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Cytoplasmic triacylglycerols and cholesteryl esters are degraded in two separate catabolic pools in cultured human fibroblasts

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The sources and the catabolic pathways of cytoplasmic pools of triacylglycerols and cholesteryl esters have been comparatively investigated in cultured fibroblasts from normal subjects and from patients affected with neutral lipid storage disease (NLSD) and Wolman disease (WD). (i) Endogenously biosynthesized triacylglycerols and cholesteryl esters were degraded extra-lysosomally since they were catabolized at similar rates in normal and in WD fibroblasts. In NLSD fibroblasts, the degradation of endogenous triacylglycerols was severely deficient, whereas that of endogenous cholesteryl esters was in the normal range. (ii) Reconstituted high density lipoproteins (HDL) containing radiolabelled [^{3}H]triolein and cholesteryl [^{14}C]oleate were taken up by cultured fibroblasts and rapidly degraded in a non-lysosomal compartment. In NLSD fibroblasts the degradation of HDL–[^{3}H]triolein was blocked whereas that of HDL–[^{14}C]cholesteryl oleate was in the normal range. These data suggest that: (i) the cytoplasmic pools of triacylglycerols and cholesteryl esters originate from HDL uptake and from endogenous biosynthesis as well, (ii) cytoplasmic (non-lysosomal) triacylglycerols and cholesteryl esters are degraded by two separate catabolic pathways.

Triacylglycerol; Cholesteryl ester; High density lipoprotein; Low density lipoprotein; Fibroblast; Neutral lipid storage disease; Wolman disease

1. INTRODUCTION

Cellular triacylglycerols (TAG) and cholesteryl esters (ChE) come from exogenous lipoproteins and from endogenous biosynthesis. These cellular TAG and ChE are metabolized in two separate subcellular compartments, the first one lysosomal, the second one cytoplasmic [1,2].

The lysosomal compartment is supplied by apoB/E containing lipoproteins [1]. The role of the lysosome in the degradation of these neutral lipids has been directly investigated by using low density lipoproteins (LDL) containing radiolabelled or fluorescent [1,2] or unlabelled [3] lipids. In the lysosomal compartment both TAG and ChE are hydrolyzed by the lysosomal acid lipase/cholesterol esterase which is deficient in Wolman disease (WD) [1,4–6].

The cytoplasmic compartment contains endogenous TAG and ChE which are biosynthesized from fatty acids taken up by cells or from products escaping the lysosome. The catabolic pathway of cytoplasmic TAG and ChE in intact cells has been investigated by 'pulse–chase' experiments with radiolabelled [7–10] or fluorescent [11] precursors [7,8]. At least three enzymatic systems have been reported to be possibly involved in the degradation of TAG and ChE. The hydrolysis of cytoplasmic TAG and ChE is catalyzed in several tissues by a single enzyme, namely the hormone-sensitive lipase [12–14]. In contrast, in tissues or cells devoid of the hormone-sensitive lipase [12], for example in liver, cholesterol esterase and triacylglycerol lipase are separate enzymes [15]. In mouse macrophagic cell lines, the conclusions are apparently conflicting, since the neutral lipase of WEHI cells seems to be identical to the hormone-sensitive lipase [13], whereas that of J774 exhibits completely different properties from the hormone-sensitive lipase [16]. Furthermore some conflicting data have been reported concerning the hypothetical involvement of the acid lysosomal lipase in the degradation of the cytoplasmic TAG and ChE [1–3,17–21].

Elsewhere radiolabelled ChE associated to high density lipoproteins (HDL) are selectively taken up by cells and degraded extralysosomally [22,23]. However, to our knowledge, the uptake and the catabolic pools involved in the degradation of HDL–TAG are unknown. Furthermore, in cultured fibroblasts, the relationship between the degradative pathways of cytoplasmic TAG and ChE is not clearly understood.

In the present paper, the catabolic pathways of cytoplasmic TAG and ChE have been compared using fibroblasts from patients affected with Neutral lipid storage disease (NLSD) and WD. NLSD is a rare inherited metabolic disease characterized by the association of muscular weakness, ichthyosis, pancreatic failure and

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Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; TAG, triacylglycerols; ChE, cholesteryl esters; NLSD, neutral lipid storage disease; LPDS, lipoprotein-depleted serum; PBS, phosphate-buffered saline.

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multisystemic triacylglycerol storage [24,25] without major impairment of the mitochondrial lipid metabolism [7,26]. TAG accumulation in NLSD cultured cells (fibroblasts and immortalized lymphoid cells) results from a severe defect in the degradation of endogenously biosynthesized TAG [7,11,27,28]. WD [29] is a rare autosomal disorder of lipid metabolism characterized by a severe acid lipase deficiency and a subsequent lysosomal storage of TAG and ChE [4–6] originating mainly from exogenous lipoproteins (LDL or/and VLDL) [1–3].

The reported data suggest that (i) endogenously biosynthesized ChE and TAG constitute two separate cytoplasmic catabolic pools; (ii) HLD–triolein and HDL–ChE are internalized by fibroblasts and constitute also two independent cytoplasmic (i.e. non-lysosomal) catabolic pools probably identical to those of endogenous ChE and TAG.

2. MATERIALS AND METHODS

2.1. *Materials*

Radiolabelled cholesteryl [^{14}C]oleate (50 mCi/mmol), [^{3}H]triolein (26 Ci/mmol) were purchased from New England Nuclear (Les Ulis, France) or Amersham (Les Ulis, France), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), bovine fatty acid-free albumin, oleic acid, triolein, pure taurocholate were from Sigma (St. Louis, MO, USA), Silica-gel G thin-layer chromatography analytical plates were from Merck (Darmstadt, Germany), RPMI 1640, Phenol red-free RPMI 1640 (PRF-RPMI), fetal calf serum, glutamine, streptomycin and penicillin were from Gibco (Cergy-Pontoise, France); Ultroser G from IBF (Villeneuve-la-Garenne, France); cell culture flasks were from Nunc (distributed by Polylabo-Block, Strasbourg, France); Hydragel was from Sebia (Issy-les-Moulineaux France); Picofluor was from Packard (Rungis, France), and the other reagents and organic solvents were from Merck or Prolabo (Paris, France).

2.2. *Cell culture*

Fibroblasts from patients affected with NLSD (Bo. and Dem.) were kindly provided by Drs. J.M. Mussini and S. Billaudel, CHU Nantes, France and by Dr. B. Winchester (Institute of Child Health, London, UK); those of WD (GM1606) were purchased from the NIGMS Human Genetics Mutant Cell Repository (Camden, NJ), those of Cholesteryl ester storage disease (DM) were kindly provided by Profs. C. Borrone and P. Durand (Istituto Gaslini, Genova, Italy) and those of normal individuals (n1, n2 and n3) were from our laboratory. Fibroblasts were grown at 37°C in 5% CO_2/95% air in RPMI 1640 medium with penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 μmol/ml) and supplemented with 10% heat-inactivated fetal calf serum or 2% Ultroser G (a synthetic lipoprotein-free serum substitute) under the previously used conditions [7,11]. Cell viability was determined by the MTT test [30].

2.3. *Lipoprotein isolation and labelling with [^{3}H]triolein and cholesteryl [^{14}C]oleate*

LDL, HDL and lipoprotein-depleted serum (LPDS) were isolated from human pooled sera by ultracentrifugation (Beckman L8-70 Ultracentrifuge) according to Havel [31], extensively dialyzed, sterilized by filtration (0.2 μm Millipore membrane) and were stored at 4°C under nitrogen (up to 3 weeks). The purity of lipoproteins was controlled by electrophoresis on Hydragel. HDL were labelled with [^{3}H]triolein and cholesteryl [^{14}C]oleate according to the procedure of Krieger et al. [32]. The final labelling was 23,000 dpm [^{3}H]triolein (1.8 nmol)/μg apoA-I and 1,700 dpm cholesteryl [^{14}C]oleate (0.4 nmol)/μg apoA-I. LDL were labelled with [^{3}H]triolein according to a procedure derived from that of Roberts et al. [33]: briefly 10 ml human serum was incubated overnight at 37°C with [^{3}H]triolein (10^{8} dpm). Then LDL were isolated by ultracentrifugation as indicated above. The final labelling was around 11,000 dpm [^{3}H]triolein/μg apoB. ApoA-I and apoB concentrations were determined by immunonephelometry (Behring system).

2.4. *Cellular uptake and metabolism of [^{3}H]oleic acid*

The optimal culture conditions to induce a large cellular biosynthesis and accumulation of radiolabelled TAG and ChE were derived from previous reports [7,9,34] and from preliminary experiments. Fibroblasts were pre-incubated for 24 h in RPMI-1640 containing 2% Ultroser G, 10% LPDS and cholesterol (130 nmol/ml), then 'pulsed' for 12 h with 30 nmol/ml radiolabelled [^{3}H]oleic acid (10^{4} dpm/nmol oleic acid). At the end of the incubation time, cells were washed twice in PBS containing 5 mg/ml bovine serum albumin (fatty acid-free) and twice in PBS, and homogenized in water. The cell-associated radioactivity was determined by liquid scintillation counting (in 5 ml Picofluor using a Packard counter model Tricarb 4530) and the lipids analyzed.

2.5. *Cellular uptake of lipoprotein–[^{3}H]triolein and –cholesteryl [^{14}C]oleate*

For determining the cellular uptake of HLD–[^{3}H]triolein –cholesteryl [^{14}C]oleate, fibroblasts were incubated for 12 h in RPMI-1640 containing 2% Ultroser G and increasing concentrations of HDL–[^{3}H]triolein and –cholesteryl [^{14}C]oleate. At the end of this pulse period, cells were carefully washed (as above indicated), harvested and the cell-associated radioactivity was counted and the lipids analyzed.

2.6. *Lipid extraction and analysis*

Cellular lipids were extracted with chloroform/methanol (2/1, v/v) according to the procedure of Folch et al. [35] and separated by thin-layer chromatography on Silicagel G plates, using the following solvent systems: chloroform/methanol/water (100/42/6, v/v/v) for the separation of the phospholipids, and petroleum ether/diethyl ether/acetic acid (80/20/1, v/v/v) for the separation of the neutral lipids. Radiolabelled lipids were counted directly on the thin-layer chromatography plate using a radiochromatoscanner Berthold (model LB 285) under the previously used conditions [7]

Protein concentrations were determined by the method of Lowry et al. [36].

3. RESULTS

3.1. *Metabolism of endogenously biosynthesized TAG and ChE*

The conditions of pre-incubation with cholesterol and pulse with [^{3}H]oleic acid have been defined in order to induce a concomitant biosynthesis and accumulation of radiolabelled TAG and ChE. The lipid concentrations used in the reported experiments did not exhibit any cytotoxic effect to the human fibroblasts during the period of the experiment (higher concentrations of cholesterol and/or oleic acid cannot be used because of their cytotoxicity to fibroblasts). At the end of the pulse, the radiolabelled TAG represented 15–30% of the lipid-associated radioactivity in normal fibroblasts and nearly 50% in NLSD fibroblasts. Radiolabelled ChE represented 7–15% of the lipid-associated radioactivity in normal and also NLSD fibroblasts.

During the 48 h chase period (in serum-free medium containing 2% Ultroser G), the cell-associated radioac-

tivity decreased slowly (Fig. 1A). The levels of radiolabelled polar lipids were almost constant during the chase (Fig. 1B). The radiolabelled TAG decreased rapidly (half-life 5 ± 1 h) in fibroblasts from normal subjects and from those with WD, whereas in NLSD fibroblasts they decreased very slowly (half-life higher than 100 h). These results are consistent with the hypothesis of a selective block of the degradation pathway of endogenously biosynthesized TAG in NLSD fibroblasts (Fig. 1C). In marked contrast, the degradation of the radiolabelled ChE was quite similar in controls and in NLSD fibroblasts (Fig. 1D). In conclusion, these data strongly suggest that, in normal human fibroblasts, endogenous TAG and ChE are degraded in the cytoplasmic compartment (since no accumulation was observed in WD cells) by two different enzymatic systems: (i) the lipase system which is defective in NLSD fibroblasts; (ii) a cholesteryl ester hydrolyzing system which is not deficient in NLSD fibroblasts.

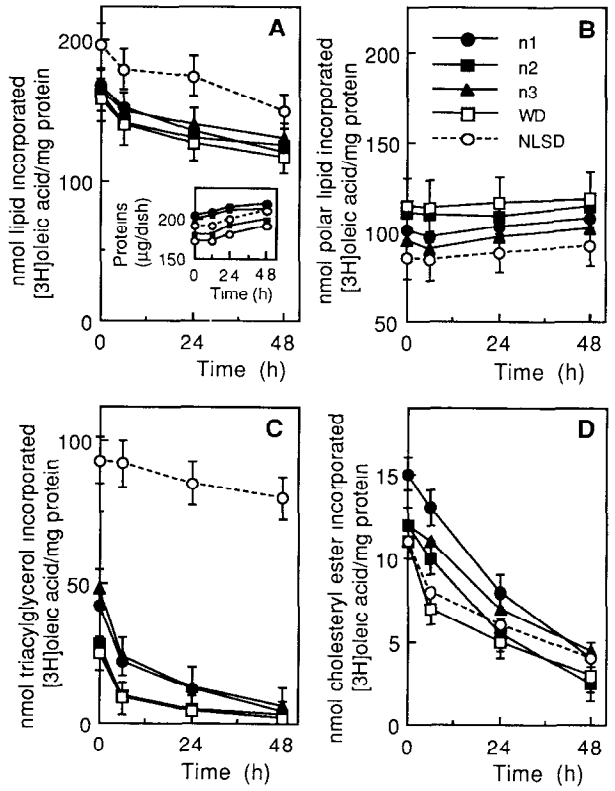

Fig 1. Time-course of the degradation and turnover of endogenously biosynthesized lipids in cultured fibroblasts from normals (filled symbols), NLSD (open circles) and WD (open squares) patients. Cultured fibroblasts were pre-incubated with cholesterol (130 nmol/ml for 24 h) and 'pulsed' with [^{3}H]oleic acid (30 nmol/ml for 12 h). Then fibroblasts were grown for 48 h (chase period) in RPMI 1640 supplemented with 2% Ultroser G (without any oleic acid or serum albumin). At the indicated times, cells were harvested and the radiolabelled cellular lipids were analyzed (thin-layer chromatography and radiochromatoscanner) as indicated in section 2. The amounts of radiolabeled lipids (in A, total lipids; in B, polar lipids; in C, TAG; in D, ChE) are expressed as nmol of [^{3}H]oleic acid incorporated in each class of lipids per mg of cell protein. In inset of A are reported the proteins per dish (Petri dishes, 3.5 cm diameter). Each point represents the mean of four separate experiments (± S.E.M.).

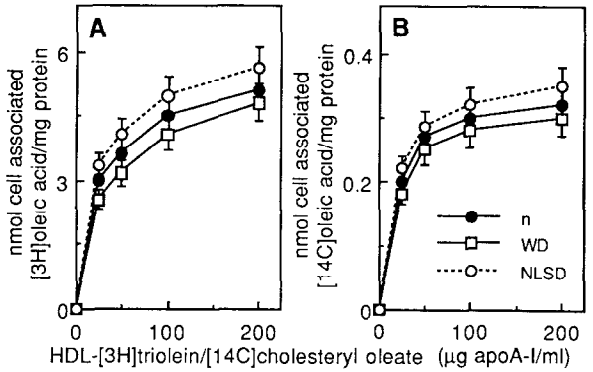

Fig. 2. Uptake of [^{3}H]triolein (A) and cholesteryl [^{14}C]oleate (B) incorporated into HDL by cultured fibroblasts from normals (filled symbols), WD (open squares) and NLSD (open circles). Fibroblasts were 'pulsed' with increasing concentrations of HDL-[^{3}H]triolein or -cholesteryl [^{14}C]oleate (up to 200 µg apoA-I/ml) for 12 h. After washing and harvesting the cells, the cell-associated radioactivity was determined by liquid scintillation counting and expressed as nmol oleic acid/mg cell protein. The results are the mean of two separate experiments (± S.E.M.).

3.2. *Uptake and degradation of HDL-[^{3}H]triolein and -cholesteryl [^{14}C]oleate*

HDL, partially delipidated and reconstituted with [^{3}H]triolein and cholesteryl [^{14}C]oleate according to the procedure of Krieger et al. [32], were used for studying the cellular uptake and subsequent degradation of [^{3}H]triolein and cholesteryl [^{14}C]oleate contained in HDL. The electrophoresis of these reconstituted HDL demonstrated that both radiolabelled lipids were really associated with HDL (data not shown). The cellular uptake of both radiolabelled lipids, [^{3}H]triolein and cholesteryl [^{14}C]oleate associated to HDL, was quite similar in all the cells used here (Fig. 2).

HDL-[^{3}H]triolein and -cholesteryl [^{14}C]oleate taken up by the fibroblasts were rapidly degraded by normal and WD fibroblasts (half-life around 6 ± 1 h for HDL-[^{3}H]triolein and less than 2 h for HDL-cholesteryl [^{14}C]oleate) (Fig. 3). In NLSD fibroblasts the degradation of the internalized HDL-[^{3}H]triolein was severely deficient (as shown by the constant level of internalized HDL-[^{3}H]triolein and by the lack of labelling of phospholipids). In contrast, the degradation of the internalized HDL-cholesteryl [^{14}C]oleate was in the normal range (Fig. 3B). Note that the level of HDL-[^{3}H]triolein undegraded at the end of the pulse was higher in NLSD than in controls cells because of the degradation block in NLSD cells. In normal cells, under the conditions used, the major part of [^{3}H]oleic acid liberated by hydrolysis of the internalized HDL-[^{3}H]triolein was reutilized in newly synthesized phospholipids. In NLSD fibroblasts, the accumulation of undegraded HDL-

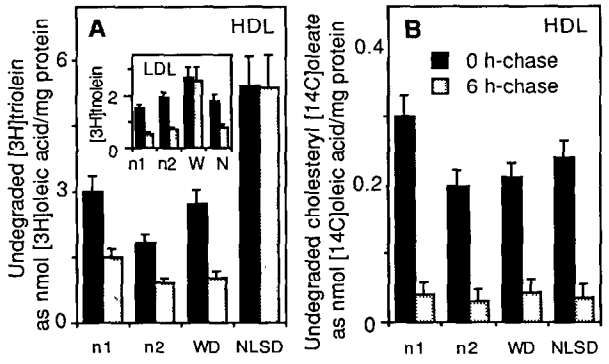

Fig. 3. Degradation of HDL–[^{3}H]triolein or –cholesteryl [^{14}C]oleate by fibroblasts from normal (n1,n2), WD (W) and NLSD (N) patients. Cultured fibroblasts were pulsed for 12 h with a fixed amount of HDL–[^{3}H]triolein or –cholesteryl [^{14}C]oleate (200 μg apoA-I/ml) under the same conditions as Fig. 2. Then cells were washed and 'chased' in the standard culture medium. Cells were harvested at the indicated times (filled bars, time 0 of the chase; stippled bars, 6 h chase), and lipids extracted and separated by TLC as indicated in Fig. 1. (A) [^{3}H]Triolein, (B) cholesteryl [^{14}C]oleate For comparison the degradation of LDL–[^{3}H]triolein performed under similar experimental conditions is shown in the inset to A. The results are the mean of three separate experiments (± S.E.M.).

[^{3}H]triolein demonstrates that HDL–[^{3}H]triolein is internalized by fibroblasts without prior hydrolysis (since in this case the liberated [^{3}H]oleic acid would be incorporated into phospholipids even in NLSD cells, as in Fig. 1).

All these results suggest that (i) HDL–[^{3}H]triolein and –cholesteryl [^{14}C]oleate are effectively taken up and internalized by cultured fibroblasts; (ii) the internalized HDL–[^{3}H]triolein and –cholesteryl [^{14}C]oleate are degraded at a normal rate in WD fibroblasts, thus in a non-lysosomal compartment (referred to as the cytoplasmic compartment); (iii) the internalized HDL–[^{3}H]triolein is degraded in normal fibroblasts but not in NLSD fibroblasts, suggesting that HDL–[^{3}H]triolein is degraded by the same catabolic pathway as endogenously biosynthesized TAG; (iv) HDL–cholesteryl [^{14}C]oleate is not blocked in NLSD cells, thus the cytoplasmic degradation pathways of cholesteryl [^{14}C]oleate and [^{3}H]triolein are independent.

In order to confirm that the catabolic pools of cytoplasmic TAG and ChE are independent of the lysosomal compartment, we have compared the degradation of radiolabelled LDL–[^{3}H]triolein in fibroblasts from normal, NLSD and WD patients. As expected, the LDL–[^{3}H]triolein degradation was blocked in WD cells, whereas it was in the normal range in NLSD cells (inset of Fig. 3A).

4. DISCUSSION

The reported data suggest that the cytoplasmic pools of TAG and ChE (i) are supplied by the endogenous biosynthesis of TAG and ChE and by the uptake of exogenous HDL–TAG and HDL–ChE; (ii) are degraded by two separate catabolic pathways, each being independent of lysosomal catabolism.

Endogenously biosynthesized TAG and ChE are degraded independently of the lysosomal compartment since they do not accumulate in WD cells. The lysosomal accumulation of neutral lipids in WD fibroblasts results from lipids taken up through the apoB/E receptor-mediated pathway which is connected to the lysosomal compartment [1–6]. HDL–TAG and HDL–ChE taken up by fibroblasts, probably through a high-affinity HDL receptor pathway [37–41], are degraded at a normal rate in WD fibroblasts, and thus are catabolized in a cytoplasmic compartment completely independent of the lysosome. This conclusion is in good agreement with previous reports [22,37,42–45], but is in disagreement with some conflicting earlier reports claiming that the degradation of HDL neutral lipids could be lysosome dependent [46–48]. Moreover, under the used conditions (serum-free medium) we did not observe any appreciable reverse transport of the radiolabelled TAG and ChE outside the cell (i.e. retro-endocytosis) since (i) no significant amount of radiolabelled lipid was recovered in the culture medium; (ii) the radioactivity was recovered in the cellular phospholipids (the radiolabelled free fatty acids liberated by the hydrolysis of HDL– [^{3}H]triolein and –cholesteryl [^{14}C]oleate being reutilized in the cellular biosynthesis of phospholipids).

In NLSD fibroblasts, the concomitant catabolic block of internalized HDL–[^{3}H]triolein and of the endogenously biosynthesized TAG strongly suggests that TAG from the two different sources (endogenously biosynthesized TAG and HDL–TAG) reach the same cytoplasmic (extra-lysosomal) catabolic pool and are degraded by the same lipase system which is deficient in NLSD cells. In contrast, endogenously biosynthesized ChE and HDL–cholesteryl [^{14}C]oleate internalized by fibroblasts, probably by the 'selective uptake' described by Pittman's group [21,22], did not accumulate in NLSD fibroblasts nor in WD fibroblasts. This suggests that these ChE are degraded in a cytoplasmic (extra-lysosomal) compartment through a catabolic pathway involving a neutral cholesteryl esterase independent of the lipase system.

In conclusion, the use of human fibroblasts from NLSD and WD patients allowed us to demonstrate that the cytoplasmic pools of TAG and ChE originate from (at least) two sources, the first one arising from endogenous biosynthesis and the second one from exogenous HDL taken up by cells. NLSD fibroblasts exhibited a severe deficiency of the degradation of endogenous and HDL–TAG as well. In contrast HDL–ChE and endogenous ChE were degraded at a normal rate in NLSD. This demonstrates the existence of two separate (non-lysosomal) catabolic pathways for cytoplasmic TAG and ChE in cultured human fibroblasts.

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