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Independence of triacylglycerol-containing compartments in cultured fibroblasts from Wolman disease and multisystemic lipid storage myopathy

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The functional relationship between the two subcellular compartments involved in catabolism of triglycerides, i.e. lysosomes and lipid-containing cytoplasmic vacuoles, has been investigated using cultured fibroblasts from patients affected with two different genetic lipid (triacylglycerol) storage disorders: Wolman disease and multisystemic lipid storage myopathy. As shown by metabolic studies in intact cultured cells, lysosomal degradation of exogenous labelled triacylglycerols (incorporated into lipoproteins and internalized via the apo B/E receptor pathway) was blocked in Wolman cells, whereas catabolism of endogenously biosynthesized triacylglycerols was in the normal range. In contrast, in fibroblasts from multisystemic lipid storage myopathy, the degradation of endogenous triacylglycerols was blocked, whereas that of exogenous triacylglycerols (i.e. from lipoproteins) was normal. This comparative study demonstrates that the lysosomal and cytoplasmic compartments are functionally independent. Enzymatic studies allow one to discriminate clearly between 3 lipases and 2 carboxylesterases the role of which is discussed.

Lipase; Triacylglycerol metabolism; Lipid storage myopathy; Wolman disease; Fluorescent lipid; (Fibroblast)

1. INTRODUCTION

The triacylglycerols stored in cultured cells can originate from extracellular lipoproteins or intracellular biosynthesis of lipids.

The cellular uptake of lipoproteins is receptor-mediated [1-3] and partially receptor-independent [4]. Internalized lipoproteins contained in the endosomal compartment can be excreted back by retro-endocytosis [5] or be able to reach the lysosomal compartment [3] in which they are rapidly degraded by acid hydrolases. In Wolman disease [6] and cholesteryl ester storage disease [7], the acid lipase deficiency results in the lysosomal catabolic block of neutral lipids and their subsequent accumulation [8].

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Endogenous triacylglycerols are synthesized, in the cytoplasmic compartment, via the phosphatidic acid pathway; high cellular influx of long-chain fatty acids results in dramatic cytoplasmic storage of triacylglycerols, which is completely reversible after stopping the cellular loading in fatty acids [9,10]. We have recently demonstrated that the catabolic block of endogenous triacylglycerols stored in cytoplasmic vacuoles [11,12] is the primary metabolic defect involved in multisystemic lipid storage myopathy [13,14].

The functional relationship between the lysosomal and cytoplasmic compartments (where neutral lipids can be accumulated) is poorly understood and a connection between these compartments has been recently suggested by Sauro et al. [15] who proposed the hypothesis of a role for lysosomal lipase in the degradation of triacylglycerols stored in the cytoplasm.

Comparative analysis of triacylglycerol catabolism in cultured fibroblasts from the above-quoted

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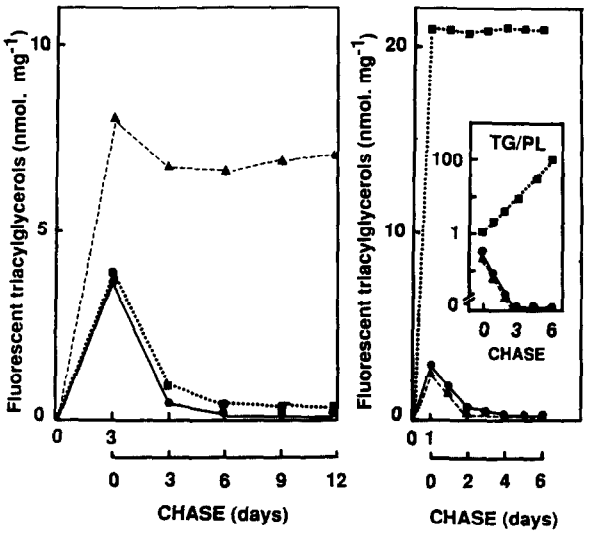

Fig.1. Metabolism of exogenous (left) and endogenous (right) fluorescent triacylglycerols in fibroblasts from Wolman disease (– – –), multisystemic lipid storage myopathy (• • •) and controls (——). (Inset) Ratio of endogenous fluorescent triacylglycerols to fluorescent phospholipids (log scale) during the chase. Pulse-chase experiments and lipid analyses were performed under the conditions indicated in section 2.

genetic diseases allows one to conclude that the lysosomal and cytoplasmic compartments are independent (no appreciable transfer of these lipids occurs between the two subcellular compartments). The characteristic properties of the cellular lipases and carboxylesterases are reported and their functional role is discussed.

2. MATERIALS AND METHODS

The sources and routes for synthesis of chemicals have been previously reported [16–19].

Cell culture of skin fibroblasts was performed in culture flasks or petri dishes, using RPMI 1640 supplemented with Ultroser (2%), fetal calf serum or human lipoproteins, under the reported conditions [14,17].

Enzyme assays for cellular lipases and carboxylesterases contained labelled substrates, either [^{3}H]triolein (10^6 dpm/ml) or 15 μmol/l fluorescent tripyrenebutanoylglycerol, 500 μmol/l unlabelled triacylglycerol, 1 g/l Triton X-100 (and for the lysosomal lipase standard assay, 2 mmol/l taurocholate), 0.2 mol/l buffer (citrate-phosphate from pH 3.5 to 7.2 and Tris-HCl from pH 7.2 to 9.0) and enzyme solution (final volume, 100 μl; incubation for 1 h at 37°C). The fatty acids liberated were quantified radiometrically and fluorometrically as in [18,19]. Proteins were determined by the method of Lowry et al. [20].

Pulse-chase experiments on exogenous triacylglycerols utilized human LDL, prepared by sequential centrifugation as reported [17] and labelled with pyrenedecanoyldioleylglycerol ($P_{10}OOG$) according to Roberts et al. [21]: $P_{10}OOG$ (500 nmol) was solubilized in DMSO (100 μl), mixed with 6 ml sterile lipoprotein-deficient serum and added to 1 ml (4 mg apo B) LDL solution; after 12 h preincubation at 37°C, fluorescent LDL were isolated by ultracentrifugation, dialyzed and mixed with RPMI 1640 (200 μg apo B per ml culture medium). This mixture was used for a 72 h pulse, then medium was removed, cells were washed with PBS containing 1% fatty acid-free serum albumin and incubated in standard culture medium for 12 days chase.

For pulse-chase experiments with fluorescent fatty acids, pyrenedecanoic acid (P_{10}) solubilized in DMSO (33 nmol/ml) was added to the culture medium (RPMI 1640 supplemented with 2% Ultroser). At the end of the pulse period (1 day), cells were washed as indicated above and chased for 6 days.

Fluorescent lipids were extracted following the procedure of Folch et al. [22], separated by TLC and fluorometrically quantified after scraping and hydrolysis as described [23].

Table 1

Distribution of fluorescent lipids in subcellular fractions of Wolman and control fibroblasts fed with P_{10} for 24 h

Subcellular fraction	Fluorescent lipids			β-Hexosaminidase
	Triacylglycerols	Phospholipids	Fatty acids	
Wolman				
Homogenate	45	40	15	1050
Lysosomes/mitochondria	5	5	90	4200
Cytosol/membranes	42	38	20	700
Controls				
Homogenate	44	40	16	980
Lysosomes/mitochondria	5	6	89	4300
Cytosol/membranes	38	41	21	600

Lipid contents are expressed as the percentage of total fluorescent lipids per subcellular fraction. β-Hexosaminidase activities are expressed as nmol · h^{-1} · mg^{-1}

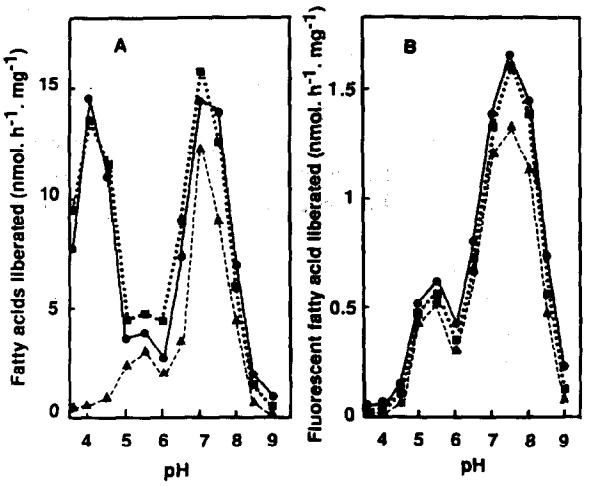

Fig.2. Hydrolysis of [^{3}H]triolein (diluted with unlabelled triolein) by lipases (A) and of tripyrenebutanoylglycerol (diluted in tributyrin) by carboxylesterases (B) from cultured fibroblasts from Wolman disease (– – –), multisystemic lipid storage myopathy (• • •) and controls (——). Assays were under the conditions described in section 2, with enzyme activities being expressed as nmol total oleic acid liberated/h and per mg cell protein (A) and nmol pyrenebutanoic acid liberated/h per mg cell protein (B).

3. RESULTS

As shown in fig.1, lipoproteins containing fluorescent triacylglycerols were taken up by controls and lipidotic cells: at the end of the pulse, the cellular content of fluorescent triacylglycerols was 2-fold greater in Wolman than in control and myopathic fibroblasts. During the chase period, the internalized fluorescent triacylglycerols were rapidly degraded in normal and myopathic cells (half-life ~2 days), whereas in Wolman cells, they underwent a slight decrease during the first 3 days (perhaps, due to retro-endocytosis) and then reached a plateau with only minor degradation (half-life ~40–60 days).

Studies of the metabolism of endogenous lipids biosynthesized from fluorescent P_{10} fatty acid, showed, at the end of the 24 h pulse period, a 7-fold higher level of fluorescent triacylglycerols in multisystemic lipid storage myopathy vs control or Wolman cells (fig.1). During the chase, the degradation of fluorescent triacylglycerols was blocked in myopathic fibroblasts, in contrast to Wolman and control cells.

Lipid analyses of the subcellular fractions (table 1) showed that in Wolman (as well as control) fibroblasts pulsed by P_{10}, the major part of the fluorescence of the lysosome-mitochondrial fraction was due to free fatty acids, without appreciable accumulation of fluorescent triacylglycerols (which were only detected in the membrane-cytosol fraction of the pulsed cells): these data demonstrate that the triacylglycerols synthesized in the cytoplasm are not transferred to the lysosomal compartment.

Enzymatic studies of the cellular lipases allowed us to discriminate clearly between 5 triacylglycerol-hydrolyzing enzymes (fig.2, table 2): 3 lipases (specific to long-chain triacylglycerols and resistant to diethyl-*p*-nitrophenyl phosphate or E_{600}) and 2 carboxylesterases (specific to short-chain triacyl-

Table 2

Comparison of characteristic properties of the lipases and carboxylesterases of cultured fibroblasts

	pH 4.0-Lipase	pH 5.5-Lipase	pH 5.5-Carboxyl-esterase	pH 7-Lipase	pH 7.5-Carboxyl-esterase
Acyl chain length specificity	long	long	short	long	short
pH optimum	4.0	5.5	5.5	7.0	7.5
Heat stability (30 min at 50°C)	85	45	25	70	20
Effectors					
Taurocholate (0.5 mmol/l)	140	115	125	110	70
Taurocholate (2.5 mmol/l)	250	55	60	85	55
p-Chloromercuribenzoate (1 mmol/l)	42	nd	100	2	84
EDTA (10 mmol/l)	120	135	35	115	50
NaCl (1 mol/l)	9	42	6	117	5
E_{600} (10^{-2} mmol/l)	90	87	15	102	5

Results are expressed as percentage of enzyme activity measured before heat treatment and in the absence of any effector

glycerols and inhibited by E_{600}). The lysosomal enzyme was severely deficient in Wolman cells (fig.2), but surprisingly none of the other lipases was deficient in the fibroblasts from multisystemic lipid storage myopathy.

4. DISCUSSION

The present data clearly demonstrate that the block of exogenous triacylglycerols is nearly complete in Wolman fibroblasts: the residual degradative activity in living cultured Wolman fibroblasts can be evaluated as being less than 5% (on the basis of the triacylglycerol half-life for Wolman vs control cells). This low level of residual catabolic activity is in agreement with the very low value for the acid lipase determined in vitro (<5% of control) (fig.2). It is also consistent with the values reported by Brown et al. [24] and Goldstein and coworkers [25] for degradation of cholesteryl esters, but significantly lower than that determined by Burton et al. [26] who probably overestimated the apparent residual activity because of the retroendocytosis process [5] occurring at the beginning of the chase. Therefore, the severe catabolic block in Wolman fibroblasts demonstrates that triacylglycerols stored in lysosomes cannot reach, to an appreciable extent, the cytoplasmic compartment where endogenously biosynthesized triacylglycerols are degraded at the normal rate (as shown by studying endogenous triacylglycerol catabolism). Conversely, endogenous cytoplasmic fluorescent triacylglycerols biosynthesized from internalized fatty acids are not accumulated in Wolman lysosomes, as indicated by the absence of labelled triacylglycerols in the fractionated lysosomes (table 1) and by their complete catabolism in Wolman fibroblasts, similarly to controls (fig.1): therefore, this demonstrates that cytoplasmic triacylglycerols do not reach the lysosomal compartment. This conclusion completely agrees with results obtained from studying metabolism of endogenous triacylglycerols (biosynthesized from labelled fatty acids) in multisystemic lipid storage myopathy fibroblasts. The latter cells exhibited severe catabolic block of these lipids and their subsequent intracytoplasmic accumulation, although lysosomal degradation was normal in these cells (as demonstrated by studies of the acid lipase and the catabolism of exogenous triacylglycerols). This shows that cytoplasmic granules containing endogenous triacylglycerols [10] are unable to fuse with lysosomes (where the catabolism of exogenous triacylglycerols is normal).

The enzymatic study demonstrates that catabolic block of lysosomal degradation of exogenous triacylglycerols is due to a deficiency in lysosomal lipase and that other lipases or carboxylesterases do not contribute to this defect. In contrast, in multisystemic lipid storage myopathy, we were unable to detect any enzyme deficiency: this suggests that another factor is probably implicated in degradation of cytoplasmic triacylglycerols, either an activator (not necessary in vitro) or a factor directing the enzyme to the substrate-containing compartment.

In conclusion, the present experiments utilizing fibroblasts from two inherited triacylglycerol storage disorders demonstrate the existence of two separate metabolic compartments of triacylglycerols (lysosomes and cytoplasmic granules): these compartments are unable to exchange their triacylglycerol contents and can be considered to be independent. Only hydrolysis products (i.e. fatty acids, glycerol and cholesterol) are able to move out of these compartments and be re-utilized in cellular biosynthetic pathways.

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REFERENCES

[1] Goldstein, J.L. and Brown, M.S. (1974) J. Biol. Chem. 249, 5153–5162.
[2] Goldstein, J.L., Ho, Y.K., Brown, M.S., Innerarity, T.L. and Mahley, R.W. (1980) J. Biol. Chem. 255, 1839–1848.
[3] Goldstein, J.L. and Brown, M.S. (1983) in: The Metabolic Basis of Inherited Diseases (Stanbury, J. et al. eds) pp. 672–712, McGraw-Hill, New York.
[4] Tabas, I., Weiland, D.A. and Tall, A. (1985) Proc. Natl. Acad. Sci. USA 82, 416–420.
[5] Greenspan, P. and St.Clair, R.W. (1984) J. Biol. Chem. 257, 1703–1713.
[6] Wolman, M., Sterk, V.V., Gatt, S. and Frenkel, M. (1961) Pediatrics 28, 742–756.

[7] Lageron, A., Caroli, J., Stralin, H. and Barbier, P. (1967) Presse Med. 75, 2785–2792.
[8] Patrick, A.D. and Lake, B.D. (1973) in: Lysosomes and Storage Diseases (Hers, G. and Van Hoff, F. eds) pp. 453–473, Academic Press, New York.
[9] Mackensie, C.J., Mackensie, J.B. and Reis, O.K. (1964) Exp. Cell Res. 36, 533–547.
[10] Schneeberger, E.E., Lynch, R.D. and Geyer, R.P. (1971) Exp. Cell Res. 69, 193–206.
[11] Chanarin, I., Patel, A., Slavin, G., Wills, E.J., Andrews, T.M. and Steward, G. (1975) Br. Med. J. 1, 553–555.
[12] Angelini, C., Philippart, M., Borrone, C., Bresolin, N., Contini, M. and Lucke, S. (1980) Ann. Neurol. 7, 5–10.
[13] Radom, J., Salvayre, R., Nègre, A., Maret, A. and Douste-Blazy, L. (1987) Eur. J. Biochem. 164, 703–708.
[14] Radom, J., Salvayre, R., Maret, A., Nègre, A. and Douste-Blazy, L. (1987) Biochim. Biophys. Acta 920, 131–139.
[15] Sauro, U.S., Klamut, H.J., Lin, C.H. and Strickland, K.P. (1985) Biochem. J. 277, 583–589.
[16] Nègre, A., Salvayre, R., Dousset, N., Rogalle, P., Dang, Q.Q. and Douste-Blazy, L. (1988) Biochim. Biophys. Acta 963, 340–348.
[17] Salvayre, R., Nègre, A., Maret, A., Radom, J. and Douste-Blazy, L. (1987) Eur. J. Biochem. 170, 453–458.
[18] Nègre, A., Salvayre, R., Maret, A., Vieu, C., Bès, J.C., Borrone, C., Durand, P. and Douste-Blazy, L. (1986) J. Inher. Metab. Dis. 9, 193–201.
[19] Nègre, A., Salvayre, R., Rogalle, P., Dang, Q.Q. and Douste-Blazy, L. (1987) Biochim. Biophys. Acta 918, 76–82.
[20] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[21] Roberts, D., Miller, N., Price, S., Crook, D., Coretse, C., La Ville, A., Masana, L. and Lewis, B. (1985) Biochem. J. 226, 319–322.
[22] Folch, J., Lees, M. and Sloane Stanley, G.N. (1957) J. Biol. Chem. 226, 497–509.
[23] Nègre, A., Maret, A., Douste-Blazy, L., Gatt, S. and Salvayre, R. (1988) Biochim. Biophys. Acta 960, 401–409.
[24] Goldstein, J.L., Dana, S.E., Faust, J.R., Beaudet, A.L. and Brown, M.S. (1975) J. Biol. Chem. 250, 8487–8495.
[25] Brown, M.S., Sabbani, M.K., Brunschede, G.Y. and Goldstein, J. (1976) J. Biol. Chem. 251, 3277–3286.
[26] Burton, B., Remy, W. and Rayman, L. (1984) Pediatr. Res. 18, 1242–1245.