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Metabolic fate of oleic acid derived from lysosomal degradation of cholesteryl oleate in human fibroblasts

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Abstract Low density lipoprotein cholesteryl [^{14}C]oleate (LDL-[^{14}C]CO) was used as a tool to label lysosomes with cholesteryl [^{14}C]oleate (CO) and to follow subsequently the metabolic processing of oleic acid released by acid lipase. Liberated [^{14}C]oleate was incorporated into glycerolipids, mainly into phosphatidylcholine. Incubations in the presence of various concentrations of exogenously added oleic acid and double label experiments showed that oleic acid derived from lysosomal degradation of CO and exogenously added oleic acid distributed in a similar fashion among triacylglycerol and various phospholipids. To further study the metabolism of LDL-derived oleic acid, experiments were performed in which fibroblasts were prelabeled with LDL-[^{14}C]CO. The subsequent processing of lysosome-derived oleic acid was followed with time without LDL-[^{14}C]CO in the medium. From these experiments it became clear that apart from the esterification into glycerolipids a substantial part of lysosome-derived oleic acid was released into the medium. The efflux of oleic acid into the medium preceded the incorporation into glycerolipids, was dependent on the composition of the extracellular medium, and was energy-independent. Our data are compatible with a mechanism in which lysosome-derived fatty acids are transported to the plasma membrane prior to transport to endoplasmic reticulum for esterification. Intra- and extracellular factors influence the distribution of lysosome-derived oleic acid among cells and medium.—**Groener, J. E. M., W. Bax, and B. J. H. M. Poorthuis.** Metabolic fate of oleic acid derived from lysosomal degradation of cholesteryl oleate in human fibroblasts. *J. Lipid Res.* 1996. **37:** 2271–2279.

Supplementary key words fatty acid • lipid transport • lysosomes • acid lipase • human fibroblasts • low density lipoprotein • cholesteryl ester

Lysosomes are acidic membrane-bound organelles containing hydrolytic enzymes for degradative processes. The metabolites formed in the lysosomes egress from the lysosomal compartment and can be re-utilized in metabolic processes in other parts of the cell. Inherited defects in individual steps of lysosomal degradation of macromolecules result in lysosomal storage of mucopolysaccharides, oligosaccharides, and (sphingo)lipids (1). The existence of the lipid storage disease indicates that complex lipids do not freely pass the lysosomal membrane. Breakdown of these lipids must occur in lysosomes and lipid end products of catabolism such as sphingosine, fatty acids, and cholesterol are expected to leave the lysosome. Little is known regarding the topology of lysosomal lipid metabolism (2), the transport of lipid metabolites over the lysosomal membrane (3), and the subsequent metabolism of these metabolites. The fact that cholesterol, derived from low density lipoprotein (LDL), accumulates inside lysosomes in Niemann-Pick type C (4, 5) is of interest and suggests that at least one of the lipid end products of lysosomal catabolism is not freely transportable across the lysosomal membrane.

LDL is taken up by the LDL-receptor and delivered to lysosomes where it is catabolized. In this process LDL-cholesteryl ester (CE) is hydrolyzed to cholesterol and fatty acids (6). While the metabolic fate of LDL-derived cholesterol has been studied extensively (5), little information is available on the metabolic fate of fatty acids formed by hydrolysis of LDL-lipids. In particular, the pathway and mechanism of transport of fatty acids over the lysosomal membrane and from lysosomes to target organelles in the cell are unknown. In general, the mechanism of transport of fatty acids over and between membranes is still a matter of discussion (7, 8). A sum-

Abbreviations: CE, cholesteryl ester; CO, cholesteryl oleate; FFA, free fatty acid; FH, familial hypercholesterolemia; LDL, low density lipoprotein; LDL-[^{14}C]CO, low density lipoprotein radioactively labeled with cholesteryl [^{14}C]oleate; LPDS, lipoprotein-deficient serum; PL, phospholipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TG, triacylglycerol.

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mary of the present understanding in lipid transport processes in eukaryotic cells has been published recently (9).

In the present study we used LDL, radioactively labeled with cholesteryl [^{14}C]oleate ([^{14}C]CO), to study the metabolic fate of oleic acid derived from lysosomal breakdown of lipids.

MATERIALS AND METHODS

Materials

Fetal calf serum (FCS) was obtained from ICN Biomedicals BV, Zoetermeer, the Netherlands. Ham's medium, penicillin, and streptomycin were purchased form Gibco Europe BV, Breda, the Netherlands. Bovine serum albumin, essentially fatty acid-free (BSA), human serum albumin, essentially fatty acid-free (HSA), and egg phosphatidylcholine were from Sigma Chemical Company, St. Louis, MO. Cholesteryl 1-[^{14}C]oleate (specific activity: 59.5 mCi/mmol) and [^{3}H]oleic acid (specific activity 10 Ci/mmol) were obtained from Amersham International, Amersham, Bucks, U.K.

Cell culture

Control human skin fibroblasts and skin fibroblasts obtained from patients afflicted with Wolman disease and familial hypercholesterolemia (FH) were used in this study. Fibroblasts from the Wolman patient were obtained from the European Human Cell Bank (Rotterdam, The Netherlands). The FH cells (GM 02000F) were purchased from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). Fibroblasts were grown and maintained in Ham's F10 medium and penicillin (10 IU/ml), streptomycin (10 IU/ml) L-glutamine (1 mM), and hypoxanthine (4 μg/ml) supplemented with 10% FCS in an atmosphere of humidified 95% air, 5% CO_2 at 37°C.

Isolation and labeling of lipoproteins

LDL was separated from freshly isolated human serum according to Redgrave, Roberts, and West (10) and subsequently labeled using the procedure as previously described (11). After labeling LDL was dialyzed against phosphate-buffered saline (PBS). LDL was stabilized by the addition of HSA (0.5%), stored at 4°C, and used within 3 weeks. Radioactivity ranged from 3000 to 4000 dpm/μg LDL-protein, specific radioactivity from 1000 to 2300 dpm/nmol CE. Lipoprotein-deficient serum (LPDS) was isolated from freshly isolated serum by ultracentrifugation at density 1.21 g/ml, 44 h, 50000 rpm, 12°C. After isolation LPDS was dialyzed against PBS and kept frozen at −20°C.

Incorporation studies

Fibroblasts were cultured in Ham's F10 medium plus 10% FCS. Four or 5 days before the experiment, cells were trypsinized, transferred to 35-mm wells (150,000 cells per well), and grown to confluency in medium supplemented with 10% FCS. Twenty four hours before the start of the experiment medium was changed for medium supplemented with 10% LPDS to enhance the uptake of LDL. The experiments were started by the addition of 1.5 ml of medium supplemented with 10% LPDS to enhance the uptake of LDL. The experiments were started by the addition of 1.5 ml of medium supplemented with 10% LPDS and radiolabeled LDL (50 μg LDL-protein/ml medium). In order to study the effect of exogenously added fatty acids, various concentrations of oleic acid bound to BSA were added. In the experiments in which [^{3}H]oleic acid was added, the specific acticity was 3000 dpm/nmol.

In other experiments cells were preincubated for 4 h in 1.5 ml medium containing 10% LPDS and LDL-[^{14}C]CO (50 μg LDL-protein/ml medium). The medium was then removed, and cells were washed and incubated for 30 min in 1.5 ml of fresh Ham's F10 (10% FCS) at 18°C. Subsequently Ham's F10 (10% FCS) was replaced and incubations were performed at 37°C for various times. In some experiments FCS was substituted by LPDS, BSA (2 mg/ml) or was omitted.

To study the effect of pharmacological agents on the metabolic fate of LDL-derived oleic acid, various agents were dissolved in the medium. NH_4Cl, NaN_3/NaF, and chloroquine were dissolved in distilled water; imipramine, progesterone, sphinganine, monensin, brefeldin A, and nigericin in ethanol; and nocodazole in dimethylsulfoxide (DMSO). To the medium were added either 5 μl solvent or 5 μl inhibitor dissolved in solvent per ml of medium. After incubations, cells were washed 3 times with PBS, incubated with trypsin for 5 min at 37°C, and pelleted by centrifugation. Cell pellets were suspended in 200 μl distilled water, sonicated for 10 sec, and the suspension was used for lipid extraction and protein estimation. In some experiments medium was saved and used for lipid extraction. Lipids were extracted with chloroform–methanol, 2:1 (v/v) and washed according to Folch, Lees, and Sloane Stanley (12). Lipids were separated by HPTLC on precoated silica gel 60 plates (Merck no. 5633) with the following solvent systems: hexane–diethylether–acetic acid 70:30:1 (v/v/v) for the neutral lipids and a 2- dimensional system: chloroform–methanol–ammonia 65:35:5 (v/v/v) and chloroform–hexane–methanol–acetic acid–water 120:70:40:30:3 (v/v/v/v/v) for the phospholipids. Lipid

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spots were detected by iodine vapor, scraped, and counted by liquid scintillation counting in 10 ml Ultima Gold (Packard Instruments, BV). When phospholipids were counted, the scraped silica gel was first suspended in 1 ml methanol. Protein concentrations were determined by the method of Lowry et al. (13).

Cholesteryl ester and cholesterol were measured by a fluorescence method described by Gamble et al. (14).

RESULTS

LDL labeled with [^{14}C]CO by use of plasma lipid transfer protein was used to study the metabolic fate of lysosome-derived oleic acid. To test whether the CE was exclusively catabolized in lysosomes, we used fibroblasts obtained from patients with acid lipase deficiency (Wolman disease). LDL-receptor-deficient cells (FH) were used to test receptor-dependent uptake of radioactive labeled LDL. The uptake and metabolism of LDL-[^{14}C]CO in control, Wolman, and FH fibroblasts are presented in **Fig. 1.** As is expected, LDL-[^{14}C]CO is effectively taken up and internalized by control and Wolman disease fibroblasts, but is not taken up by FH fibroblasts (Fig. 1a). The amount of radioactivity in fibroblasts derived from the patient with Wolman disease was found to be significantly higher than in control fibroblasts. In contrast to control fibroblasts, in fibroblasts derived from a patient with Wolman disease CO was not degraded and all cell-associated radioactivity was found to be present in cholesteryl ester (data not shown). This confirms that LDL-[^{14}C]CO is taken up via the LDL-receptor pathway and is exclusively degraded in lysosomes by acid lipase. Figure 1b shows that liberated [^{14}C]oleate is utilized for lipid biosynthesis. The released oleate is mainly incorporated into phospholipids, predominantly phosphatidylcholine. Smaller amounts of [^{14}C]oleate are found in triacylglycerol and other phospholipids. A minor part of the liberated oleate (approximately 10%) is still present as free fatty acid. The amount of radioactivity associated with cholesteryl ester increases during the first 4–7 h of the incubation and thereafter slightly decreases, suggesting a low re-incorporation of LDL-derived oleic acid into cholesteryl ester.

In order to investigate how lysosome-derived oleic acid is utilized in glycerolipid metabolism as compared to exogenously added oleic acid, experiments were performed in which the incorporation of [^{3}H]oleate, added to the medium in different concentrations, into triacylglycerol, phospholipids, and cholesteryl ester was measured. The results are presented in **Table 1.** The distribution of exogenously added oleic acid among triacylglycerol and the major phospholipids depends on its concentration in the medium. At the concentration of 0.01 mM, oleic acid is predominantly incorporated into phosphatidylcholine. At the higher concentration of 0.1 mM, relatively more oleic acid was incorporated into triacylglycerol and relatively less into phospholipids. At both concentrations only a small fraction (<6.5%) of the cell-associated oleic acid was incorpo-

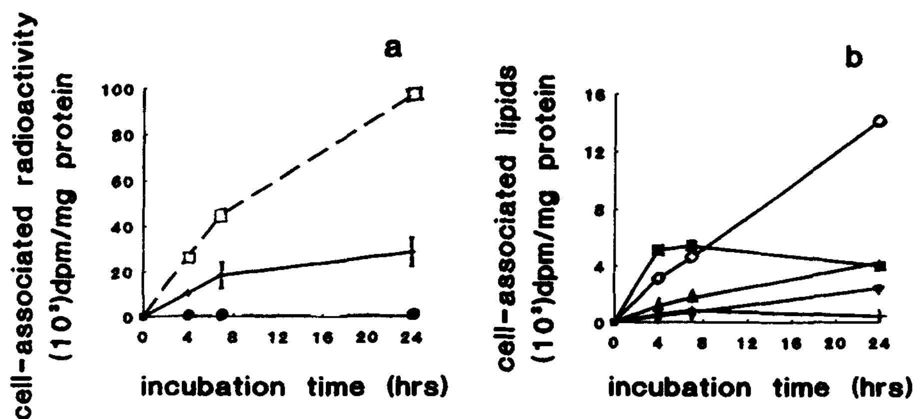

Fig. 1. Uptake (a) and metabolism (b) of LDL-cholesteryl [^{14}C]oleate by cultured fibroblasts. Fibroblasts from control subjects (————, n = 3), from a patient with Wolman disease (□——□), and from a patient with familial hypercholesterolemia (●——●) were incubated for 24 h in Ham's medium containing 10% LPDS followed by incubation in medium containing 10% LPDS and LDL-[^{14}C]CO (50 μg LDL-protein/ml). (a) Cells were harvested at the times indicated and the cell-associated radioactivity was determined by liquid-scintillation counting. The amount of cell-associated radioactivity was determined (dpm/mg protein). (b) Cell-associated radioactivity in various lipids in control fibroblasts. Cholesteryl ester (■——■), phosphatidylcholine (○——○), phosphatidylethanolamine (▼——▼), triacylglycerol (▲——▲), and free fatty acids (+——+). Similar results were obtained in 3 independent experiments.

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TABLE 1. Incorporation of [^{3}H]oleate into triacylglycerol, phospholipids, and cholesteryl ester

Oleate	Cell-Associated [^{3}H]Oleate	Incorporation of [^{3}H]Oleate into CE	(PL and TG)	Distribution of Label TG	PC	PE	(PI + PS)
mM	*10^2 dpm/mg protein*			*% of total radioactivity in (PL + TG)*			
0.01	567 ± 21	36 ± 3	456 ± 117	20.9 ± 8.7	53.7 ± 8.5	6.7 ± 1.1	8.0 ± 0.6
0.10	2760 ± 393	108 ± 36	2076 ± 255	39.8 ± 8.0	39.1 ± 5.4	5.5 ± 1.0	6.7 ± 0.4

Control fibroblasts were incubated with 0.01 or 0.10 mM [^{3}H]oleate for 7 h in medium containing 10% LPDS and LDL (50 µg LDL-protein/ml). Cells were harvested, lipids were extracted, and the incorporation of [^{3}H]oleate into glycerolipids was measured after thin-layer chromatography. Cell-associated [^{3}H]oleate and incorporation of [^{3}H]oleate into CE, PL, and TG are expressed as dpm/mg protein. CE, cholesteryl ester; PL, phospholipids; TG, triacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine. Means ± SD of 3 different experiments are presented.

rated into cholesteryl ester (Table 1). In addition, fibroblasts were incubated in the presence of LDL-[^{14}C]CO and different concentrations of unlabeled oleic acid. **Table 2** presents the results. Exogenously added oleic acid markedly affects the distribution of lysosome-derived oleic acid among the various glycerolipids. In addition, 0.1 mM oleic acid had a significant effect on the amount of cell-associated radioactivity. Comparison of the data from Tables 1 and 2 shows that the distribution of lysosome-derived oleate and exogenously added oleate among the various glycerolipids at each concentration is similar, suggesting that they enter a common pool used for esterification. Further evidence for a common pool of oleic acid used for glycerolipid synthesis was obtained from double label experiments in which the ratio of the incorporation of exogenously added [^{3}H]oleic acid and lysosome-derived [^{14}C]oleic acid was calculated. Results of two representative experiments are presented in **Table 3.** Although the actual amount of radioactive fatty acid incorporated into glycerolipids varies from experiment to experiment and between different cell lines, the ratio of incorporated [^{3}H]oleate and [^{14}C]oleate into triacylglycerol and the major phospholipids within each experiment is constant. A lower ratio was found in free fatty acid in both experiments.

TABLE 3. Ratio of [^{3}H]oleic acid/[^{14}C]oleic acid (dpm/dpm) incorporated into glycerolipid and free fatty acids

	TG	PC	PE	PI + PS	FFA
Exp.I	7.5	8.3	8.3	7.4	3.2
Exp.II	2.7	2.7	3.1	2.8	1.5

Control fibroblasts were incubated for 7 h in medium containing 10% LPDS, LDL-[^{14}C]CO (50 µg LDL-protein/ml), and 0.01 mM [^{3}H]oleate. Cells were harvested, lipids were extracted, and radioactivity in glycerolipids was measured after thin-layer chromatography. Two representative experiments with two different control fibroblast lines are presented. Abbreviations are as in Table 1; FFA, free fatty acids.

All the experiments presented so far were conducted with LDL-[^{14}C]CO present in the medium. In order to study the release of oleic acid from the lysosome and its subsequent metabolism in more detail, we performed studies in which cells were prelabeled with LDL-[^{14}C]CO for 4 h. The cells were then washed and further incubated up to 24 h without LDL-[^{14}C]CO in the medium. The distribution of newly released oleic acid

TABLE 2. Effect of various concentrations of exogenously added oleic acid on the incorporation of [^{14}C]oleate, derived from lysosomal breakdown of CE, into triacylglycerol and phospholipids

Oleate	Cell-Associated LDL-[^{14}C]CO	Incorporation of [^{14}C]Oleate into PL and TG	Distribution of Label TG	PC	PE	(PI + PS)
mM	*10^2 dpm/mg protein*		*% of total radioactivity in (PL + TG)*			
—	263 ± 89	111 ± 196	21.9 ± 10.0	52.6 ± 5.9	7.8 ± 1.1	7.4 ± 1.2
0.01	245 ± 98	89 ± 17	27.5 ± 10.5	50.2 ± 8.4	6.5 ± 0.7	7.9 ± 1.7
0.10	168 ± 72[a]	53 ± 12	42.3 ± 12.6	35.6 ± 7.5	5.8 ± 1.7	8.3 ± 2.2

Control fibroblasts were incubated for 7 h in medium containing LDL-[^{14}C]CO (50 µg LDL-protein ml medium), 10% LPDS, and 0.01 or 0.10 mM oleate. Cells were harvested, lipids were extracted, and the incorporation of liberated [^{14}C]oleate into glycerolipids was measured after thin-layer chromatography. Cell-associated LDL-[^{14}C]CO and incorporation of oleate were expressed as dpm/mg protein. Abbreviations as in Table 1. Means ± SD of 3 different cell lines are presented.

[a]Significantly different from the uptake without exogenously added oleate.

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among cellular and media lipids was determined at various time points during the incubation time. The hydrolysis of cholesteryl ester was linear up to 2–3 h. After 3 h, 50 ± 11% (mean of three different cell lines) of the endocytosed LDL-CO was hydrolyzed. After 24 h, 12 ± 2% of the radioactivity originally present in CE was still present in CE. This low percentage of radioactivity in CE after 24 h incubation suggests that lysosome-derived oleic acid is incorporated into cholesteryl ester only to a low extent. At the same time it strongly suggests that after the 4 h preincubation the radioactivity associated with CE is predominantly (pre)lysosomal. Further evidence for this point will be presented below. **Figure 2** presents the data on the distribution of radioactivity in pellet and medium. During the incubation radioactivity decreased in the pellet with time and appeared in the medium (Fig. 2a). This radioactivity in the medium was already found after 15–30 min of incubation. The sum of the radioactivity in pellet and medium was constant over the whole incubation period and was equal to the amount of radioactivity associated with the pellet after the prelabeling period at t = 0. In the pellet radioactivity decreased in cholesteryl ester and increased in phospholipids and triacylglycerol (Fig. 2b). Lipid extraction of the medium showed that apart from a small and constant amount of radioactive cholesteryl ester, the radioactivity in the medium was present in the free fatty acid fraction (Fig. 2b). The sum of the radioactivity in the lipid extracts from medium and cell pellets was, at all measured time points, more than 90% of the radioactivity originally present in the pellet. Very small amounts of radioactivity were found in the water phase after extraction of the media, suggesting that only a low amount of lysosome-derived oleic acid could have been oxidized under the assay conditions used. Figure 2c presents the changes in radioactivity in pellet PL and TG and the radioactivity in medium free fatty acid (FFA) during the first 3 h in more detail. The rapid appearance of ^{14}C-labeled fatty acid in the medium preceded the increases in the radioactivity in PL and TG in the pellet.

To further examine the release of LDL-derived oleic acid into medium, we tested whether the composition of the medium affected the distribution of LDL-derived oleic acid among cells and medium. **Table 4** presents the results. No significant effect of the media on the hydrolysis of cholesteryl ester was noticed. However, the release of fatty acids was strongly influenced by the medium. The distribution of the newly released LDL-derived oleic acid among medium fatty acid and cell (PL + TG + FFA) was expressed as the ratio of radioactivity in FFA in the medium divided by the sum of the increase in radioactivity in cellular (PL + TG + FFA) and the medium FFA. By far the highest ratio was found

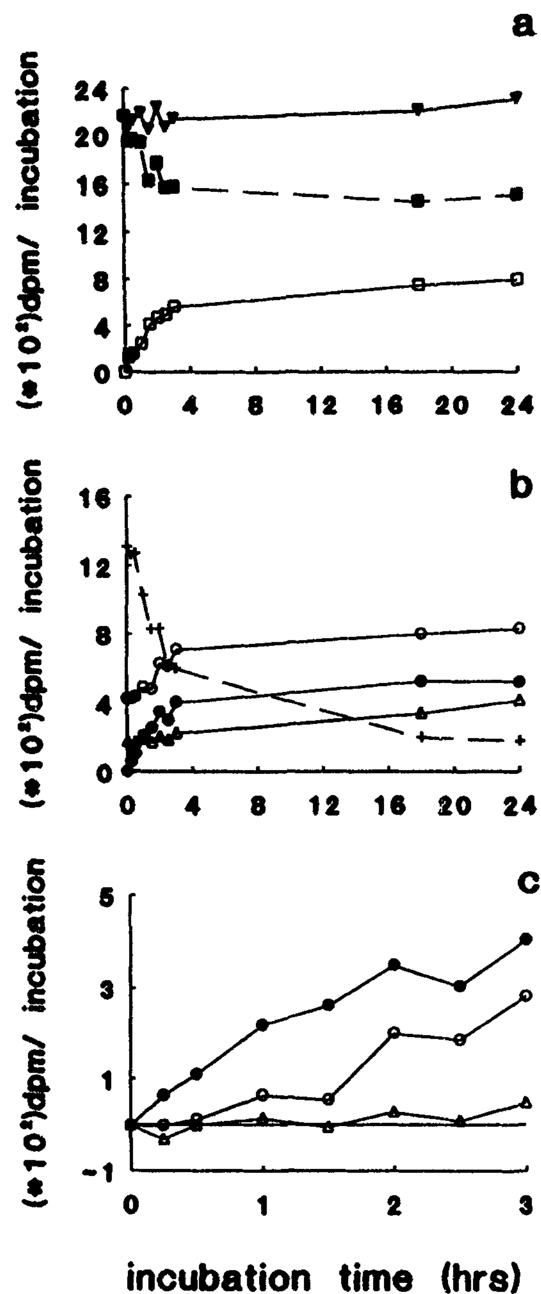

Fig. 2. Metabolic fate of LDL-derived oleic acid. Fibroblasts were preincubated for 4 h in medium containing LDL-[^{14}C]CO (50 μg LDL-protein/ml). Then cells were washed, incubated for 30 min at 18°C, followed by incubation at 37°C in fresh medium containing 10% FCS. Time 0 means the time at which fresh medium is added to the cells. After various times medium was removed and cells were harvested. Radioactivity was measured in various lipid fractions in pellet and medium. Panel a: radioactivity in cell pellet (■——■), radioactivity in medium (□——□), and total radioactivity in pellet plus medium (▼——▼). Panel b: radioactivity in pellet CE (+——+), in pellet PL (○——○), in pellet TG (△——△), and medium FFA (●——●). Panel c: Time curve for the first 3 h. Changes in radioactivity in pellet PL (○——○), pellet TG (△——△), and in medium FFA (●——●) as compared to t = 0 are plotted.

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TABLE 4. Effect of composition of the medium on the efflux of LDL-derived oleic acid

Medium	Time	Cell-CE	Cell PL + TG + FFA	Medium FFA	Ratio
	h		*dpm*		
	0	1315	355		
10% FCS	1.5	865	533	212	0.55
	3.0	666	656	297	0.50
	24.0	117	1122	539	0.41
10% LPDS	1.5	862	388	318	0.91
	3.0	619	462	567	0.84
	24.0	34	617	877	0.77
BSA, 2 mg/ml	1.5	866	537	173	0.49
	3.0	619	697	235	0.41
	24.0	61	933	479	0.47
No protein[a]	1.5	541	611	17	0.06
	3.0	315	741	17	0.04
	24.0	45	830	18	0.04

Control fibroblasts were preincubated for 4 h in medium containing 10% LPDS and LDL-[^{14}C]CO (50 μg LDL-protein/ml). Then cells were washed and incubated for 30 min in Ham's F10 (10% FCS) medium at 18°C. The experiment was started with the addition of fresh Ham's F10 containing either 10% FCS, 10% LPDS, 2 mg BSA/ml, or no protein. After various times the medium was removed and cells were harvested. Radioactivity was measured in pellets and medium as described in Materials and Methods. Abbreviations as in Tables 1 and 3. The ratio is calculated as radioactivity in medium FFA divided by the sum of the increase in radioactivity in cellular (PL + TG + FFA) plus the radioactivity in FFA medium. Means of duplicate incubations of a typical experiment are presented. Duplicate incubations varied within 10%.

[a]Under conditions in which no protein was present in the medium, the recovery of cell protein was slightly less than with protein in the medium. The recovery of cell-associated radioactivity was proportionally decreased.

when 10% LPDS was present in the medium, a much lower ratio was found in the presence of 10% FCS and 2 mg/ml BSA. When no protein was added to the medium, no fatty acids were found in the medium. Apart from the effect on release of fatty acids into the medium, the incorporation of oleic acid into triacylglycerols was decreased in the presence of medium supplemented with BSA (2 mg/ml). This effect was particularly seen after 24 h of incubation (data not shown).

We next tested whether agents known to block the export of cholesterol from the lysosome (progesterone, sphinganine, imipramine), agents known to effect lysosomal function (NH_4Cl, chloroquine, monensin, and nigericin), an agent known to affect vesicle transport (brefeldin A), an agent known to disrupt the cytoskeleton (nocodazole), and metabolic energy poisons (NaN_3/NaF) influence lysosomal transport and metabolic fate of LDL-derived oleic acid. **Table 5** summarizes the results of these experiments. Radioactivity in the various lipid fractions in cells and media was measured 1.5 and 3 h after addition of the fresh medium. Agents known to effect LDL metabolism at certain points in its

TABLE 5. Effect of various pharmacological agents on the metabolic fate of LDL-derived oleic acid

Addition	Time	Cell-CE	Cell PL + TG + FA	Medium FFA	Ratio
	h		*dpm*		
Exp. 1	0	1032	318		
Control	1.5	625	465	269	0.85
	3.0	466	540	334	0.60
NH_4Cl	1.5	765	296	158	nc
	3.0	715	354	138	0.79
Imipramine	1.5	852	434	130	0.53
	3.0	631	429	201	0.64
Progesterone	1.5	799	357	177	0.82
	3.0	719	429	208	0.65
Sphinganine	1.5	922	338	75	0.79
	3.0	820	360	127	0.75
Exp. 2	0	1092	387		
Control	1.5	711	600	224	0.51
	3.0	558	642	278	0.52
Monensin	1.5	948	405	65	nc
	3.0	956	407	56	nc
Chloroquine	1.5	1096	426	50	nc
	3.0	1058	422	72	nc
Nocodazole	1.5	661	551	220	0.57
	3.0	576	548	273	0.63
NaN_3/NaF	1.5	735	410	284	0.93
	3.0	688	387	381	1.00
Exp. 3	0	1315	355		
Control	1.5	864	494	212	0.60
	3.0	681	625	296	0.52
Brefeldin A	1.5	941	481	204	0.62
	3.0	691	634	335	0.56
Nigericin	1.5	1103	269	48	nc
	3.0	1108	268	52	nc

Control fibroblasts were preincubated for 4 h with Ham's F10 (10% LPDS) medium containing LDL-[^{14}C]CO (50 μg LDL-protein/ml). Then cells were washed and incubated for 30 min in Ham's F10 (10% FCS) medium at 18°C. The experiment was started with the addition of Ham's F10 supplemented with 10% FCS and one of the following additions: none, 20 mM NH_4Cl, 10 μM imipramine, 10 μg/ml progesterone, 10 μM sphinganine, 10 μM monensin, 20 μM chloroquine, 20 μg/ml nocodazole, 10/20 mM NaN_3/NaF, 4 μg/ml brefeldin A, 20 μM nigericin. After 1.5 and 3 h the medium was removed and cells were harvested. Radioactivity was measured in pellets and medium as described in Materials and Methods. Abbreviations as in Tables 1 and 3. Means of duplicate incubations are presented. Duplicate incubations varied within 10%; nc, not calculable.

catabolic pathway (NH_4Cl, chloroquine, monensin, and nigericin) caused a total or partial (NH_4Cl) inhibition of hydrolysis of cholesteryl oleate. These experiments show that under conditions of no further lysosomal hydrolysis of LDL-[^{14}C]CO e.g., in the presence of nigericin, chloroquine, and monensin, only very small amounts of free fatty acids were recovered from the medium, indicating that the source of free fatty acids in the medium measured during the incubation was lysosomal cholesteryl ester and not the glycerolipids formed during the pre-incubation. In addition, these experiments confirmed that most of the [^{14}]CO associated with the cells after the 4 h preincubation was (pre)-lysosomal. Imipramine, sphinganine, and progesterone, agents

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known to block cholesterol export from the lysosome, did not seem to have a major effect on the release of fatty acids from the lysosome; however, these three agents partially inhibited LDL-CE catabolism. Agents that affect the cytoskeleton (nocodazole) or vesicle transport (brefeldin A) did not have an effect on the hydrolysis of LDL-CO nor on lysosomal release or the subsequent metabolic fate of the released oleic acid. The metabolic energy poisons (NaN_3/NaF) did not have an effect on the hydrolysis of LDL-[^{14}C]CO but inhibited incorporation of oleic acid into phospholipids and triacylglycerol. A concomitant increase in the amount of oleic acid in the medium was found. In addition to the above mentioned effect, progesterone stimulated the incorporation of oleic acid into triacylglycerol at the expense of incorporation into phospholipids (data not shown).

DISCUSSION

In order to study the metabolic fate of fatty acids released from lysosomes we used LDL labeled with [^{14}C]cholesteryl oleate as a source of fatty acids. We first showed that the LDL labeled with [^{14}C]cholesteryl oleate by the use of plasma lipid transfer protein is taken up by the LDL receptor and that, subsequently, cholesteryl ester is exclusively catabolized by lysosomal acid lipase. For this purpose we used LDL-receptor and acid lipase-deficient cell lines (Fig. 1a).

The metabolic fate of oleic acid was studied under two different experimental conditions. One set of experiments was performed under conditions of continuous labeling of the cells with LDL-[^{14}C]CO present in the incubation medium. In the other set of experiments the cells were prelabeled with LDL-[^{14}C]CO and the metabolism of [^{14}C]oleate was subsequently studied in a medium devoid of exogenous LDL. The results of the experiments in the presence of LDL showed a predominent incorporation of lysosome-derived oleic acid into phosphatidylcholine (Fig. 1b and Table 2) and to a lesser extent into triacylglycerol. Similar results were recently reported in studies with macrophages by Li, Fuhrman, and Aviram (15) for the incorporation of linoleate into phosphatidylcholine. The distribution of lysosome-derived oleic acid and exogenously added oleic acid among various glycerolipids in the cell is strongly influenced by the concentration of oleic acid added to the cells (Tables 1 and 2), suggesting that lysosome-derived oleic acid and exogenously added oleic acid enter a common pool of fatty acid used for glycerolipid synthesis. This suggestion was strengthened by the results of double label experiments (Table 3). Obviously, from these experiments no conclusion can be drawn as to whether lysosome-derived oleic is used for cholesteryl ester synthesis in the endoplasmic reticulum. However, the data on the amount of radioactivity associated with CE (Fig. 1b) and the data on the incorporation of exogenously added oleic acid into cholesteryl ester and the common pool of oleic acid used for glycerolipid synthesis (Tables 1 and 3) suggest that only a low amount of lysosome-derived oleic acid is used for cholesteryl ester formation. A concentration-dependent incorporation of fatty acids into various glycerolipids in cultured fibroblasts, isolated hepatocytes, and smooth muscle cells has been reported (16–18). In a recent paper Teruya et al. (19) suggested that in endothelial cells the source of oleic acid, either LDL-cholesteryl ester or LDL-triacylglycerol, may be a factor determining the metabolic fate of lysosome-derived oleic acid. Whether this also holds true for other cell types needs further research.

The experiments with cells prelabeled with LDL-[^{14}C]CO show that a considerable portion of lysosome-derived oleic acid is released into the medium. This oleic acid must have originated from lysosomal hydrolysis of cholesteryl ester because under conditions where lysosomal hydrolysis is blocked (in the presence of monensin, nigericin, and chloroquine) there is hardly any release of labeled fatty acid into the medium. To our knowledge efflux of lysosome-derived oleic acid has not been reported before. The time course (Fig. 2c) shows that the release of oleic acid into the medium is an early event and precedes the incorporation of oleic acid into glycerolipids. Therefore it seems justified to hypothesize that the lysosome-derived oleic acid might be transported to the plasma membrane prior to either transport to the endoplasmic reticulum or release into the medium. This hypothesis is strengthened by the finding that under conditions of equal hydrolysis of lysosomal cholesteryl ester, the composition of the medium effects the metabolic fate of oleic acid (Table 3). Oleic acid is either released into the medium to an extent depending on the type of acceptors present in the medium or used for esterification in the absence of acceptors. The role of serum albumin in the uptake of long chain fatty acids by mammalian cells has been studied thoroughly although the exact mechanism is as yet unknown (20). A similar role of albumin might also be of importance in the efflux process described in the present study.

We tested several agents for their ability to inhibit passage of oleic acid from the lysosome. Imipramine (21, 22), progesterone (23), sphinganine (24) are agents known to cause accumulation of cholesterol in lysosomes. In our experiments these agents partially inhibited LDL-CE hydrolysis but did not cause any intracellu-

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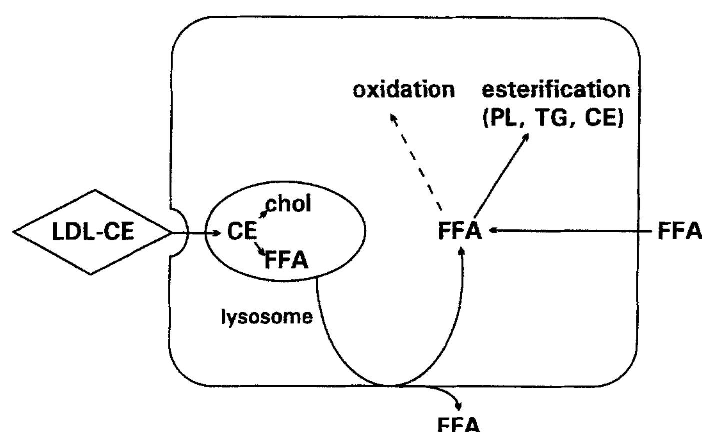

Fig. 3. Schematic presentation of metabolic fate of LDL-derived fatty acid in human fibroblasts. Fatty acid acid, originating in LDL-CE taken up by the cell by the LDL-receptor and formed in the lysosome by acid lipase, is transported to the plasma membrane; from there it is secreted into the medium or re-enters the cell for various metabolic processes. Abbreviations: chol, free cholesterol; CE, cholesteryl ester; FFA, free fatty acids; LDL, low density lipoprotein.

lar accumulation of oleic acid. A similar effect was found for NH_4Cl. The cytoskeleton-disrupting agent, nocodazole, and an inhibitor of vesicle transport, brefeldin A, had no effect on the metabolic fate of LDL-derived oleic acid. We found no evidence that the cytoskeleton or vesicle transport were involved in transport of lysosome-derived oleic acid. The effect of the energy poisons (NaN_3/NaF) (25) is of interest (Table 4). Under these conditions in which cholesteryl ester hydrolysis is unaffected and glycerolipid synthesis is inhibited, LDL-derived oleic acid did not accumulate in the cell but was released into the medium, pointing again to an important role of the plasma membrane in the distribution of LDL-derived oleic acid between medium and cell. The process of fatty acid transport from lysosome to the medium seems to be energy-independent. Efflux of lysosome-derived fatty acids into the medium involves passage over the lysosomal membrane, transport through the cytosol to the plasma membrane, and transport over the plasma membrane followed by removal of fatty acids from the plasma membrane. Except for the passage of fatty acids over the lysosomal membrane, the individual steps of fatty acid transport have been studied for many years, but the exact mechanisms are unknown. Fatty acid transport across the plasma membrane of various cell types has been considered as a passive diffusion process, as a protein-mediated transport process, or as both (7–9, 20). Cytosolic fatty acid binding proteins (FABPs) are thought to be involved in the transport of fatty acids from plasma membrane to cell organelles (7–9, 18, 26). Transport of long chain fatty acid across intracellular membranes has been studied for mitochondria and peroxisomes (27, 28). It is generally accepted that unesterified fatty acids constitute a minor fraction of lipid in the cell but may play a central role in metabolic and regulatory processes. Apart from providing substrates for energy production and lipid synthesis, unesterified fatty acids have been reported to participate in processes like signal transduction pathways (29) and cell regulation (30). Thus, cellular levels of free fatty acids must be tightly controlled. Little information is known about the involvement of lysosome-derived fatty acids in these processes.

In contrast to the few studies on the metabolic fate of lysosome-derived fatty acids, a large number of studies on the metabolic fate of lysosome-derived cholesterol have been published (see review, ref. 5). It is of interest that these studies do suggest that lysosome-derived cholesterol is also transported to the plasma membrane prior to its movement to endoplasmic reticulum (31) where it is converted to cholesteryl ester.

Our current hypothesis on the metabolic fate of LDL-cholesteryl ester-derived fatty acids in human fibroblasts is summarized schematically in **Fig. 3.** In this scheme oleic acid is transported to the plasma membrane where it is either released into the medium or re-enters the cell and is transported to the endoplasmic reticulum for glycerolipid synthesis.

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