Mode of transport of fatty acid to endothelial cells influences intracellular fatty acid metabolism

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Abstract Fatty acids are transported to cells from a variety of different moieties in the plasma. In this study, using oleate and human umbilical vein endothelial cells, we asked whether the vehicle that delivers fatty acid to cells has an influence on its metabolism upon its incorporation into the cell. For oleate vehicles, we compared free oleate bound to albumin with oleate in low density lipoprotein (LDL) which was delipidated and reconstituted with either radiolabeled triolein or cholesteryl oleate. Using approximately physiologic concentrations of LDL and free oleate, we demonstrated by three lines of evidence unique patterns of cellular oleate metabolism for oleate delivered as triolein within LDL, for oleate delivered as cholesteryl oleate within LDL, and for oleate delivered as free oleate bound to albumin. In fact, the difference was most marked between cholesteryl oleate and triolein, even though the oleate in cholesteryl oleate and triolein was delivered in identically reconstituted LDL particles, which were presumably incorporated into the cells and degraded in lysosomes in a similar fashion. First, we demonstrated that oleate delivered as free oleate or as triolein in reconstituted LDL was desaturated and elongated to fatty acid metabolites, but cholesteryl oleate in reconstituted LDL was not similarly metabolized. The elongated and desaturated metabolites of oleate were preferentially esterified in cellular triglyceride when oleate was delivered as free oleate, but they were preferentially esterified in phospholipids when oleate was delivered as triolein in LDL. Second, we observed that there was a difference in the distribution of oleate among phospholipids when oleate was delivered as cholesteryl oleate in reconstituted LDL versus triolein in reconstituted LDL. When the oleate was delivered as triolein in reconstituted LDL, there was greater esterification in diacyl phosphatidylethanolamine, in phosphatidylserine, and in phosphatidylinositol. When oleate was delivered as cholesteryl oleate in reconstituted LDL, there was greater esterification in diacyl phosphatidylcholine. Third, there was a marked preference for oleate delivered from triolein in LDL over cholesteryl oleate in LDL for esterification into the *sn*-1 position of plasmalogens as a vinyl ether-linked fatty acid. These data indicate that mode of transport of fatty acid to cells influences fatty acid metabolism upon its incorporation into the cell, even when the fatty acid is delivered from the core of the same lipoprotein.—**Teruya, J., J. Cluette-Brown, Z. M. Szczepiorkowski, and M. Laposata.** Mode of transport of fatty acid to endothelial cells influences intracellular fatty acid metabolism. *J. Lipid Res.* 1995. **36:** 266–276.

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There are many metabolic options for fatty acids that have been newly incorporated into the cell: β-oxidation, desaturation and elongation, esterification into triglycerides, cholesteryl esters, and phospholipids that distribute among different subcellular membranes, and oxygenation with export from the cell (1).

In the plasma, there are many vehicles that deliver fatty acids to cells. Among these are the fatty acid-containing phospholipids (PL), triglycerides (TG), and cholesteryl esters (CE) in the four major plasma lipoproteins, and a much smaller pool of free fatty acids bound to albumin. Thus, fatty acids can be delivered to cells from at least 13 different vehicles. We speculated that a correlation exists between the vehicle that delivers the fatty acid to the cells and intracellular fatty acid metabolism. The concept that a unique sequence of metabolic events in the cell is associated with each of the many different vehicles in the plasma that deliver fatty acids to cells is an untested hypothesis. Addressing this question requires the reconstitution of lipoproteins with complex lipids containing radiolabeled fatty acids of sufficient specific activity to allow metabolic studies to be performed after incorporation of fatty acids into the cell, and the availability of a cell culture system with sufficient incorporation of reconstituted lipoproteins via specific cell surface receptors.

There are several mechanisms whereby the fatty acids enter cells, with the mechanism of entry largely dependent on the lipoprotein in which the fatty acid-containing

Abbreviations: PL, phospholipid; TG, triglyceride; CE, cholesteryl ester; LDL, low density lipoprotein; VLDL, very low density lipoprotein; TLC, thin-layer chromatography; DPE, diacyl phosphatidylethanolamine; DPC, diacyl phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine; rLDL, reconstituted LDL; [^{3}H-cholesteryl linoleate]rLDL, LDL reconstituted with [^{3}H]cholesteryl linoleate; [^{3}H-triolein]rLDL, LDL reconstituted with [^{3}H]triolein; [Cholesteryl ^{14}C-oleate]rLDL, LDL reconstituted with cholesteryl [^{14}C]oleate; HPLC, high pressure liquid chromatography; BCA, bicinchoninic acid.

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PL, TG, or CE is located. Low density lipoprotein (LDL) particles are endocytosed via LDL receptors, and their PL, TG, and CE enter the cell intact, from which free fatty acids are released intracellularly (2). Chylomicrons and very low density lipoproteins (VLDL) can deliver free fatty acids from TG without total particle engulfment as extracellular lipoprotein lipase releases free fatty acids from TG in these lipoproteins, and the free fatty acids then enter cells (3). Chylomicrons and VLDL can also deliver lipid to cells by receptor-mediated endocytosis via receptors recognizing apolipoprotein E (4). For a variety of lipoproteins, outer shell phospholipids of lipoproteins have been shown to exchange with cell membrane phospholipids (5) and in this manner deliver fatty acids to cells. Lipids other than phospholipids can also be delivered to cells from lipoproteins by means other than receptor-mediated endocytosis or lipid exchange, such as the selective uptake of cholesteryl esters from HDL and LDL (6–8). Plasma free fatty acids can cross the plasma membrane directly with a portion of such transport possibly mediated by membrane transport proteins (1). In light of the great diversity of fatty acid vehicles and their associated mechanisms of fatty acid entry into cells and the many metabolic options for fatty acids in the cells, we specifically asked whether the vehicle from which oleate, as a representative fatty acid, is delivered to endothelial cells influences *1)* its desaturation and elongation, and subsequently the complex lipids into which these fatty acid metabolites are esterified; *2)* the distribution of oleate among cellular phospholipids; and *3)* the extent of oleate incorporation into the vinyl ether-linked *sn*-1 position of ethanolamine and choline plasmalogens. To address these questions, we isolated and delipidated LDL from human plasma, reconstituted the LDL core with radiolabeled triolein or radiolabeled cholesteryl oleate, and then incubated either these reconstituted lipoproteins or free oleate bound to albumin with human umbilical vein endothelial cells. Remarkably, the largest metabolic differences in the cells were observed between cholesteryl oleate in LDL and triolein in LDL as fatty acid vehicles, even though these moieties were both within the core of identical LDL particles, which were presumably incorporated into cells via the same receptor and degraded in the same lysosomes. Our data demonstrate that differences in the fatty acid vehicle resulted in a marked difference in the desaturation and elongation of oleate, the phospholipid distribution of oleate, and the extent of esterification into the vinyl ether-linked *sn*-1 position of plasmalogens. Thus, the route of delivery of fatty acids to cells has a major impact upon the metabolism of the fatty acid upon its incorporation into the cells. This observation suggests that cellular uptake and metabolism of fatty acid is dependent both on the concentration of fatty acid in the plasma and the vehicle that delivers the fatty acid to the cells.

MATERIALS AND METHODS

LDL isolation from fresh frozen plasma

Plasma LDL was isolated according to a modification of the method of Hatch (9). N-ethylmaleimide (5 mM) was added to fresh frozen plasma to inhibit the activity of lecithin:cholesterol acyltransferase. Residual platelets and platelet fragments were removed by centrifugation for 15 min at 12,000 *g*. The plasma was then dialyzed overnight against 0.15 M NaCl–2.0 mM Na_2EDTA prior to ultracentrifugation. A stock solution of 0.2 M NaCl–2.0 mM Na_2EDTA (d 1.006 g/ml) was layered onto 17 ml dialyzed plasma and the sample was centrifuged in a Beckman 50.2 Ti rotor for 30 min (4°C) at 26,000 *g* to remove chylomicrons. The 0.2 M NaCl–2.0 mM Na_2EDTA solution (d 1.006 g/ml) was layered onto the plasma a second time and the plasma was centrifuged for 18 h (4°C) at 30,000 *g* to remove VLDL. The infranatant was adjusted to d 1.025 g/ml by adding specific volumes of a stock solution of 0.2 M NaCl–2.0 mM Na_2EDTA (d 1.006 g/ml) and a stock solution of 2.6 M NaCl–3.0 M KBr–2.0 mM Na_2EDTA (d 1.350 g/ml). A 0.22 M NaCl–0.11 M KBr–20 mM Na_2EDTA stock solution (d 1.019 g/ml) was layered onto the plasma and the plasma was centrifuged for 18 h (4°C) at 75,000 *g* for isolation of intermediate density lipoprotein. The infranatant was adjusted to d 1.075 g/ml using 1.350 g/ml and 1.006 g/ml stock solutions. A solution of 0.62 M NaCl–0.52 M KBr–2.0 mM Na_2EDTA (d 1.066 g/ml) was layered onto the plasma and the plasma was centrifuged for 18 h (4°C) at 105,000 *g* to isolate LDL. Purity of native LDL prior to its reconstitution with labeled lipids was demonstrated by the appearance of one band with the mobility of LDL in a Titan gel lipoprotein electrophoresis system (Helena Laboratories).

Preparation of reconstituted LDL

LDL was reconstituted using the method of Krieger et al. (10). Briefly, purified LDL (1.5–2.5 mg protein) was dialyzed against 0.3 mM Na_2EDTA, pH 7.4, overnight, and then mixed with 25 mg of starch. The mixture was lyophilized and subsequently incubated with 5 ml heptane at −10°C for 1 h to remove lipids in the core of the LDL lipoprotein. After centrifugation at 1,000 *g* for 10 min at −10°C to remove the supernatant, the pellet was incubated with 5 ml heptane at −10°C for 30 min. The heptane was removed after centrifugation as before, and labeled lipids in heptane were added to the delipidated LDL for 1 h at −10°C to reconstitute the lipid core of the LDL particle. The carrier heptane was evaporated under nitrogen in an ice bath, and 1 ml 10 mM tricine buffer (pH 8.4) was added overnight at 4°C to suspend the LDL particles in aqueous medium. After microcentrifugation for 20 min, the supernatant was removed and filtered through an 0.8 μm syringe filter (Micron Separations).

Total protein content of the reconstituted LDL (rLDL) suspension in buffer was approximately 1 mg/ml, as measured by the method of Lowry et al. (11). Total cholesterol (cholesterol and cholesteryl ester) in LDL reconstituted with cholesteryl oleate was approximately 0.04 mg/ml, as measured using the method of Rosenthal, Pfluke, and Buscaglia (12). Reconstituted LDL containing radiolabeled triolein or radiolabeled cholesteryl oleate was subjected to lipoprotein electrophoresis. At the end of the electrophoresis, an autoradiogram of the gel was made. The gel was cut into 3-mm slices that were counted for radioactivity. In all cases, a single sharp band of radioactivity with the mobility of LDL was found on the gel. This band was distinctively separated from bands of acetylated LDL and LDL oxidized by incubation with endothelial cells for 24 h.

Cell culture

Human umbilical vein endothelial cells were harvested according to the method of Jaffe et al. (13) and cultured in Medium 199 (Gibco) containing 20% fetal calf serum (Gibco), 170 U/ml penicillin–170 μg/ml streptomycin (Gibco), 1.6 mM L-glutamine (Gibco), 90 μg/ml heparin (Sigma), and 30 μg/ml of endothelial cell growth supplement (Collaborative Research) (14). Fetal calf serum has been shown to contain little or no cholesteryl ester transfer protein activity (15). The identity of the cells was confirmed by immunofluorescence using anti-Factor VIII antibody (Atlantic Antibodies) and fluorescein-conjugated anti-IgG (Sigma) (16). Cells were plated in 35-mm or 60-mm diameter petri dishes (Corning) at initial cell counts of 5×10^5 and 7×10^5 per dish, respectively. The endothelial cells were used for experiments either as primary cultures or after one or two passages. In all incubation experiments, prior to incubation with rLDL or [^{14}C]oleate, the endothelial cells were incubated in delipidated medium (medium containing 20% delipidated fetal calf serum, 170 U/ml penicillin–170 μg/ml streptomycin, and 1.6 mM L-glutamine) for 48 h. This has been shown to increase uptake of rLDL by inducing synthesis of the LDL receptor (17). Delipidated fetal calf serum was prepared by the method of Capriotti and Laposata (18).

Time course of uptake of rLDL and free oleate

[^{14}C]oleate (Du Pont, New England Nuclear, 53.8 mCi/mmol) and unlabeled oleate (approximately 40 mM total fatty acid) were dried completely under nitrogen, and warmed (37°C) delipidated medium was added for 30 min to resuspend the fatty acid. The solution was then sterile-filtered through an 0.2-μm syringe filter (Nalgene). The oleate concentration was finally adjusted to 28 μM (physiologic concentration of plasma free oleate in nonfasting individuals (19)) by dilution with sterile delipidated medium after the filtration. For other experiments, the oleate concentration was adjusted to 10, 25, 50, or 100 μM in a similar fashion. For studies on the time course of rLDL and fatty acid uptake, delipidated medium (0.8 ml) and 0.2 ml of [^{3}H-cholesteryl linoleate]rLDL (DuPont, New England Nuclear, originally 90 Ci/mmol) (0.2 mg/ml protein, final concentration, 8,934 dpm/μg protein in rLDL), or [^{14}C]oleate (28 μM, final concentration, 15,714 dpm/nmol) were added to the dishes. After 0, 4, 8, 24, and 48 h of incubation, the medium was removed from selected dishes and the cell monolayer was washed 4 times with ice-cold Dulbecco's phosphate-buffered saline. The cells from each dish were then scraped into 1 ml phosphate-buffered saline using a rubber policeman. Total radioactivity associated with the cells was determined by counting 50 μl of the cell suspension. The percent uptake was calculated as ((dpm in cell suspension) ÷ (total dpm added to the cells)) × 100.

Fatty acid analysis of cells incubated with rLDL or free oleate

Delipidated medium (1.6 ml) and 0.4 ml of either [^{3}H-triolein]rLDL (DuPont, New England Nuclear, originally 26.8 Ci/mmol) (0.2 mg/ml protein, final concentration, 9,130 dpm/μg protein in rLDL), [cholesteryl ^{14}C-oleate]rLDL (DuPont, New England Nuclear, originally 57.0 mCi/mmol) (0.2 mg/ml protein, final concentration, 6,055 dpm/μg protein in rLDL), or 28 μM nonesterified oleate (final concentration, 15,714 dpm/nmol) were added to 60-mm diameter petri dishes for 24 h. Very similar masses of oleate were made available to the cells when the oleate was presented as triolein in rLDL (2,277 nmol oleate per 10^6 endothelial cells in the dish) and as cholesteryl oleate in rLDL (2,679 nmol oleate per 10^6 endothelial cells in the dish). These amounts represent approximately physiologic concentrations of esterified fatty acid in LDL triglyceride and cholesteryl ester. The 28 μM concentration of nonesterified oleate was selected because it was also physiologic, with the understanding that matching the concentration of nonesterified oleate with the concentration of esterified oleate in rLDL in vitro would have necessitated use of nonesterified oleate concentrations that were highly nonphysiologic. The cells were harvested as described above. Cell number per dish was determined by counting an aliquot of the cell suspension stained with 0.1% crystal violet using a hemocytometer. Total lipid was extracted from the cell suspension according to the method of Cohen et al. (20). Concentrated lipid extracts were spotted onto silica gel 60 thin-layer chromatography (TLC) plates (Merck), and the plates were developed in a system of petroleum ether–diethyl ether–acetic acid (94:6:1.25). Lipid standards (phosphatidylcholine, triolein, and cholesteryl oleate) were visualized by spraying the plates with 0.012% rhodamine 6G (Sigma) in water. Fatty acid methyl esters were prepared from the TLC scrapings of PL, TG, and CE using

methanolic KOH, and the fatty acid methyl esters were extracted three times with hexane–diethyl ether (1:1) as described by Lefkowith et al. (21). [^{3}H]triolein used for reconstitution of LDL was > 99.5% intact with essentially no free oleate in the preparation at the time it was used in these experiments.

HPLC and measurement of radioactivity

Fatty acid methyl ester-containing extracts were dried completely under nitrogen and then resuspended in 0.07 ml of a fatty acid methyl ester standard mix (18:1 n–9, 18:2 n–6, 18:3 n–6, 20:1 n–9, 20:3 n–9, 20:4 n–6, 22:1 n–9, and 24:1 n–9) in acetonitrile. Fatty acid methyl esters were separated on a 4.6 mm ID × 250 mm, Microsorb 5 μm C18 column (Rainin) with a guard column containing an 0.5 mm filter element, using a solvent system consisting of acetonitrile and 0.017 M H_3PO_4 (pH 2.5) in a ratio 90:10 (v/v) for 60 min and 100:0 (v/v) for 55 min, with a flow rate of 1 ml/min. The absorbance was measured at 205 nm. One-ml fractions were collected directly into scintillation vials for measurement of radioactivity.

Experiments to test binding of rLDL to the LDL receptor

In one set of experiments, LDL was freshly isolated as mentioned above and dialyzed against 1,000 volumes of 0.15 M NaCl–0.2 mM Na_2EDTA for 12 h prior to incubating it with the endothelial cells. Delipidated medium (0.8 ml) and 0.2 ml of either freshly isolated LDL (approximately 0.4 mg/ml protein, final concentration) or saline as a control were added to 35-mm diameter petri dishes for 8 h. Then 0.8 ml of delipidated medium and 0.2 ml of [^{3}H-triolein]rLDL (0.2 mg/ml protein, final concentration, 9,130 dpm/μg protein in rLDL) were added for 18 h after removing the previous medium. In this experiment, because of the limited uptake of LDL, the unlabeled native LDL was removed before the addition of radiolabeled rLDL to avoid dilution of the radiolabeled rLDL with native LDL. The cells were harvested and counted for radioactivity as described in the time course experiments.

In a series of direct competition experiments, endothelial cells were plated in 35-mm petri dishes and allowed to reach approximately 95% confluence. The cells were rinsed four times with warm phosphate-buffered saline after which a 1290 μl total volume of serum free medium, buffer, and various combinations of native LDL, acetyl-LDL, and reconstituted LDL with cholesteryl ester in the core were added. When present, the final concentration of native LDL was 9.9 mg/dl; the final concentration of acetyl-LDL was 9.9 mg/dl; and the reconstituted LDL containing cholesteryl ester was 0.39 mg/ml. The cells were incubated for 14 h with the various mixtures. After this time, the medium was removed and the cells were rinsed twice with 500 μl of warm phosphate-buffered saline. While on ice the cells were scraped from the dish into 1 ml of cold phosphate-buffered saline. The cell suspension was the analyzed for protein content which was determined using the bicinchoninic acid (BCA) assay. Radioactivity of the cell suspension was measured by liquid scintillation counting. The ratio of native LDL or acetyl LDL to reconstituted LDL in these experiments was 25 to 1.

Determination of percent incorporated triolein derived from unhydrolyzed triolein in rLDL and determination of percent incorporated cholesteryl oleate derived from unhydrolyzed cholesteryl oleate in rLDL

For the triglyceride experiments, after a 24-h incubation in delipidated medium, endothelial cells were incubated for 24 h with either [^{3}H-oleate-labeled triolein] rLDL (DuPont, New England Nuclear, originally 26.8 Ci/mmol) (0.26 mg/ml protein, final concentration 1,073 dpm/μg protein in rLDL or with [^{3}H-glycerol-labeled triolein] rLDL (American Radiolabeled Chemicals, Inc., originally 10 Ci/mmol) (0.26 mg/ml protein, final concentration 1,565 dpm/μg protein in rLDL). The cells were harvested and lipids were extracted (18), and PL, TG, CE, cholesterol, and free fatty acids were isolated by TLC using a sequential two-solvent system, (solvent I: diethyl ether–petroleum ether–acetic acid 130:70:1 (v/v/v); solvent II: petroleum ether–diethyl ether 15.6:1 (v/v). The percent distribution of dpm among PL, TG, CE, cholesterol, and free fatty acids was determined by scraping the spots from the TLC plate containing these lipids and measuring the radioactivity in the spots by liquid scintillation counting. The same strategy was used in the cholesteryl ester experiments using [^{14}C-oleate-labeled cholesteryl oleate] rLDL (DuPont, New England Nuclear, originally 55 mCi/mmol) (0.46 mg/ml protein, final concentration 4,572 dpm/μg protein in rLDL) and [^{3}H-cholesterol-labeled cholesteryl oleate] rLDL (DuPont, New England Nuclear, originally 68.4 Ci/mmol) (0.46 mg/ml protein, final concentration 3,795 dpm/μg protein in rLDL).

Measurements of oleate distribution among phospholipids and oleate incorporation into the *sn*-1 position of plasmalogens

rLDL [^{3}H-triolein] or rLDL [cholesteryl ^{14}C-oleate] in identical amounts or free oleate was added to endothelial cells in 60-mm diameter petri dishes for 8, 16, or 24 h. The cells were harvested as described above and total cellular lipid was extracted in chloroform–methanol–1 N HCl 50:50:0.3. This acidic extraction completely hydrolyzes the vinyl ether-linked fatty acid from ethanolamine and choline plasmalogens (22) and leads to the formation of fatty aldehydes from the ether-linked fatty acids as well as the formation of lysophospholipids. For phospholipid distribution experiments, concentrated lipid extracts were

spotted onto silica gel 60 TLC plates together with a standard phospholipid mixture (diacyl phosphatidylethanolamine (DPE), diacyl phosphatidylcholine (DPC), phosphatidylserine (PS), phosphatidylinositol (PI), lysophosphatidylethanolamine (LPE), and lysophosphatidylcholine (LPC)). The plates were developed by two-dimensional TLC using chloroform–methanol–NH_4OH–water 65:35:5:0.6 in the first dimension, and then chloroform–acetone–methanol–acetic acid–water 45:19:10:15:4 in the second dimension. Lipids were stained by I_2 vapor, and after sublimation of I_2, each spot was scraped directly into a scintillation vial for measurement of radioactivity. To determine the extent of oleate incorporation into the vinyl ether-linked, acid-labile *sn*-1 position of plasmalogens, concentrated lipid extracts were spotted onto silica gel 60 TLC plates together with standards of *cis*-13-octadecenal (Sigma), cholesteryl oleate, triolein, and the standard phospholipid mixture (DPE, DPC, PS, PI, LPE, and LPC). The lipids were extracted in chloroform–methanol–1 N HCl 50:50:0.3. The plates were developed by two-dimensional TLC using hexane–diethyl ether–acetic acid 70:30:1 in the first dimension, and then in hexane–diethyl ether–acetic acid 94:6:1.25 in the second dimension. Isolated fatty aldehydes and PL visualized by I_2 vapor were scraped directly into scintillation vials after I_2 sublimation for measurement of radioactivity. The radioactivity associated with fatty aldehydes was expressed as percent of dpm in total phospholipids.

Statistics

Statistical analyses were performed using the Student's *t*-test (two-tailed, unpaired). Best fit lines were determined using least squares regression analysis.

RESULTS

Initial studies

We first determined the time course of rLDL uptake and free oleate uptake into human umbilical vein endothelial cells. This was done in order to determine the optimum time period over which to perform subsequent incubations. As has previously been reported by Krieger et al. (10), the uptake of rLDL into cultured cells over 24 h is relatively limited, and a long enough incubation period must be used to allow incorporation of sufficient dpm for subsequent metabolic studies. Much previous work in our laboratory with cultured fibrosarcoma cells (23) and HepG2 cells (24) maintained in medium containing delipidated serum and measurements of the fatty acid composition of endothelial cells incubated 36 h in medium containing delipidated serum indicate that the endothelial cells in the present studies do not meet any of the criteria for essential fatty acid deficiency after incubation in delipidated serum. After the incubation in delipidated medium for 48 h to increase the number of LDL receptors on the endothelial cells, the endothelial cells were incubated for up to 48 h with approximately physiological concentrations of the [^{3}H-cholesteryl linoleate]rLDL (0.2 mg/ml protein, final concentration), and the percent uptake was determined by the number of dpm in harvested cells. The total incorporation of [^{3}H-cholesteryl linoleate]rLDL into endothelial cells over 48 h was 3.6% of total dpm added with sufficient cellular dpm by 24 h for future experiments (data not shown). In uptake experiments with a physiologic concentration of nonesterified oleate (28 μM), approximately 15% of the nonesterified oleate was incorporated into endothelial cells at approximately 24 h (data not shown).

To assess the capacity of the reconstituted LDL for binding to the LDL receptor on endothelial cells, we used [^{3}H-triolein]rLDL and freshly isolated unlabeled LDL in an indirect competition experiment. When endothelial cells were preincubated with freshly isolated native LDL for 8 h, the uptake of [^{3}H-triolein]rLDL into the cells was approximately 20% of control (rLDL uptake after saline incubation: 5,549 ± 1,184 dpm per dish versus rLDL uptake after native LDL incubation: 1,176 ± 283 dpm per dish, n = 2, mean ± SEM). A direct competition experiment was also performed in which native LDL was incubated with reconstituted LDL containing radiolabeled cholesteryl ester in its core in a 25/1 ratio (native LDL/rLDL). Using this ratio of native LDL to rLDL, the uptake of radiolabeled cholesteryl ester decreased from 100.0 ± 4.0% to 61.5 ± 0.9% (mean ± SEM, n = 3) as a result of competition with the unlabeled native LDL. Acetyl LDL, which can decrease uptake of reconstituted LDL by entering cells, delivering cholesterol, and decreasing the number of surface LDL receptors by a mechanism that is not directly competitive, lowered the uptake of rLDL to only 75.7 ± 1.3 (mean ± SEM, n = 3). Because endothelial cells are known to bind substantial quantities of LDL for transcytosis, in which LDL is passed intact in vesicles through endothelium for transfer to underlying tissue without interacting with the LDL receptor (25, 26), native LDL at a 25 × concentration of rLDL was able to inhibit some but not all of the rLDL uptake. However, taken together, the results of the direct and indirect competition studies and the electrophoretic analysis of reconstituted LDL all support the conclusion that the rLDL used in this study was not oxidized and could compete with native LDL for LDL receptor binding. This conclusion is the same as that reached by Krieger et al. (10) in their original manuscript describing LDL reconstitution, as they also demonstrated that the reconstituted LDL prepared according to this method is intact, can bind to LDL receptors, and is incorporated into the cells.

Distribution of incorporated oleate among cellular lipid pools versus mode of transport

In the first series of experiments, using approximately

physiologic concentrations of LDL and free oleate, we compared the delivery of oleate from LDL, reconstituted with triolein or cholesteryl oleate, with free oleate bound to albumin. As shown in **Table 1,** all three moieties delivered oleate into the cellular phospholipid pool. To become associated with phospholipid, the oleate from triolein and cholesteryl oleate first had to be hydrolyzed to free oleate, then converted to oleoyl-CoA, and esterified into phospholipid. The hydrolysis step would not be necessary for cellular oleate incorporated as free oleate. Oleate from triolein within reconstituted LDL was also delivered to cellular triglyceride pools. We determined in our studies that essentially all (> 95%) of the radiolabeled triglyceride in the cells represented triolein that was not degraded. Similarly, > 95% of the cholesteryl oleate that was found in the cells after incubation with cholesteryl oleate in LDL represented cholesteryl oleate that was not degraded upon incorporation into the cells. This was proven because the percent distribution of dpm incorporated into cellular TG (among PL, TG, CE, and free fatty acid) was similar when triolein in rLDL was labeled in the glycerol or the fatty acid moiety (82% vs. 95%, respectively). Thus, the fatty acid pool whose metabolic fate is most influenced by the vehicle delivering the fatty acid to the cells is the fatty acid that is hydrolyzed from the TG or CE. This liberated fatty acid can then be elongated, desaturated, or not metabolized and later reesterified into a variety of different fatty acid-containing lipids in the cells.

Free oleate was incorporated much more effectively than both lipoprotein-associated moieties. Thus, at approximately physiologic concentrations, in experiments that involved separate incubations with each of the three moieties tested, the incorporation of free oleate was several orders of magnitude more efficient than the incorporation of oleate from triolein or cholesteryl oleate within reconstituted LDL. When the concentration of free oleate was lowered 2.8-fold (28 μM to 10 μM) to permit a similar amount of oleate uptake from albumin-bound oleate as was found for rLDL-TG and rLDL-CE, the distribution of oleate among cellular lipids did not change significantly from studies in which higher amounts of free oleate were used. Thus, the higher amount of oleate incorporated from the nonesterified pool was not a factor in its distribution among cellular lipids. As expected, when the concentration of free oleate greatly exceeded 28 μM (50–100 μM), the percent of free oleate incorporated into cellular TG increased (% of total incorporated oleate esterified into TG: 10 μM, 5.3 ± 0.2; 25 μM, 7.6 ± 0.8; 50 μM, 19.5 ± 0.4; 100 μM, 26.5 ± 1.1; n = 3, mean ± SEM. The remainder for each entry was esterified into PL.) It should be noted that oleate in triolein and cholesteryl oleate was presented in comparable amounts to the cells (2,277 vs. 2,679 nmol/10^6 endothelial cells, respectively) and thus, any differences between these two moieties in metabolism should not reflect differences in the mass of oleate made available to the cells. It should also be noted that in our studies, oleate was the only fatty acid present in the core of the reconstituted LDL particles unlike native LDL. As this was true for both rLDL reconstituted with triolein and rLDL reconstituted with cholesteryl oleate, this should not influence comparisons between these two moieties despite the fact that it differs from the in vivo situation. The influence of the phospholipid-associated fatty acids in the outer shell of the LDL particle was not assessed. However, as the LDL delipidations were prepared from the same lot of native LDL for triolein and cholesterol oleate reconstitutions, this also should not influence comparisons between the two different particles.

TABLE 1. Quantitative summary of oleate delivery to cells from different vehicles

	Cellular Phospholipids	Cellular Triglycerides	Cellular Cholesteryl Esters
		nmol/10^6 cells	
Baseline cellular concentration	180.3 ± 10.0	16.6 ± 2.1	<0.5
		Increments above baseline cellular concentrations	
Transfer of oleate from rLDL-containing triolein to:	8.0 ± 2.4	19.9 ± 3.1 (non-hydrolyzed TG from rLDL)	N.D.
Transfer of oleate from rLDL-containing cholesteryl oleate to:	13.4 ± 1.1	N.D.	18.3 ± 1.1 (non-hydrolyzed CE from rLDL)
Transfer of albumin bound oleate to:	32.2 ± 2.2	17.5 ± 2.3	N.D.

Endothelial cells in 60-mm or 100-mm diameter petri dishes were incubated with medium containing 20% delipidated fetal calf serum for 48 h. Phospholipids, triglycerides, and cholesteryl esters (nmol per 10^6 cells) were measured as described in Methods. rLDL containing radiolabeled triolein (0.2 mg/ml protein, final concentration) or cholesteryl oleate (0.2 mg/ml protein, final concentration), or 28 μM free oleate (final concentration) were incubated with endothelial cells, and the mass of oleate delivered from these moieties to cells was quantitated as described in Methods. Values represent mean ± SEM of four determinations; N.D., none detected.

Desaturation/elongation studies

In the first of three series of experiments to test whether mode of oleate transport influences cellular oleate metabolism, we assessed whether mode of oleate transport influences desaturation and elongation of cellular oleate. Free oleate delivered from albumin and oleate from triolein of rLDL were effectively elongated and desaturated to 20:3Δ5,8,11, which was then esterified into PL and TG (**Fig. 1A**). Cholesteryl oleate, on the other hand, delivered no oleate or oleate metabolites to cellular TG. The sensitivity for detecting oleate metabolites in PL originating from [^{14}C]cholesteryl oleate was not sufficient to allow reproducible measurement. Therefore, no conclusion could be made regarding esterification of elongated and desaturated metabolites of oleate into phospholipids from cholesteryl oleate in rLDL (N.D. in Fig. 1). The elongated and desaturated metabolite of oleate, 20:3Δ5,8,11, was detected in both cellular TG and PL when oleate was delivered as free oleate and triolein in rLDL. Interestingly, however, the 20:3Δ5,8,11 was preferentially esterified in TG over PL when delivered as free oleate, but it was preferentially esterified in PL over TG when delivered as triolein in rLDL. Qualitatively similar results were obtained for the immediate elongation product of oleate, 20:1Δ11 (Fig. 1B). Collectively, the results shown in Fig. 1 indicate that the delivery vehicle influences both the degree of desaturation/elongation and the TG and PL distribution of the elongated and desaturated metabolites. For each vehicle, both of the PL versus TG comparisons in Fig. 1A and in Fig. 1B were statistically significant ($P < 0.008$ for all four). As mentioned previously, the endothelial cells were not essential fatty acid-deficient after only 48 h in delipidated medium. It has been previously demonstrated that Δ6 desaturase, the rate-limiting step in the Δ6desaturase/elongase/Δ5 desaturase pathway, becomes activated after exposure to delipidated medium for as little as 5 h (21). In these studies with fibrosarcoma cells (21), the primary substrate for Δ6 desaturase was the endogenous unlabeled 18:2 n-6 (linoleate) with conversion to unlabeled 20:4n-6 (arachidonate). However, in the present studies with endothelial cells, the activity of this pathway was assessed using radiolabeled oleate as a substrate for Δ6 desaturase. Thus, this experimental design allowed us to activate the Δ6desaturase/elongase/Δ5desaturase pathway and demonstrate that the metabolism of the n-9 fatty acid oleate was dependent on the vehicle delivering the oleate to the cells.

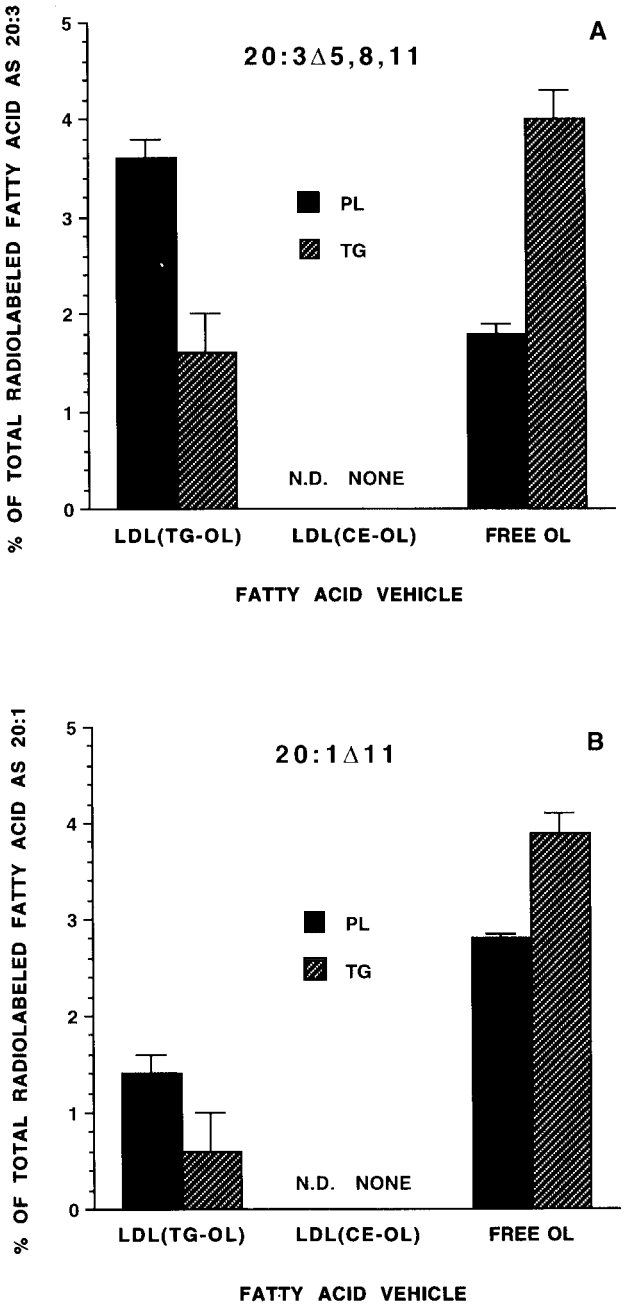

Fig. 1. Metabolism of oleate incorporated into endothelial cells via [^{3}H-triolein]rLDL, [cholesteryl ^{14}C-oleate]rLDL, and nonesterified [^{14}C]oleate. Endothelial cells in 60-mm diameter dishes were incubated with either [^{3}H-triolein]rLDL, [cholesteryl ^{14}C-oleate]rLDL, or nonesterified [^{14}C]oleate for 24 h. Lipids were extracted from the cells, PL, TG, and CE moieties were separated by TLC, and then the fatty acid composition in PL, TG, and CE was analyzed by HPLC. Panels A and B show radiolabeled 20:3Δ5,8,11 and radiolabeled 20:1Δ11, respectively, as percent of total radioactive fatty acid in PL, TG, or CE. Each bar represents the mean ± SEM from two experiments (n = 4).

Phospholipid distribution studies

In the second series of metabolic experiments we assessed whether mode of oleate transport to the cells influenced its distribution among cellular phospholipids. In these experiments we compared the distribution of oleate from triolein within rLDL versus cholesteryl oleate within rLDL. Comparisons between free oleate bound to albumin and esterified oleate within rLDL must take into account the many differences between the vehicles (such

as the effect of phospholipid-associated fatty acids in the rLDL outer shell). However, these differences do not exist for comparisons between triolein and cholesteryl oleate in similarly reconstituted LDL particles. Presumably, these moieties are transported to the cells by way of the same specific LDL receptors, and both are degraded in the same lysosomes, leading to the generation of free oleate from triolein and cholesteryl oleate. Given the similarities in the intracellular transport and degradation of triolein and cholesteryl oleate within the core of reconstituted LDL, we were surprised to find a consistently significant difference in the distribution of oleate among phospholipids when the oleate was delivered from triolein versus cholesteryl ester (**Fig. 2**). Relative to each other, the oleate delivered from triolein to phospholipids was preferentially esterified in diacyl phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol, whereas the oleate delivered from cholesteryl oleate was preferentially delivered to diacyl phosphatidylcholine. (All TG-oleate vs. CE-oleate comparisons were statistically significant, $P < 0.05$.) The distribution of oleate among phospholipids when the oleate was delivered as nonesterified oleate bound to albumin was very similar to its distribution among phospholipids when delivered as cholesteryl oleate in rLDL. In these experiments, the lipids were extracted from the cells with acid in the extraction solvents. This permitted an analysis of the diacyl moieties of PC and PE free from the alkenylacyl choline and ethanolamine fractions, because the latter degrade to lysophospholipids in the presence of acid. The small alkylacyl choline and ethanolamine fractions were not removed from the diacyl forms in the analysis.

Fatty aldehyde studies

We next asked whether there was a difference in esterification of oleate into the vinyl ether-linked *sn*-1 position of ethanolamine and choline plasmalogens when the oleate was delivered to the endothelial cells as triolein or cholesteryl ester within rLDL. In a study involving isolated cardiac myocytes, oleate esterification into the *sn*-1 position of plasmalogens was found to be quantitatively similar to stearate esterification, with vinyl ether-linked palmitate approximately 2.5-fold the amount of stearate or oleate (27). Thus oleate esterification into the vinyl ether-linked *sn*-1 position of plasmalogens is a quantitatively significant process. In these experiments we incubated endothelial cells with triolein or cholesteryl oleate within reconstituted LDL particles for 24 h, and then harvested the cells and extracted the lipids in a mixture that contained 1 N HCl to hydrolyze the vinyl ether-linked fatty acid, with its subsequent conversion to a fatty aldehyde. The fatty aldehyde was then isolated by two-dimensional TLC using a fatty aldehyde standard to locate the spot on the thin-layer plate. As shown in **Fig. 3,** the dpm in the fatty aldehyde spot (relative to the dpm in total phospholipids) was significantly higher when the ole-

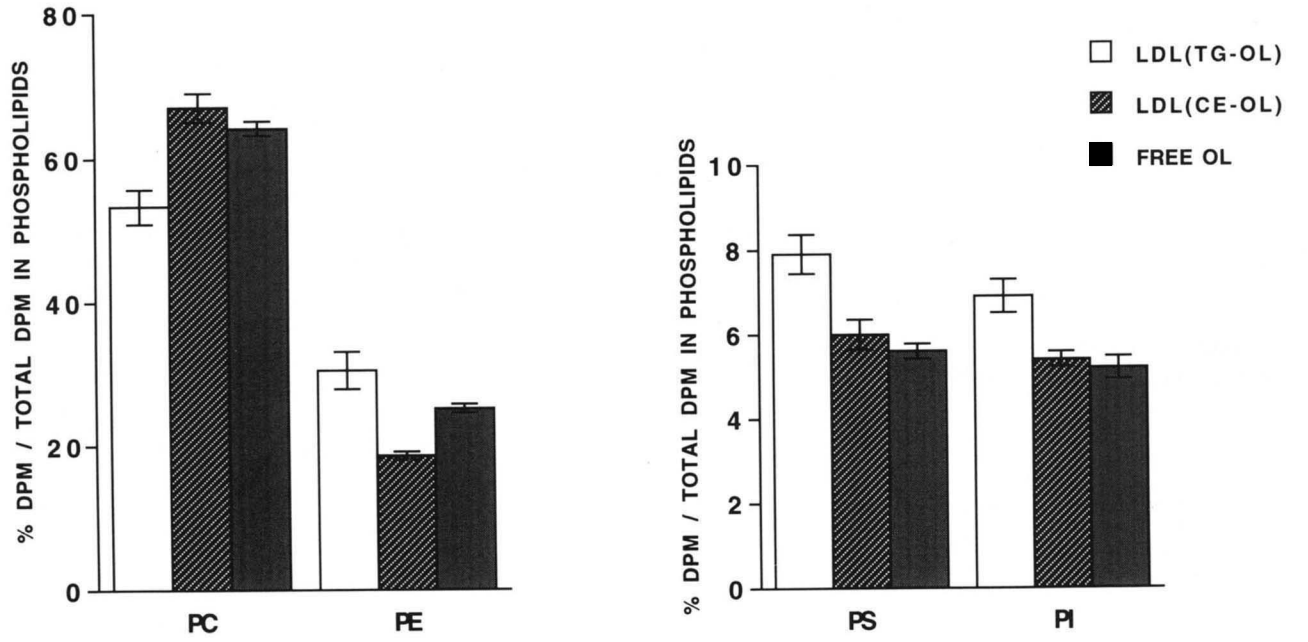

Fig. 2. Phospholipid distribution of oleate and metabolites delivered via [^{3}H-triolein]rLDL. Endothelial cells in 60-mm diameter dishes were incubated with either rLDL containing radiolabeled triolein (TG-OL) (0.2 mg/ml protein, final concentration), cholesteryl oleate (CE-OL) (0.2 mg/ml protein, final concentration) or free oleate (28 μM) for 24 h. Lipids were extracted from the cells; DPE, DPC, PS, and PI were isolated by two-dimensional TLC; and the radioactivity associated with each was determined. Each value is expressed as dpm in DPE, DPC, PS, or PI relative to dpm in total phospholipids. Each value represents the mean ± SEM (n = 3, except PS and PI at 24 h in which n = 6). For each delivery vehicle, 100% represents the sum of the % dpm in the four phospholipids.

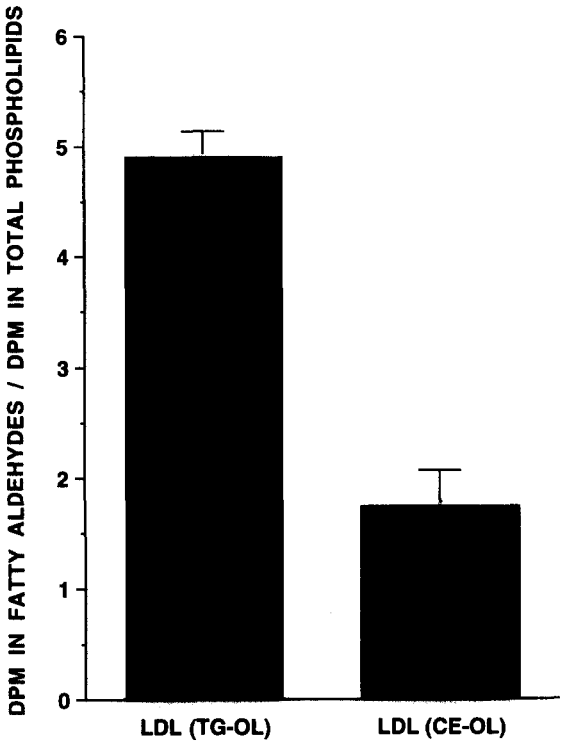

Fig. 3. Incorporation of oleate and metabolites delivered via [^{3}H-triolein]rLDL and [cholesteryl ^{14}C-oleate]rLDL into ethanolamine and choline plasmalogens as vinyl ether-linked fatty acid. Endothelial cells in 60-mm diameter dishes were incubated with either [^{3}H-triolein]rLDL or cholesteryl ^{14}C-oleate]rLDL for 24 h. Lipids were extracted from the cells, fatty aldehydes were isolated by two-dimensional TLC, and the radioactivity associated with fatty aldehydes was determined. Each value is expressed as dpm in fatty aldehydes relative to dpm in total phospholipids. Each bar represents the mean ± SEM (n = 3).

ate was delivered as triolein rather than as cholesteryl oleate within rLDL (P = 0.001). The dpm in the fatty aldehyde spot (relative to the dpm in total phospholipids) when the oleate was delivered as nonesterified oleate bound to albumin was essentially identical to the result for delivery via cholesteryl oleate in rLDL. This series of experiments provided a third piece of evidence that oleate from triolein and cholesteryl oleate in LDL, despite similarities in intracellular transport and degradation in lysosomes, has a different metabolic fate, depending upon the mode of oleate transport to the cell.

DISCUSSION

Figure 4 illustrates the unique metabolic pathways in the cell we observed for oleate delivered as triolein within LDL, as cholesteryl oleate within LDL, or as free oleate bound to albumin. By assessing fatty acid desaturation/elongation, phospholipid distribution of newly incorporated fatty acid, and the extent of incorporation of fatty acid into the *sn*-1 ether-linked position of plasmalogens, we have demonstrated that the mode of transport of fatty acid to the cell influences its subsequent intracellular metabolism. We have performed our experiments with a representative fatty acid. We suspect cellular fatty acid trafficking is a remarkably complex process in the cell for all fatty acids and, as clearly indicated by our results, it depends to a significant extent on the vehicle that delivers the fatty acid to the cells.

The observed differences in the metabolism of oleate presented to the cells as free oleate bound to albumin

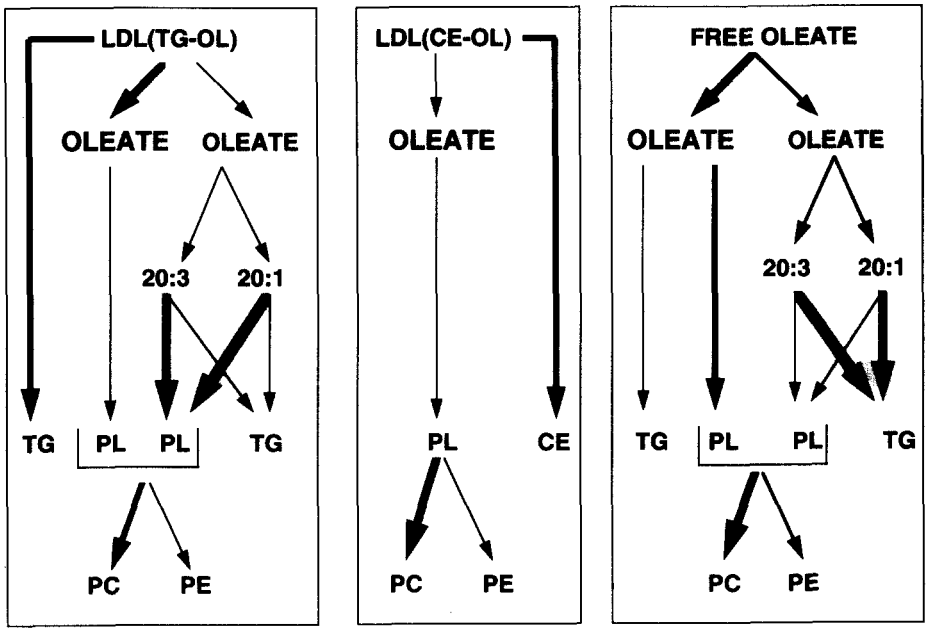

Fig. 4. Summary diagram. The unique metabolic pathways in the cell for oleate delivered as triolein within LDL (LDL (TG-OL)), as cholesteryl oleate within LDL (LDL(CE-OL)), and as free oleate.

versus oleate esterified within triglyceride or cholesteryl esters of rLDL have several possible explanations. First, the uptake of fatty acids from lipoproteins is receptor-mediated, as opposed to the uptake of free oleate bound to albumin, which is predominantly non-receptor-mediated uptake (1). Second, free oleate enters the cell and in a single metabolic step is converted to fatty acyl-CoA for esterification into cellular complex lipids. On the other hand, oleate delivered as triolein and cholesteryl oleate must first be hydrolyzed from the triolein or cholesteryl oleate, and then be converted to fatty acyl-CoA for esterification. This extra metabolic step may account for some of observed differences in the cellular metabolism of oleate. Third, given the higher efficiency of uptake of free oleate relative to esterified oleate in lipoproteins, it is possible that the oleate delivered from albumin reaches higher free oleate concentrations in the cell than the oleate delivered from triglyceride and cholesteryl ester. A higher free oleate concentration in the cell may affect certain of the metabolic pathways for fatty acids in the cells. Fourth, as mentioned, phospholipid-associated fatty acids in the outer shell of rLDL may have contributed to observed metabolic differences between oleate delivered as free oleate versus esterified oleate in rLDL. Finally, given the structural diversity in cellular fatty acid binding proteins (28), which can be separated into different groups using ion-exchange chromatography (29), one might speculate that there is a subpopulation of fatty acid binding proteins that directs oleate to different metabolic pathways if it presents as free fatty acid versus esterified fatty acid.

More surprising than the metabolic differences between oleate delivered as free oleate versus esterified oleate in rLDL were the significant differences in oleate metabolism when the oleate was delivered as triolein versus cholesteryl oleate within rLDL. Both the triolein and cholesteryl oleate were presumably incorporated into the cell in the core of similar if not identical LDL particles, through the same receptor-mediated pathway, and hydrolyzed in an identical lysosomal environment. There may be several possible mechanisms operating that could explain the observed differences. We tested one of these mechanisms directly. We had speculated that the rates of hydrolysis of oleate from cholesteryl oleate and from triolein in the lysosomes were different. It has been shown in a variety of cell types (23, 30) that free oleate incorporated into cells is first incorporated into PC and subsequently incorporated into PE. Thus, if there was a difference in the rate of hydrolysis of oleate from cholesteryl oleate and triolein, we thought that this might be reflected in a different rate of incorporation of oleate into PC and PE from these two vehicles. Over the time course that we tested, namely, 8, 16, and 24 h, there was, however, no change in PC to PE esterification for either cholesteryl oleate or triolein (data not shown). It is possible that a difference in the rate of lysosomal hydrolysis of oleate from triolein versus cholesteryl oleate does exist, but that this difference occurs before 8 h. Eight hours was the shortest time period that could be tested in our studies because the number of dpm incorporated into the cells for incubations in less than 8 h was prohibitively small. A second possible mechanism to explain observed differences in oleate metabolism when oleate was delivered as triolein versus cholesteryl oleate in LDL is that there may be compartmentation of hydrolysis events within the lysosome, such that triglyceride hydrolysis occurs in an anatomically distinct location from the site of hydrolysis of cholesteryl ester. If there is a difference in the compartmentation of hydrolysis events within the lysosome, this could lead to differences in the esterification of oleate into PC or PE, which may be distributed differentially among the inner and outer leaflets of the lysosomal membrane. It is not known whether there is an asymmetry of fatty acid distribution between inner and outer leaflets of the lysosomal membrane, as has been clearly demonstrated for the plasma membrane (31). Because compartmentation studies within lysosomes are not technically possible at the present time, this hypothesis cannot be tested.

There were marked differences in oleate desaturation and elongation when oleate was delivered as triolein versus cholesteryl oleate within LDL. It is possible that a difference exists in the accessibility of the oleate hydrolyzed from triolein versus cholesteryl oleate for the desaturase and elongase enzymes in the cell. The subcellular distribution of these two enzymes is known to be microsomal, but there may be a more focal distribution of these enzymes within the microsomes such that oleate that exits the lysosomal particle has differential access to these enzymes, depending upon whether or not it was originally esterified within triolein or cholesteryl oleate. Because subcellular fractionation of endothelial cells to this extent is not yet feasible, this speculation cannot be assessed. A similar "greater accessibility to enzymes" argument can be offered to explain our observation that oleate from triolein is more effectively esterified as a vinyl ether-linked fatty acid in plasmalogens than oleate from cholesteryl oleate. The precursor for the *sn*-1 fatty acid of plasmalogens is a fatty alcohol and, therefore, the oleate from triolein may be more efficiently converted to oleoyl alcohol for esterification as the *sn*-1 fatty acid in plasmalogens.

In summary, our findings support the novel observation that cellular uptake and metabolism of fatty acid is in part dependent on the vehicle that delivers the fatty acid to the cells.

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