The Turnover of Cytoplasmic Triacylglycerols in Human Fibroblasts Involves Two Separate Acyl Chain Length-dependent Degradation Pathways*

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Cultured fibroblasts from patients affected with the genetic metabolic disorder named neutral lipid storage disease (NLSD) exhibit a dramatic accumulation of cytoplasmic triacylglycerols (Radom, J., Salvayre, R., Nègre, A., Maré, A., and Douste-Blazy, L. (1987) Eur. J. Biochem. 164, 703-708). We compared here the metabolism of radiolabeled short-, medium- and long-chain fatty acids in these cells. Short/medium-chain fatty acids (C4-C10) were incorporated into polar lipids (60-80%) and triacylglycerols (20-40%) at a lower rate (5-10 times lower) than long-chain fatty acids. Pulse-chase experiments allowed to evaluate the degradation rate of cytoplasmic triacylglycerols in normal and NLSD fibroblasts and to discriminate between two catabolic pathways of cytoplasmic triacylglycerols. Short/medium-chain (C4-C10) triacylglycerols were degraded at a normal rate in NLSD fibroblasts, whereas long-chain (C12 and longer) triacylglycerols remained undegraded. These data are confirmed by mass analysis. The use of diethylparanitrophenylphosphate (E600) and parachloromercuribenzoate (PCMB) inhibitors allows to discriminate between the two triacylglycerol degradation pathways. E600 inhibited selectively the in situ degradation of short/medium-chain triacylglycerols without inhibition of the degradation of long-chain triacylglycerols, whereas PCMB inhibited selectively the in situ hydrolysis of long-chain triacylglycerols without affecting the degradation of long-chain triacylglycerols. This was correlated with the in vitro properties of cellular triacylglycerol-hydrolyzing enzymes characterized by their substrate specificity and their susceptibility to inhibitors; the neutral lipase specific to long-chain triacylglycerols is inhibited by PCMB, but not by E600, in contrast to short/medium-chain lipase, which is inhibited by E600 but not by PCMB. The data of in vitro and in situ experiments suggest the existence in fibroblasts of two separate acyl chain length-dependent pathways involved in the degradation of cytoplasmic triacylglycerols, one mediated by a neutral long-chain lipase and another one mediated by a short/medium-chain lipase.

In human fibroblasts, triacylglycerols are degraded in two separate and independent subcellular (lysosomal and cytoplasmic non-lysosomal) compartments (1). In the lysosomal compartment, triacylglycerols and cholesteryl esters of low density lipoprotein and very low density lipoprotein taken up by cells are degraded by the acid lysosomal lipase (2, 3), which is genetically deficient in Wolman disease (4). In the cytoplasmic compartment of fibroblasts, triacylglycerols are degraded by a lipase system (yet poorly characterized) different from the other known cellular (hormone-sensitive lipase) or secretory lipases (1, 5) and different from the cholesteryl ester degradation pathway (6). As shown by using radiolabeled oleic acid or pyrene-containing fluorescent fatty acids, this degradative pathway of cytoplasmic triacylglycerols is deficient in the neutral lipid storage disease (NLSD)1 (1, 5, 7–10), a rare inherited metabolic disease generally characterized by the association of muscular weakness, ichthyosis, and multisystemic triacylglycerol storage (11, 12). We have recently reported that the pool of cytoplasmic triacylglycerols accumulated in fibroblasts from neutral lipid storage disease is constituted by triacylglycerols endogenously biosynthesized (7–10) and by triacylglycerols taken up from high density lipoproteins (13). An increased biosynthesis of phosphatidylethanolamine has also been reported (14).

Comparative studies of the uptake and metabolic utilization of short-chain and long-chain fluorescent pyrene fatty acids in cultured lymphoblasts (15) and in fibroblasts (16) have shown that short-chain (pyrene-butanoic) acid was incorporated into phospholipids but not in triacylglycerols, whereas long-chain (pyrene-decanoic and pyrene-dodecanoic) fatty acids were incorporated into phospholipids and triacylglycerols. In lymphoblasts from neutral lipid storage disease pulsed with short-chain pyrene fatty acid, we detected no significant accumulation of fluorescent triacylglycerols (15). These results led us to speculate that replacing natural long-chain fatty acids by short-chain fatty acids could be of interest to slow down the triacylglycerol accumulation in neutral lipid storage disease cells. However, the conclusions obtained with fluorescent pyrene fatty acids cannot be directly transposed to natural fatty acids because natural and pyrene fatty acids exhibited several known metabolic differences (5, 8, 17). The apparent lack of incorporation of short-chain fluorescent fatty acid (pyrene-butanoic) in triacylglycerols and of accumulation of pyrene-butanoic containing triacylglycerols in NLSD cells could be due either to a lack of biosynthesis of pyrene-butanoic-containing triacylglycerols (despite the biosynthesis of pyrene-butanoic-

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containing glycerophospholipids) or to a rapid degradation of pyrene-butanoate-containing triacylglycerols by a cytoplastic pathway independent of the neutral lipase system (deficient in intact NLSD cells). This latter hypothesis is supported by the existence in fibroblast homogenates of five different enzymes able to hydrolyze in vitro long- or short-chain fluorescent triacylglycerols. Both groups of enzymes referred to as long-chain lipases or short/medium-chain lipases, respectively, can be discriminated by their enzymatic properties (heat stability, effect of inhibitors) (1, 18), with short/medium-chain lipases exhibiting properties of (nonspecific) carboxylesterases (19).

This prompted us to investigate the metabolism of short- and long-chain fatty acids in cultured fibroblasts from controls and from neutral lipid storage disease to examine the metabolic fate of short-chain fatty acids and their potential influence on the triacylglycerol accumulation in neutral lipid storage disease.

The data reported here showed that, in comparison to long-chain fatty acids, short/medium-chain fatty acids 1) are incorporated into cellular lipids at a much lower rate, 2) induce a lower accumulation of triacylglycerols in NLSD cells, and 3) contained in triacylglycerols are hydrolyzed at a normal rate through a catabolic pathway involving a short/medium-chain fatty acid lipase activity not deficient in situ in NLSD cells (in contrast to the defect of the long-chain lipase activity).

**MATERIALS AND METHODS**

Chemicals—[1-14C]Octanoic acid (55 Ci/mmol), [1-3H]Iododecanoic acid (25 Ci/mmol), [3H]Palmitylic acid (60 Ci/mmol), [1-14C]Leic acid (10 Ci/mmol), and [1-3H]Iodoic acid (26 Ci/mmol) were from Du Pont NEN (Les Ulis, France); bovine fatty acid-free albumin, tridecen-3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium iodide, diethyl-p-nitrophenylphosphate (E600), [1-14C]Butanoic acid (25 Ci/mmol), [1-3H]Hexanoic acid (10 Ci/mmol), [1-14C]Decanoic acid (100 Ci/mmol), and unlabelled fatty acids (butanoic, hexanoic, octanoic, decanoic, dodecanoic, palmitic, and oleic acids) were from Sigma; [1-14C]Leic acid (47 Ci/mmol) and LM-1 autoradiographic emulsion for light microscopy was from Amersham (Les Ulis, France); Silica Gel G and RP18 thin layer chromatography plates were from Merck (Darmstadt, Germany); RPMI 1640 with Glutamax®, fetal calf serum, streptomycin, and penicillin, from Amersham (Les Ulis, France); Silica Gel G and RP18 thin layer chromatography plates were from Merck (Darmstadt, Germany); RPMI 1640 with Glutamax®, fetal calf serum, streptomycin, and penicillin were from Seromed (Strasbourg, France); Ultroser G was from IBF (Villeneuve-la-Garenne, France); Instafluor was from Packard (Warrer-rville, IL); Aquasafe 300 Plus was from Zinsser Analytic (Mainheaden, United Kingdom); and the other reagents were from Merck or Prabro (Paris).

**Cell Cultures**—Skin fibroblasts were obtained from normal individuals (n0, n2), from two patients affected with NLSD (Bo. and Dem.) and with Wolman disease (GM1606). NLSD fibroblasts (N1 and N2) were kindly provided by Drs. J. M. Mussini and S. Billaudel (Nantes, France) and by Dr. B. Wineson (London). GM1606 cells were purchased from the NIGMS, National Institutes of Health, Human Genetic Mutant Cell Repository (Camden, NJ). Other fibroblasts from normal subjects (n0, n2) were from our laboratory. Lymphoblastoid cell lines were obtained by Epstein-Barr virus (895/8), and transformation of blood B lymphocytes from normal individuals (n0 and n4) or from a patient affected with NLSD (Bo) was as previously indicated (5). Fibroblasts and lymphoblasts were grown at 37°C in 5% CO2, 95% air in RPMI 1640 medium with penicillin (100 units/ml) and streptomycin (100 µg/ml) and supplemented with 10% fetal calf serum or 2% Ultroser G or H Y (lipoprotein-free serum substitutes for fibroblasts and lymphoblasts, respectively) under the previously used conditions (14, 15). Cell cultures were always used in the same growth state since the level of fatty acid incorporation into cellular lipids may be influenced by the growth rate of cells (18); fibroblasts were used at confluency, and lymphoblasts were maintained in exponential phase.

Cell viability was assessed by 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide test (20) or by trypsin blue test and by morphological examination as previously used (21), which shows that the number of necrotic and apoptotic cells did not exceed 8–10% during the metabolic experiments.

Pulse-Chase Experiments with Fatty Acids—The culture conditions defined to induce a large cellular biosynthesis and accumulation of radiolabeled triacylglycerols were derived from data previously reported (7, 8) and from preliminary experiments. Before studies were initiated, fibroblasts and lymphoblasts were grown in a lipoprotein-free medium (RPMI 1640 containing 2% Ultroser G) for the period of time indicated in the figure legends.

Cells were pulsed with fatty acids, used at the concentrations indicated in the text, generally 30 nmol/ml and 106 dpm, solubilized in Me2SO (0.5% final concentration), and preincubated with the culture medium (RPMI 1640 with 2% Ultroser G) for 30 min at 37°C before addition to the medium containing radiolabeled fatty acids. Before the end of the pulse period (12 or 24 h, as indicated in the text), the fatty acids were removed by washing the cells twice with phosphate-buffered saline containing 10 mmol/ml bovine serum albumin (essentially fatty acid-free) and twice with phosphate-buffered saline. One batch of cells was harvested at the end of the pulse (time 0 of the chase); the other batch was grown in a fresh medium containing no additional fatty acids and harvested at the indicated time of this chase period. Under the experimental conditions used here, fatty acids and Me2SO had no adverse effect on cell viability.

**Lipid Extraction and Analysis**—At the indicated time, cells were washed twice with phosphate-buffered saline supplemented with 10 mmol/ml bovine (essentially fatty acid-free) serum albumin and twice with phosphate-buffered saline. Lymphoblasts were pelleted by centrifugation (2,000 x g for 10 min), and fibroblasts were harvested by scraping with a rubber policeman. Cells were homogenized in 1 ml of distilled water by sonication (2 cycles of 15 s, Soniprep 150). A aliquot (50 µl) was used for protein determination, and another (50 µl) was used for counting the total cellular radioactivity. The remaining aliquot (50 µl) was used for lipid analysis by two procedures: A) Lipids of the samples extracted by the Folch procedure (22) and separated by TLC on Silica Gel G plates, using petroleum ether/diethyl ether/acetic acid (80/20/1 (v/v/v)) for the separation of the neutral lipids and chloroform/methanol/water (100/426/2 (v/v/v)) for the separation of the phospholipids. Lipid spots were visualized by iodine vapors, and the radiolabeled lipids were determined directly on the TLC plate by using a TLC-radiochromatography scanner Berthold under the previously used conditions (9, 10). Alternatively (when using low levels of radiolabeled lipids), triacylglycerols (the major radiolabeled neutral hydrophobic lipids) were separated from polar lipids by a solvent partition system derived from the Dole's procedure (23) as previously used (13). Briefly, the chloroformic phase of the lipid extract was washed with water and was evaporated under nitrogen, and the 1.25 ml of alkaline Dole's mixture (isopropanol/2 M sodium carbonate, pH 10.5, NaOH-glycine buffer, 40/10, 0.75 ml of heptane, and 1 ml of water were added to the lipid residue. After mixing and centrifuging, the phases were separated and backwashed once with the fresh phases (the upper phase with 2 ml of fresh lower phase and the lower phase with 1 ml of fresh upper phase). Under these conditions, the recovery of triacylglycerols in the heptane phase was better than 98%, and the contamination of the aqueous phase by triacylglycerols was negligible (lower than 0.1%). These data were confirmed by the thin layer chromatography analysis (and quantification by TLC-radiochromatography scanner Berthold) of radiolabeled lipids contained in each phase (data not shown). The radioactivity was determined in a liquid scintillation counter in Picofluor® using a Packard β counter (Tricarb 4530).

Mass analysis of cellular lipids was performed by gas liquid chromatography. After adding triptenadecanoin (50 µg used as standard), cellular lipids were extracted according to the Folch procedure. After drying of the chloroformic phase, lipids were partitioned in the Dole's biphasic solvent system under the above indicated conditions. After evaporation of the heptane phase, neutral lipids were solubilized in 50 µl of hexane, and triacylglycerols were determined by gas liquid chromatography under the previously used conditions (24) (Carlo Erba GC 8000: 6 m CP-Sil 5CB, 0.32-mm diameter capillary column; oven temperature programmed from 220 to 350°C, 5°C per min, flame ionization detector 370°C, carrier gas: N2, 35 ml/min). For the determination of the chloroformic phase of the Folch extract) were evaluated by their phosphorus content determined according to the method of Chen et al. (25). Alternatively, gross triacylglycerol mass was estimated by a method derived from efferson et al. (26) (briefly, after TLC separation of neutral lipids on Silica Gel G plates as above indicated (petroleum ether/diethyl ether/acetic acid, 80/20/1 (v/v/v)) for the determination of the chloroformic phase of the Folch extract) were evaluated by their phosphorus content determined according to the method of Chen et al. (25).

In Vivo Enzymatic Assays and Preparation of [1-14C]Decanoic-containing Triacylglycerols—Radiolabeled [1-14C]Decanoic-containing triacylglycerols were biosynthesized in cultured fibroblasts or lymphoblasts pulsed for 24 h with [1-14C]Decanoic acid (30 nmol/ml, 70,000 dpm/ml)
in the presence of 1 μmol/liter E600 (used to block the degradation of short-chain triacylglycerols, see Fig. 6). Then, cells were washed twice with phosphate-buffered saline and homogenized in water by sonication. Neutral lipids were extracted by partition in the Dole's solvent system (21) and [14C]decanoic-containing triacylglycerols were purified by preparative thin layer chromatography on silica gel G (solvent system: ethyl ether/diethyl ether/acidic acid, 80/20/1). The specific radioactivity of the [14C]decanoic-containing triacylglycerols (70,000 dpm/nmol) was calculated on the basis of that of the [14C]decanoic acid incorporated into the culture medium (assuming that the non-labeled and radiolabeled decanoic acids were incorporated into triacylglycerols at the same rate).

Enzymatic Assays—Enzyme solutions were prepared by homogenizing fibroblasts or lymphoblasts in distilled water and by sonication (3 cycles of 10 s, using MSE sonicator, Soniprep 150). The standard enzymatic assays of the enzymes hydrolyzing [14C]decanoic-containing triacylglycerols (referred to as carboxyl esterases) contained 5 nmol of radioactivity of the [14C]decanoic-ac containing triacylglycerols and 50 nmol of egg phosphatidylcholines dispersed in the 0.2 M citratephosphate buffer, pH 7.2, and the enzyme preparation (100 μg of protein) in a final volume of 200 μl. The standard assay for determining lipase activity contained 10 nmol/assay and 105 dpm [3H]triolein (10,000 dpm/nmol), 0.1% Triton X-100, 0.2 M citratephosphate buffer, pH 7.2, and the enzyme solution (100 μg of protein) in a final volume of 200 μl. When varying the pH of the assay, we used citratephosphate buffers (from pH 3.5 to 7.0) and Tris-HCl (from pH 7.2 to 9.0). At the end of the incubation time (2 h at 37 °C, under the standard conditions), the liberated fatty acids were extracted according to the procedure of Belfrage and Vaughan (27), and the radioactivity extracted in the aqueous phase was determined by liquid scintillation counting (in Aquasafe-300-Plus, using a Packard β counter, Tricarb 4530). Enzyme activities of the homogenates were generally expressed as nmol of fatty acid liberated per hour and per milligram of cell protein. Protein concentrations were determined using the method of Lowry et al. (28).

Cell Microautoradiography—Fibroblasts, grown on microscopy cover glasses, were pulsed for 12 h with [14C]decanoic or [14C]oleic acid (30 nmol/ml and 2.5 μCi/ml). After washing the cells under the previously described conditions, one batch was immediately used for microautoradiography, and another batch was chased for an additional 48-h period in RPMI containing 2% Ultraser G before use for microautoradiography. Cells were fixed for 20 min at 4 °C in cetylalcoholate buffer 0.1 M, pH 8.0, containing 2% glutaraldehyde, then washed twice with distilled water; cover glasses were then immersed in LM-1 autoradiographic emulsion for light microscopy, under the procedure indicated by the manufacturer. After 1-3 days (at 4 °C in the dark), microautoradiographies were developed and examined by light microscopy (Leica Diaplan).

RESULTS

The Accumulation of Triacylglycerols in NLSDFibroblasts is Largely Dependent on the Presence of Lipids in the Culture Medium—As shown in Fig. 1, triacylglycerol levels are higher in NLSDFibroblasts than in normal cells, in agreement with previously reported results (7–10). But these data also show that the rate of accumulation of triacylglycerols in NLSDFibroblasts is largely dependent on the intake of extracellular lipids. NLSDFibroblasts grown in culture medium supplemented with 10% fetal calf serum exhibit a large accumulation of triacylglycerols, whereas the triacylglycerol accumulation was relatively lower in NLSDFibroblasts grown in a serum-free medium for 15 days (when triacylglycerol levels are related to the cell proteins) (Fig. 1). This led us to hypothesize that the rate of triacylglycerol accumulation in NLSDFibroblasts may be slowed down by modifying the lipid composition of the culture medium. Moreover, as we have previously reported that short-chain pyrene fatty acids did not induce any storage of fluorescent triacylglycerols in NLSDFibroblasts (because of the lack of incorporation of short-chain fluorescent fatty acid analogs into triacylglycerols) (15), we studied the metabolism of natural (non-fluorescent) fatty acids with various acyl-chain lengths and compared their influence on the triacylglycerol storage in NLSDFibroblasts.

Incorporation of Radiolabeled Fatty Acids with Various Acyl Chain Lengths into Cellular Lipids—When fibroblasts were grown for 12 h in a medium containing a fixed concentrations of radiolabeled fatty acids (30 nmol/ml, 33,000 dpm/nmol) with various chain lengths, the level of the radiolabeled fatty acids incorporated into cellular lipids at the end of this pulse period was largely dependent on the acyl chain length (Fig. 2). Short- and medium-chain fatty acids used here (from butanoic to dodecanoic acid) were incorporated into cellular lipids at a lower rate (5–10 times lower) than the long-chain fatty acids (palmitic and oleic acids). Under the conditions used here (pulse for 12 h with 30 nmol/ml fatty acid), incorporation of radiolabeled fatty acids into triacylglycerols of normal cells ranged between 20 and 40% (of total radiolabeled lipids) (Fig. 3). A large part (60–80%) of the cell-associated fatty acids was incorporated into phospholipids (Fig. 3). Medium-chain and long-chain fatty acids were incorporated in the main classes of phospholipids, but [3H]oleic acid incorporated in phosphatidylethanolamine was higher than that of [14C]octanoic acid (Table I). Under the used conditions, we observed no significant difference between the phospholipid classes of NLSDFibroblasts and controls. At the end of the pulse, the radioactivity was localized in both the plasma membrane and the cytoplasm (Fig. 4, A, C, E, and G).

It is noteworthy that, under the used experimental conditions of the pulse (12 or 24 h), elongation of C10 incorporated into cellular lipids was negligible. Reversely, we did not detect any appreciable amount of short derivatives of [3H]oleic acid incorporated into cellular lipids. Similar data were observed with lipids extracted at the end of the chase (data not shown). These data allow to conclude that the chain lengths of the main part of fatty acids incorporated into cellular lipids remain unaltered during the time of the experiments reported here.

Study of the Degradation of Endogenously Biosynthesized Triacylglycerols Containing Fatty Acids with Various Chain Lengths (Figs. 3 and 4)—Pulse-chase experiments clearly showed that the short- and medium-chain radiolabeled fatty acids (from C4 to C10) incorporated into triacylglycerols were degraded at a similar rate in NLSDFibroblasts and in control fibroblasts (Fig. 3, A–D). In contrast, triacylglycerols containing radiolabeled C12 and longer fatty acids (C16 and C18:1) were degraded only in normal cells but not in NLSDFibroblasts (Fig. 3, E–H). The data, summarized in Fig. 3, D and H, show the clear-out discrimination between the group of fatty acids that accumulated in triacylglycerols of NLSDFibroblasts (C12 and longer fatty acids) and the group of short/medium-chain fatty acids that did...
experiments.

When cells were labeled with \([^{14}C]\)oleic acid, the cyto-

philicfeaturesareinagreementwiththemetabolicstudies

persistent only in cell membranes (Fig. 4, and on the basis of 33,000 dpm/nmol). Mean ± S.E. of four experiments.

not accumulate (C4–C10).

These conclusions were also supported by morphological (mi-

From controls and patients with NLSD and Wolman disease at the end of the pulse period. Cells were incubated for 12 h in RPMI 1640 medium supplemented with 2% Ultroser G and fatty acids (radio-

labeled fatty acids, 10^6 dpm/ml, were mixed with non-labeled fatty acid, 30 nmol/ml, i.e. 33,000 dpm/nmol). At the end of the pulse, fibroblasts were washed and harvested. Lipids were extracted by the procedure of Folch, and the radioactivity of the chloroformic phase was determined by liquid scintillation counting. The level of radiolabeled fatty acids incorporated into cellular lipids was calculated assuming that the radio-

labeled and non-labeled fatty acids have the same metabolic fate (and on the basis of 33,000 dpm/nmol). Mean ± S.E. of four experiments.

As the accumulation of long-chain triacylglycerols in NLSD

cells is due to a defect of the degradation pathway, it was suggested that, in the cytoplasmic compartment, short/medium-chain triacylglycerols could be degraded by an enzymatic system specific to short/medium-chain triacylglycerols in NLSD cells (Fig. 5D).

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Mass Evaluation of Medium-chain and Long-chain Triacyl-

glycerol Metabolism (Fig. 5)—To confirm by mass quantitation of medium- and long-chain triacylglycerols the results obtained with radiolabeled fatty acids, we used immortalized lympho-

blasts from normal subjects and from a patient affected with

NLSD. We used lymphoblasts for mass quantitations because

lymphoblasts are rapidly growing cells (dou-

bling time, 24–36 h) and grow in suspension, thus permitting to obtain relatively easily large amounts of cell material (nec-

essary for mass quantitation and analysis of lipids), and 2) NLSD lymphoblasts exhibit the same block of triacylglycerol degradation as fibroblasts (5).

Lymphoblasts grown in the presence of 30 nmol/ml C10 or C18:1 (for 24 and 12 h, respectively) showed that the levels of triacylglycerols, at the end of the pulse, were much lower in

cells grown in the presence of C10 than in cells grown in the presence of C18:1 (Fig. 5). In normal cells, the levels of triacyl-

glycerols were considerably reduced at the end of the 48-h chase (Fig. 5, A and B). In NLSD cells grown in the presence of oleic acid, the triacylglycerol level was almost unchanged (Fig. 5B), whereas it was reduced by about 50% in cells fed with C10 (Fig. 5A). Analysis of the triacylglycerol species in NLSD cells fed with C10 showed that triacylglycerols with a carbon number lower than 45 (triaclylglycerols containing C10 or short/medium-chain fatty acid) were degraded during the chase, whereas triacylglycerols with a carbon number higher than 48 (long-chain triacylglycerols) were not degraded (Fig. 5A). In
Acyl Chain Length Dependence of Triacylglycerol Degradation

TABLE I
Phospholipid composition of fibroblasts and lymphoblasts from NLSD patients and from normal subjects grown in the presence of radiolabeled [14C]decanoic or [3H]oleic acids

| Fatty acids | Cells (subjects) | PS/Pl | Sm | PC | PE |
|------------|----------------|-------|----|----|----|
| C8:0       | Fibroblasts    |       |    |    |    |
|            | NLSD Control 1 | 3 ± 0.5 | 17 ± 3 | 67 ± 7 | 9 ± 1 |
|            | NLSD Control 2 | 2 ± 0.3 | 13 ± 3 | 65 ± 9 | 8 ± 2 |
|            | Lymphoblasts   |       |    |    |    |
|            | NLSD Control 1 | 3 ± 0.5 | 11 ± 2 | 54 ± 6 | 11 ± 2 |
|            | NLSD Control 2 | 2 ± 0.5 | 18 ± 2 | 68 ± 7 | 10 ± 2 |

| C18:1      | Fibroblasts    |       |    |    |    |
|            | NLSD Control 1 | 2 ± 0.3 | 11 ± 2 | 54 ± 5 | 29 ± 4 |
|            | NLSD Control 2 | 2 ± 0.4 | 10 ± 2 | 58 ± 3 | 24 ± 4 |
|            | Lymphoblasts   |       |    |    |    |
|            | NLSD Control 1 | 3 ± 0.4 | 11 ± 1 | 56 ± 5 | 27 ± 3 |
|            | NLSD Control 2 | 3 ± 0.3 | 10 ± 2 | 51 ± 6 | 29 ± 5 |

![Fig. 4](source.jpg)

**FIG. 4.** Microautoradiography of fibroblasts from a normal subject (A, B, E, F) and from NLSD (C, D, G, H) pulsed for 12 h with radiolabeled fatty acids (30 nmol/ml and 2.5 μCi/ml), [14C]decanoic acid (A-D), or [3H]oleic acid (E-H) and chased for an additional 48 h in a fatty acid-poor medium, as described under “Materials and Methods.” Microautoradiographies were performed (under the procedure described under “Materials and Methods”; exposure times were 4 and 1.5 days for [14C]decanoic acid and [3H]oleic labeled cells, respectively) after fixing the cells, either at the end of the pulse period (upper panel, A, C, E, G) or at the end of the chase period (lower panel, B, D, F, H). Magnification, 800×.

Cells were incubated at 37°C for 24 h (pulse period) in RPMI 1640 supplemented with 2% Ultroser G and radiolabeled fatty acids (10⁶ dpm/ml and 30 nmol/ml non-labeled fatty acid) as described under “Materials and Methods.” At the end of the pulse period, cells were washed and harvested, and the lipids were extracted and analyzed by TLC as described under “Materials and Methods.” Mean ± S.E. of three experiments. PS/Pl, phosphatidylycerine/phosphatidylglycerol; Sm, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

![Fig. 5](source.jpg)

**FIG. 5.** Mass analysis of cellular triacylglycerols in pulse-chase experiments with a fixed concentration of decanoic (A) or oleic acid (B) in lymphoblasts from NLSD patient or normal subject. Cells were grown in a serum-free RPMI medium for 15 days, under the standard conditions of Fig. 1. Then, cells were pulsed with 30 nmol/ml decanoic acid for 24 h or oleic acid for 12 h. After washing, one batch was harvested for analysis (p), and another batch was grown in lipid-free medium (i.e. serum-free and fatty acid-free medium) for a 24-h chase period and washed and harvested for analysis (ch). Lipids were extracted and analyzed either by gas liquid chromatography or by thin layer chromatography as described under “Materials and Methods.” Hatched and black bars represent triacylglycerols with carbon number <45 and >48, respectively (note that no significant level of triacylglycerols with the carbon number <45 was detected in cells fed with oleic acid (panel B)). Mean ± S.E. of three experiments. In insets are shown the triacylglycerol TLC spots of the relative cells treated as indicated above.

Involvement of Two Separate Enzymatic Pathways in the Degradation of Short-chain and Long-chain Triacylglycerols (Figs. 6 and 7)—The above reported data showed that the in situ degradation of C10-containing triacylglycerols is not defective in NLSD cells in contrast to the degradation of long-chain triacylglycerols, which is genetically deficient. This led us to formulate the hypothesis that these two types of triacylglycerols are probably degraded through the two different degradation pathways. This suggests the existence of two cytoplasmic enzymes, a short/medium-chain lipase and a long-chain lipase, respectively. This hypothesis was supported by two types of experiments: 1) by blocking selectively in situ each degradation pathway by irreversible inhibitors and 2) by evaluating in vitro the activity of each enzyme in homogenates of cells pretreated by these irreversible inhibitors. These inhibitors have been selected, in preliminary experiments, on the basis of their selective inhibitory effect in vitro on candidate enzymes, namely lipases hydrolyzing long-chain fluorescent triacylglycerols and lipases hydrolyzing short-chain fluorescent triacylglycerols (this latter enzyme exhibiting properties of nonspecific carboxylesterases) (1, 18). The organophosphorous compound E600 is an irreversible inhibitor of various esterases in vitro (29, 30), among them the short/medium-chain lipase, but it did not inhibit the long-chain lipase (1, 18). Conversely, the sulfhydryl-reactive compound p-chloromercuribenzoate
Acyl Chain Length Dependence of Triacylglycerol Degradation

**FIG. 6. Effect of E600 and PCMB on the in situ degradation of triacylglycerols endogenously biosynthesized from radiola beled decanoic and oleic acids by fibroblasts from normal sub jects.** During the pulse period, cells were incubated at 37°C for 12 h in RPMI 1640 medium supplemented with 2% Ultroser G and radiolabeled fatty acids, decanoic (A, C) or oleic (B, D) acids (10^6 dpm/ml and 30 nmol/ml non-labeled fatty acids). At the end of the 12-h pulse period (time 0 of the chase), total radioactivity levels, in cell batches used without (control) or with E600 and without (control) or with PCMB, were 0.67 ± 0.04, 0.69 ± 0.06, 0.70 ± 0.04, and 0.71 ± 0.05 with radiolabeled decanoic acid and 6.1 ± 0.4, 6.8 ± 0.8, 6.3 ± 0.6, and 6.2 ± 0.7 (as 10^6 dpm/mg cell protein) with radiolabeled oleic acid, respectively. After washing, cells were incubated in RPMI 1640 containing 2% Ultroser G for a 48-h chase period. Inhibitors, E600 (1 μM/liter) (A, B) or PCMB (10 μM/liter) (C, D), were added to the culture medium only during the chase period. Mean ± S.E. of three experiments.

**FIG. 7. In vitro determination of activities of enzymes degrad ing radiola beled decanoic acid- or oleic acid-containing triacyl glycerols (A–D and E, respectively) in fibroblast homogenates (prepared in distilled water as described under “Materials and Methods”).** A, linearity of the enzyme activity versus time (standard assay conditions using 100 μg of protein of the homogenate per assay); B, linearity of the enzyme activity versus enzyme concentration (ex pressed as μg of homogenate protein/assay; incubation time, 2 h); C, effect of increasing concentrations of the substrate (100 μg of protein/ assay; incubation time, 2 h); D and E, hydrolytic activity when varying pH from 3.5 to 9.0 using decanoic- or oleic-containing triacylglycerols as substrates (D and E, respectively). Mean ± S.E. of three experiments.

(PCMB) is an irreversible inhibitor of the neutral long-chain lipase, but it did not inhibit the short/medium-chain lipase in vitro (1). Preliminary experiments allowed to show that optimal (i.e., effective in inhibiting the degradation pathways) concentrations of these inhibitors can be used without any adverse effect on the incorporation of radiolabeled fatty acid into cellu lar lipids (Table II) and without any cytotoxic effect to cultured cells (data not shown).

When intact normal cells were incubated in the presence of 1 μM/liter E600, the in situ hydrolysis of short/medium-chain triacylglycerols was completely blocked (Fig. 6A), whereas that of long-chain triacylglycerols was not affected (Fig. 6B). In contrast, when intact normal cells were incubated in the presence of 10 μM/liter PCMB, the in situ hydrolysis of long-chain triacylglycerols was severely impaired (Fig. 6C), whereas that of medium-chain triacylglycerols was not altered (Fig. 6D). The separate inhibition of the degradation pathways of short/medi um- and long-chain triacylglycerols by these two inhibitors supports the hypothesis of two separate degradation pathways.

The activities of the two candidate enzymes were evaluated in homogenates of cells treated with the irreversible inhibitors under conditions of Fig. 6, using in vitro assays containing either [14C]decanoic-containing triacylglycerols (prepared from cells loaded either with radiolabeled [14C]decanoic acid or [3H]triolein). The enzyme hydrolyzing C10-containing triacylglycerols was inhibited in cells treated by E600, whereas the enzyme hydrolyzing triolein was not inhibited in the same cells (Fig. 8, C and D). Reversely, in cells treated with PCMB, the enzyme hydrolyzing triolein was inhibited, whereas the enzyme hydrolyzing C10-containing triacylglycerols retained its activity (Fig. 8, C and D).

These results support the hypothesis that cytoplasmic C10-containing triacylglycerols are degraded in situ by an E600-sensitive short-chain lipase (not deficient in NLSD cells), which is clearly discriminated from the neutral lipase activity (sp ecific to long-chain triacylglycerols) involved in the liberation of long-chain fatty acids of cytoplasmic triacylglycerols and defi dent in NLSD cells.

**TABLE II**

Effect of E600 and PCMB on the incorporation of radiolabeled [14C]decanoic or [3H]oleic acids into lipids of normal fibroblasts

| Fatty acids | Inhibitor | Radiolabeled lipids |
|-------------|-----------|---------------------|
| [14C]Decanoic acid | + | 22 ± 3 | 6.3 ± 1.1 |
|                       | + E600    | 20 ± 3 | 7.1 ± 1.5 |
|                       | + PCMB    | 23 ± 4 | 7.8 ± 0.9 |
| [3H]Oleic acid       | +         | 151 ± 12 | 31 ± 4  |
|                       | + E600    | 147 ± 10 | 35 ± 4  |
|                       | + PCMB    | 155 ± 16 | 30 ± 3  |

**DISCUSSION**

The data previously reported (8–10, 31, 32) and those reported here clearly show that the cytoplasmic triacylglycerol accumulation in cultured fibroblasts is largely dependent on the extracellular lipids, since it is supplied by (at least) two separate pathways, the first one being the intracellular pathway of triacylglycerols endogenously biosynthesized from the cytoplasmic pool of fatty acids (8–10) and the second one (probably minor) resulting from the cellular uptake of triacylglycerols contained in high density lipoproteins (13). Both sources of triacylglycerols have been shown to participate to the storage of cytoplasmic triacylglycerols in NLSD (8–10, 13).
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The hypothesis of the existence of two separate hydrolytic pathways is also supported by experiments with irreversible inhibitors specific to each degradation pathway and able to inhibit the enzyme activities in situ and in vitro as well. E600 inhibited concomitantly the degradation of short/medium-chain triacylglycerols in situ and the short-chain lipase activity in vitro but not the long-chain lipase in vitro or the in situ degradation of long-chain triacylglycerols. Reversely, the sulhydryl reagent PCMB inhibited the in situ degradation of long-chain triacylglycerols and the long-chain lipase activity in vitro, but not the in situ degradation of short/medium-chain triacylglycerols and the short-medium-chain lipase activity. Moreover, the inhibition of the enzymes (activities determined in vitro by using their respective substrates) persisting after lysis of cells treated with irreversible inhibitors, E600 and PCMB (this study), and the in vitro studies of enzymatic properties previously reported (1, 40–42) are consistent with the idea that the short-chain lipase is different from the long-chain lipase. The enzymatic properties (specificity to long-chain triacylglycerols, heat stability, susceptibility to PCMB but not to E600) of the neutral long-chain lipase (1, 5, 18, 42) are similar to those of the microsomal neutral lipase (43–45), which has not been cloned to date, to our knowledge, and is different from the hormone-sensitive lipase (no activation of triacylglycerol degradation by dibutyryl-cAMP) (data not shown) and other known lipases by its enzymatic properties in vitro (1, 5). The neutral short/medium-chain lipases exhibit some enzymatic properties (specificity to various short-chain lipophilic esters, heat lability, susceptibility to organophosphorous compound E600, but relative resistance to PCMB) similar to those of carboxylesterases (1, 40–42), some of them having been cloned in liver or other tissues (45–48). As the enzymes are not identified at the molecular level, the alternative hypothesis that there is one lipase with differential substrate specificity cannot be excluded.

Finally, from the data observed in intact cells, i.e. degradation at a normal rate of short-chain triacylglycerols in NLSD (in contrast to the accumulation of long-chain triacylglycerols), it is suggested to use diets enriched with short/medium-chain lipids (and poor in long-chain lipids) to tentatively slow down the long-chain triacylglycerol accumulation in NLSD patients.

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