treated as in Figure 1 (mean ± SE, $n = 4$); *$P < 0.05$ significantly reduced; **$P < 0.05$, significantly increased compared with the control.

DISCUSSION

That dietary FA activate FA β-oxidation in hepatocytes has been well established (27–29), but such a direct effect in adipocytes had not been determined in a controlled setting *in vitro*. Our results demonstrate that the β-oxidation of endogenous FA in adipocytes was increased by treatment with EPA but was not affected by oleic acid (Fig. 1A). This is consistent with a previous study showing that feeding rats with n-3 FA, but not monounsaturated FA, stimulated mitochondrial oxidation in adipose tissue (30). Clofibrate, the activating ligand for PPARα, also did not affect FA oxidation. Octanoic acid, on the other hand, inhibited the oxidation of endogenous FA (Fig. 1A). EPA also increased adipocyte mitochondrial CPT-1 activity, whereas oleic acid, octanoic acid, and clofibrate did not (Fig. 1B). EPA, as well as oleic acid and clofibrate, did not affect *de novo* FA synthesis (Table 1). Both EPA and oleic acid increased net TAG synthesis (Table 1). Octanoic acid, on the other hand, inhibited *de novo* FA synthesis but did not affect basal TAG synthesis (Table 1). However, within the 24-h incubation period, none of these changes had a significant effect on total cellular TAG contents.

EPA is known to be an activating ligand for PPARα, the nuclear transcription factor that regulates the expression of FA catabolic genes, including CPT-1 (31–35). An EPA-induced increase in CPT-1 expression is considered to be one of the main mechanisms for increased CPT-1 activity in hepatocytes (6). One would expect that EPA-induced CPT-1 activity in adipocytes might also be a result of ligand activation of PPARα, leading to increased transcription of this enzyme (36). However, we did not detect significant effects of EPA on CPT-1 at either the mRNA or the protein level (Fig. 2). The mRNA levels of PPARα and a few of its downstream targets, including acyl-CoA oxidase and acyl-CoA synthase, were also unchanged by the treatment with EPA or other additives used in this work (data not shown). Therefore, we conclude that under our experiment conditions, EPA-induced CPT-1 activity is not likely mediated through the PPARα pathway. The low abundance of PPARα expressed in adipocytes as compared with its high abundance in hepatocytes might partly explain the discrepancy in the effects of EPA on CPT-1 expression between these two cell types. Even clofibrate, a synthetic PPARα-specific ligand, failed to induce significant changes in CPT-1 expression (Fig. 2), consistent with findings that fenofibrate, another synthetic PPARα-specific ligand, had minimal effects on the expression of PPARα target genes in either rat adipose tissue or 3T3-L1 adipocytes (37).

On the other hand, although expressed to a limited extent, PPARα has been demonstrated to be functional in adipocytes (23,36,38), and might be regulated by supra-high dosages of synthetic ligands (39). Therefore, the lack of effects of EPA, or oleic acid and clofibrate, on CPT-1 mRNA in adipocytes might not be entirely due to the low expression levels of PPARα in these cells. An alternative possibility is that ligand stimulation of PPARα might already have been saturated in adipocytes because of its constant exposure to high levels of endogenous FA. Most of these FA can also be activating ligands of PPARα (34). In this regard, moderate variations in exogenous FA within a short period of time might not be sufficient to alter the transcriptional activity of PPARα in adipocytes. Hence, an alternative mechanism needs to be considered to interpret the EPA-mediated increase in CPT-1 activity and the associated increase in FA oxidation.

Mitochondrial CPT-1 activity can be modified by the fluidity of the membranes in which it resides, as shown by an earlier study (40). Because mitochondrial lipids contain a low proportion of cholesterol, membrane fluidity is largely controlled by the acyl chain unsaturation of the phospholipids. As shown in Figure 3, exogenous EPA was incorporated into mitochondrial membrane lipids with a simultaneous decrease in palmitoleic acid (16:1). These changes in the mitochondrial membrane lipids are expected to alter membrane structure and cell functions (2,11), as we also confirmed (Fig. 1A, 1B). The direct cause(s) of changes in CPT-1 activity, however, merit further investigation.

In addition to its effects on β-oxidation and CPT-1 activity, EPA has been shown to inhibit lipogenesis and reduce TAG synthesis in hepatocytes (41). In contrast, we found that EPA did not inhibit *de novo* FA synthesis and even increased TAG synthesis in adipocytes, as evidenced by the increased synthesis of the TAG-glycerol moiety (Table 1). Similar results were found when cells were treated with oleic acid but not octanoic acid (26). Such an increase in TAG synthesis is likely caused by an increased availability of exogenous substrates, which implies that EPA can be used for lipid synthesis in adipocyte analogs to oleic acid.

A quantitative comparison of the results in Table 1 shows that the amount of FA or TAG synthesized *de novo* during the 24-h incubation period accounted for only a small fraction of the total storage of TAG, implying that the majority of cellular

TAG was preformed before the incubation was initiated. Hence, although EPA and oleic acid both increased TAG synthesis, they did not substantially change the total TAG storage. In addition, since *de novo* FA synthesis occurred at a slower rate than net TAG synthesis, most of the FA substrate used for TAG synthesis in these cells might come from intracellular FA recycling or from exogenously added FA.

In summary, we showed that EPA increases CPT-1 activity and β-oxidation in adipocytes. Since FA oxidation normally accounts for only a very small part of the fat disposition in adipocytes (18), we did not detect an acute effect of EPA on lipid synthesis and storage in adipocytes, as previously established in hepatocytes. Our results support the hypothesis that the hypolipidemic effect of EPA or fish oil found *in vivo* might be primarily a liver-mediated systemic effect. However, a small but steady increase in β-oxidation might gradually modulate fat storage in adipocytes on a long-term basis.

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