Brefeldin A enables synthesis of active lipoprotein lipase in *cld/cld* and castanospermine-treated mouse brown adipocytes via translocation of Golgi components to endoplasmic reticulum

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Brown adipocytes cultured from newborn combined-lipase-deficient (*cld/cld*) mice and castanospermine (CST)-treated 3T3-L1 adipocytes synthesize lipoprotein lipase (LPL) which is inactive and retained in the endoplasmic reticulum (ER) [Masuno, Blanchette-Mackie, Chernick and Scow (1990) *J. Biol. Chem.* **265**, 1628–1638; Masuno, Blanchette-Mackie, Schultz, Spaeth, Scow and Okuda (1992) *J. Lipid Res.* **33**, 1343–1349]. Brefeldin A (BFA), which is known to block protein transport from ER and translocate Golgi components to ER, was used here to study the effect of translocated Golgi enzymes on LPL retained in ER of *cld/cld* and CST-treated mouse brown adipocytes. Brown adipocytes cultured from newborn normal mice contained 3000–5000 m-units of LPL activity/mg of DNA and secreted 35 m-units of LPL activity/mg of DNA per h. BFA at 10 µg/ml doubled LPL activity in normal cells within 2 h as it stopped completely secretion of active LPL. LPL in mouse cells has two N-oligosaccharide chains per subunit. Analyses with SDS/PAGE and immunoblotting showed that about one-third of LPL subunits in untreated normal cells were totally endo-β-N-acetylglucosaminidase (endo H)-resistant, one-third were partially endo H-resistant, and one-third were totally endo H-sensitive. BFA decreased to zero the proportion of subunits which were totally endo H-resistant, while it increased the proportion which were partially endo H-resistant. Thus, BFA blocked processing of one oligosaccharide chain per subunit to endo H-resistance. Sucrose-gradient centrifugation studies showed that BFA increased the proportion of LPL subunits in

normal cells which were present as active dimers. LPL activity in *cld/cld* adipocytes was 120 m-units/mg of DNA and that in normal adipocytes treated with CST was 430 m-units/mg of DNA. Most LPL subunits in such cells were totally endo H-sensitive and some were partially endo H-resistant, but none were totally endo H-resistant. Some of the subunits, in both *cld/cld* and CST-treated cells, were present as inactive LPL dimers. BFA increased LPL activity in *cld/cld* cells to 2100 m-units/mg of DNA and that in CST-treated cells to 2600 m-units/mg of DNA within 2 h. BFA increased in both groups the proportion of LPL subunits which were partially endo H-resistant. BFA also increased the proportion which were present as active dimers. Immunofluorescence studies in normal and *cld/cld* adipocytes showed that BFA caused retention of LPL in large tubular and spherical structures and in ER, but not in Golgi. When BFA was withdrawn and protein synthesis was blocked with cycloheximide, LPL in normal cells was transferred to Golgi within 30 min and disappeared within 60 min, whereas LPL in *cld/cld* cells was retained in large vesicles and ER. The findings indicate that BFA enabled synthesis of active LPL in *cld/cld* and CST-treated cells via translocation of Golgi components to ER. Also, *cld/cld* cells synthesized LPL which could be processed to active lipase and the enzymes needed for activation of the lipase were present in Golgi of such cells. Production of inactive LPL in *cld/cld* adipocytes probably results from their inability to transport LPL from ER to Golgi.

INTRODUCTION

Lipoprotein lipase (LPL) is required for uptake of triacylglycerol from blood by extrahepatic tissues [1–4]. It is synthesized by parenchymal cells [5–9] and transferred to the luminal surface of capillaries where it hydrolyses blood triacylglycerol to fatty acid and monoacylglycerol for transport into cells [10–12]. LPL is attached to capillary endothelium by proteoglycan sulphate and can be released into the bloodstream by injection of heparin [3,13]. Catalytic activity of LPL is enhanced by apolipoprotein C-II [14].

Active LPL is a non-covalent homodimer of N-linked glycosylated subunits [15]. LPL in mouse and rat has two N-linked oligosaccharide chains per subunit [7,8,16–20]. These chains may be immature high-mannose-type, mature complex-

type, or a mixture. The ability of endo-β-N-acetylglucosaminidase (endo H) to remove high-mannose-type chains from proteins [21] has been used to determine the types of oligosaccharide chains attached to LPL subunits [6,8,16–18]. On the basis of these findings, LPL subunits have been divided into three groups: totally endo H-resistant subunits have two complex-type chains, partially endo H-resistant subunits have one complex-type chain and one high-mannose-type chain, and totally endo H-sensitive subunits have two high-mannose-type chains [8,16–18].

Production of active LPL normally involves synthesis and glycosylation of LPL subunits in endoplasmic reticulum (ER), trimming of glucose and mannose residues from LPL oligosaccharide chains in ER, trimming of mannose residues and conversion of LPL oligosaccharides into complex types in Golgi,

Abbreviations used: LPL, lipoprotein lipase; ER, endoplasmic reticulum; endo H, endo-β-N-acetylglucosaminidase; CST, castanospermine; BFA, brefeldin A; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, NitroBlue Tetrazolium; NP-40, Nonidet P-40; DMEM, Dulbecco's modified Eagle's medium containing 25 mM glucose; TBS, Tris-buffered saline (24.8 mM Tris/HCl, 2.68 mM KCl and 137 mM NaCl, pH 7.4).

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dimerization of LPL subunits, and acquisition of high affinity for heparin by the lipase [5–9,16–19,22]. Processing of LPL oligosaccharides to complex types, however, is not necessary for synthesis of active lipase [6,17,19].

Combined-lipase deficiency (*clد*) is a recessive mutation on mouse chromosome 17 which causes severe functional deficiencies of lipoprotein and hepatic lipases [23–25]. Mice homozygous for this deficiency (*clد/clد*) develop extreme hypertriacylglycerolaemia (10000 mg/dl in 12 h), have tissues devoid of fat, and die within 3 days of birth [23,26]. Early studies showed that heart, diaphragm muscle and brown adipose tissue of *clد/clد* mice contained 2–6 times normal amounts of LPL protein and these tissues could incorporate [³⁵S]methionine into LPL, but the lipase was inactive [27,28]. Other studies showed that brown adipocytes cultured from *clد/clد* mice synthesized inactive high-mannose-type LPL which was retained in ER [8]. The structural gene for LPL, located on mouse chromosome 8 [20], is normal [29] and functional in *clد/clد* mice [28,30]. Whether the *clد* mutation affects primarily glycosylation of LPL, trimming of LPL oligosaccharides chains in ER, transport of LPL from ER to Golgi, or some other process, has not been resolved [8,28].

Processing of oligosaccharide chains of glycoproteins is initiated by the removal of the outermost glucose residue by glycosidase I in ER [31]. Inhibition of this enzyme with castanospermine (CST) [31,32] resulted in the synthesis of LPL which was inactive, had untrimmed oligosaccharide chains and was retained in ER [18,19,22]. Blockade of protein transport from ER with carbonyl cyanide *N*-chlorophenylhydrazone also caused synthesis and retention of inactive LPL in ER [5]. These findings, as well as those in *clد/clد* cells, suggested that retention of LPL in ER might block processing of LPL to an active form.

Brefeldin A (BFA) is known to block protein transport from ER, dismantle Golgi, and cause translocation of Golgi enzymes to ER in several types of cells [33–37]. This paper describes experiments in which BFA was used to study the effect of translocated Golgi components on LPL retained in ER of *clد/clد* and CST-treated newborn mouse brown adipocytes.

EXPERIMENTAL

Materials

Tri[9,10(n)-³H]oleoylglycerol and ¹⁴C-methylated protein *M_r* standards were from Amersham. ENHANCE® was from Du Pont-New England Nuclear. L-[³⁵S]Methionine and cycloheximide were from ICN Biomedicals. Dulbecco's modified Eagle's medium (DMEM) and methionine-free DMEM were from Biofluids. Prestained protein *M_r* standards for SDS/PAGE, SDS, mercaptoethanol, dithiothreitol, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and NitroBlue Tetrazolium (NBT) were from Bio-Rad. CST, endo H and leupeptin were from Boehringer Mannheim. PANSORBIN® cells were from Calbiochem. BFA was from Epicentre Technologies. Nonidet P-40 (NP-40), Triton X-100 and Mops were from Fluka Chemical Corp. Fetal bovine serum was from Gibco. Insulin, triiodothyronine, deoxycholate, antipain, benzamide and aprotinin were from Sigma. Collagenase (type I) was from Worthington. Chicken antibodies (IgG) against bovine LPL were kindly given by Dr. Thomas Olivecrona, University of Umeå, Umeå, Sweden. Rat monoclonal antibodies against mouse Golgi membrane protein and against mouse lysosomal membrane protein, developed by Dr. J. August Thomas, were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology, Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A. and the Department of Biology, University of Iowa, Iowa City, IA, U.S.A. Rabbit

antibodies (IgG) against chicken IgG, alkaline phosphatase-conjugated rabbit antibodies (IgG) against chicken IgG, rhodamine-labelled affinity-purified rabbit anti-(chicken IgG) and fluorescein-labelled affinity-purified goat anti-(rat IgG) were from Jackson ImmunoResearch. All other chemicals were of the highest quality commercially available.

Solution A, for lysis of cells, contained 1% NP-40, 20 mM Mops, 0.2 mM MgCl₂, 100 mM NaCl and protease inhibitors (1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml antipain and 10 µg/ml benzamide), pH 7.6. Solution B, for dissolving samples for SDS/PAGE, contained 62.5 mM Tris, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.001% Bromophenol Blue, pH 6.8. Solution C, containing 10 mM Mops and 1.5 mM magnesium acetate, pH 7.45, and solution D, containing 400 mM Mops, 700 mM KCl, 40 mM magnesium acetate and 1 mM dithiothreitol, pH 7.45, were used for preparation of the post-nuclear membrane fraction. Solution E, for washing immunoprecipitates, contained 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris and 0.02% NaN₃, pH 8.5.

Culture medium contained 10% (v/v) fetal bovine serum, 17 µM D-pantothenic acid, 33 µM D-biotin, 100 µM ascorbic acid, 0.5 nM insulin, 0.5 nM triiodothyronine, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 0.25 µg/ml amphotericin B in DMEM [8].

Animals

The mice used in this study were derived from a colony bearing the combined-lipase deficiency (*clد*) mutation [23]. The very high plasma triacylglycerol concentration in *clد/clد* mice suckled more than 12 h, > 10000 mg/dl, gives the blood a creamy pink colour, in contrast with the non-creamy red colour in unaffected mice [8]. Because of the close association of the *clد* mutation with mutations affecting tail length, about 95% of the mice born with combined lipase deficiency had no tail [8,23]. Thus, suckled mice which were tailless and had creamy pink blood were classified as defective (*clد/clد*), whereas those which had a tail and non-creamy red blood were classified as normal. The mice were raised in animal facilities of NIDDK, NIH, Bethesda, MD, U.S.A. from stock kindly supplied by Dr. Karen Artzt of the Patterson Laboratory, University of Texas, Houston, TX, U.S.A. The mice were fed NIH Open Formula Rat and Mouse Ration (NIH-7).

Culture of brown adipocytes

Primary cultures of brown adipocytes were grown from the stromal-vascular fraction of brown adipose tissue taken from the interscapular region of suckled 12–24-h-old *clد/clد* and unaffected mice. The stromal-vascular fraction was prepared, using collagenase digestion, by the method of Masuno et al. [8]. A sample of 30 mg of tissue (from 2–3 mice) usually provided sufficient cells to seed one 60-mm-diam. culture plate. The cells were grown in an atmosphere of 5% CO₂ in air at 37 °C. The incubation medium was changed every 2 days before confluence and every day after confluence. The cells became confluent 4–5 days after seeding and were used for experiments on days 2–4 of confluence.

Assay of LPL activity

LPL activity associated with cells was measured in aqueous extracts of dried-defatted (acetone/ether) powders of cells [8,38]. LPL activity in culture medium was measured in an aliquot of medium filtered through 0.22-µm-pore-size Millex-GS filter units.

LPL activity was measured by hydrolysis of serum-activated emulsified trioleoylglycerol (labelled with tri[9,10(n)- ^3H]oleoylglycerol) to fatty acids at pH 8.1 [8]. A 1 m-unit lipolytic activity represents the release of 1 nmol of fatty acid/min. DNA in cells was measured fluorometrically by the method of Hinegardner [39], using calf thymus DNA as standard.

LPL activity can be calculated as the difference in lipolytic activity in the presence and in the absence of serum (source of apolipoprotein C-II) [40]. LPL activity accounted for > 88 % of the lipolytic activity of cells and medium measured in the presence of serum, except when lipolytic activity was very low. Therefore, lipolytic activity measured with serum was used as an estimate of LPL activity.

Endo H-digestion and immunoblotting of LPL subunits

Processing of LPL oligosaccharides was analysed by digesting LPL in cell lysates with endo H, separating LPL subunits by SDS/PAGE, and visualizing the subunits by immunoblotting.

Cells grown in 6-well culture plates were harvested after experiment with 0.5 ml of solution A and sonicated. The sonicate was centrifuged for 20 min at 12000 *g*. An aliquot of the supernatant was mixed with 1 % SDS in 0.1 M sodium citrate (pH 5.5), boiled for 6 min and centrifuged at 3000 *g* for 10 min. An aliquot (50 μl) of the supernatant was mixed with 5 μl of 2 % (v/v) mercaptoethanol in 1 M sodium citrate (pH 5.5) and 5 μl of protease inhibitor mix (1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml antipain and 10 mg/ml benzamide). The mixture was incubated with or without 3 μl of 1 unit/ml endo H for 24 h at 37 °C, the reaction was stopped by the addition of an equal volume of double-strength solution B, and the mixture was boiled for 5 min.

LPL subunits in the mixture were separated by SDS/PAGE [41] with 9 % resolving and 3 % acrylamide stacking gel, and transferred to nitrocellulose membrane. The membranes were blocked with 2 % (w/v) BSA, and incubated for 24 h with chicken antiserum to bovine LPL and then for 4 h with alkaline phosphatase-conjugated rabbit antiserum to chicken IgG. Bands containing LPL subunits were marked with black–purple precipitate produced by the action of the immunobound alkaline phosphatase on BCIP/NBT substrate solution.

Preparation of post-nuclear membrane lysates

Post-nuclear membrane lysates were prepared from adipocytes according to procedures published by Vannier et al. [7,42] and correspondence with Dr. Christian Vannier (European Molecular Biology Laboratory, Heidelberg, Germany). At the end of the experiment, cells in three 25 cm² culture flasks were quickly chilled to 0 °C, washed with solution C, scraped from the flasks, and suspended together in 3 ml of solution C. All steps were performed at 0–4 °C. Dithiothreitol was added to a final concentration of 1 mM, and the cells were homogenized in a Dounce homogenizer by 20 strokes of pestle A and 40 strokes of pestle B. A volume of 1 ml of solution D was added and the homogenate was immediately centrifuged at 600 *g* for 5 min. The supernatant was removed and centrifuged in a Beckman SW 60 Ti rotor at 100000 *g* for 60 min. The pellet was suspended in a 3:1 mixture of solutions C and D, and centrifuged again at 100000 *g* for 60 min. The resultant pellet, the post-nuclear membrane fraction, was lysed by adding 150 μl of solution B and mixing gently for 30 min. The post-nuclear membrane lysate was centrifuged at 15000 *g* for 30 min.

Zonal (sucrose-gradient) centrifugation

An aliquot of the post-nuclear membrane lysate was mixed with markers, BSA ($M_r = 65400$, $s = 4.3$ S) [43] and baker's yeast alcohol dehydrogenase ($M_r = 150000$, $s = 7.6$ S) [44], and layered on top of 3.6 ml of a linear gradient of 5–20 % (w/v) sucrose in 0.1 % NP-40, 20 mM Mops, 0.2 mM MgCl_2 and 100 mM NaCl (pH 7.6) overlaid on 0.2 ml of 40 % (w/v) sucrose in the same buffer solution. The samples were centrifuged in a Beckman SW 60 Ti rotor at 54000 rev./min for 9 h at 4 °C. Fractions of 0.23 ml were collected from the top of the centrifuge tube and each fraction was analysed for LPL protein and LPL activity. Molecular masses of the various forms of LPL isolated by zonal centrifugation were estimated from a plot of the relative distance travelled during centrifugation versus $\log M_r$ of the sedimentation markers, BSA and alcohol dehydrogenase [44,45].

Measurement of LPL protein

The relative amount of LPL protein present in fractions of post-nuclear membrane lysates collected after centrifugation was calculated from the amount of ^{125}I -radioactivity associated with LPL bands on immunoblots made with chicken IgG against bovine LPL, rabbit IgG against chicken IgG, and ^{125}I -Protein A. LPL was separated from other proteins by SDS/PAGE [41] with 9 % resolving gel and 3 % stacking gel, and then transferred to a nitrocellulose membrane according to the manual of Bio-Rad. Completeness of transfer to the membrane was checked with prestained protein standards run at the same time. Non-specific binding sites were blocked by incubating the membrane with 2 % (w/v) BSA for 1 h. The membrane was then incubated overnight with 0.5 μg of chicken IgG against bovine LPL in a solution of 0.5 % Tween-20 in Tris-buffered saline (TBS). The membrane was thoroughly washed with Tween–TBS, incubated with rabbit IgG against chicken IgG for 4 h, and washed again with Tween–TBS to remove excess rabbit antibodies. The membrane was then incubated with 3 μCi of ^{125}I -Protein A for 1 h, and washed with Tween–TBS. The amount of radioactivity associated with LPL bands was measured with a Phosphorimager® (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The relative amount of LPL protein in each fraction was calculated from the amount of ^{125}I -radioactivity associated with LPL on immunoblots of each fraction and that on immunoblots of all fractions.

Incorporation of [^{35}S]methionine into LPL

Normal and *cld/cld* brown adipocytes grown in 6-well culture plates were incubated for 17.5 h on day 3 of confluence with 0 or 200 $\mu\text{g}/\text{ml}$ of CST in 3 ml of medium [18]. The medium was removed, the plate was quickly rinsed twice with ice-cold PBS, 2 ml of methionine-deficient DMEM with or without inhibitor was added to the plate, and the plate was returned to the incubator. The methionine-deficient DMEM contained 0.5 nM insulin, 0.5 nM tri-iodothyronine, 17 μM D-pantothenic acid, 33 μM D-biotin, 100 μM ascorbic acid and 10 % (v/v) fetal bovine serum which had been dialysed overnight against methionine-deficient DMEM. After 30 min, 180 μCi of [^{35}S]methionine was added to each plate and the cells were incubated for 10 min. The medium was immediately removed and the plate was rinsed twice with ice-cold PBS. The cells were harvested with a plastic cell scraper into 0.5 ml of solution A, and sonicated briefly at 0 °C. The suspension was then centrifuged for 20 min at 12000 *g*, and the supernatant below the fatty layer was removed.

A 50–100 μl aliquot of the supernatant was used for immuno-

precipitation of LPL. Non-specific immunoprecipitates were removed by adding 30 μ l of a 20% suspension of PANSORBIN® cells complexed with rabbit IgG against chicken IgG, letting the mixture stand at 4 °C for 2 h, except for occasional vortexing, and centrifuging the mixture at 12000 *g* for 3 min. An aliquot of the supernatant was incubated overnight with 1 μ l of PBS containing 0.5 μ g of chicken IgG against bovine LPL. LPL-antibody complexes were precipitated by adding 30 μ l of a 20% suspension of PANSORBIN® cells complexed with rabbit IgG against chicken IgG, letting the mixture stand at 4 °C for 4 h, except for occasional vortexing, and centrifuging the mixture at 12000 *g* for 3 min. The precipitates containing ³⁵S-labelled LPL were washed 6–7 times with solution E.

³⁵S-labelled LPL was dissolved by boiling the precipitate in 1% SDS/0.1 M sodium citrate buffer (pH 5.5) for 6 min. The solution was centrifuged, and aliquots of the supernatant were mixed with mercaptoethanol/sodium citrate and protease inhibitor and incubated with or without endo H as described above.

³⁵S-labelled LPL subunits in the mixtures were resolved by SDS/PAGE with a 9% resolving gel and a 3% stacking gel. ¹⁴C-labelled molecular-mass markers were run with each gel. Gels containing ³⁵S-labelled LPL were fixed with 10% acetic acid/30% methanol, impregnated with ENHANCE® and dried on a sheet of cellophane with vacuum. Autoradiographs were obtained by exposing Kodak X-Omat film to gels for periods up to 14 days at –70 °C.

Immunolocalization of LPL, Golgi and lysosomes in cells

A double-antibody technique was used for localizing by immunofluorescence LPL, Golgi and lysosomes in cells [8,17,18]. The primary reactants used were antibodies raised in chicken against bovine LPL, and monoclonal antibodies raised in rat against mouse Golgi membrane protein or against mouse lysosomal membrane protein. The secondary reactants used were rhodamine-labelled affinity-purified rabbit anti-(chicken IgG) and fluorescein-labelled affinity-purified goat anti-(rat IgG). Cells were fixed with 3% paraformaldehyde, free aldehyde groups were quenched in blocker solution containing 1.5 mg/ml glycine, and cells were permeabilized in blocker solution containing 0.1% saponin before being incubated with primary antibodies. The blocker solution for LPL localization contained 2.5 mg/ml of rabbit IgG in PBS, and that for Golgi and lysosome localizations contained 2.5 mg/ml of rat IgG in PBS. Control cells for non-specific staining were incubated with chicken serum or rat IgG, and the appropriate secondary antibody.

RESULTS

Effect of BFA on LPL activity in normal, *cld/cld* and CST-treated adipocytes

Primary cultures of brown adipocytes from newborn mice were used to study the effect of BFA on LPL synthesis. The cells were studied on days 3–4 of confluence.

Adipocytes from normal mice contained 3000–5000 m-units of LPL activity/mg of DNA and released spontaneously 35 m-units of LPL activity/mg of DNA per h to the incubation medium (Table 1, Figure 1). BFA at 10 μ g/ml increased LPL activity in cells to 7000–10000 m-units/mg of DNA in 2 h and stopped completely secretion of LPL activity. The increase in LPL activity in treated cells was evident at 30 min and continued for at least 4 h (Figure 1).

Adipocytes cultured from *cld/cld* mice contained 120 m-units of LPL activity/mg of DNA, 4% of that in normal cells, and

Table 1 Effect of BFA on cellular and medium LPL activity of normal, *cld/cld* and CST-treated brown adipocytes

Normal and *cld/cld* cells were incubated with or without 10 μ g/ml BFA for 2 h on day 4 of confluence. CST-treated cells were normal cells treated on day 3 of confluence with 200 μ g/ml CST for 18 h and then incubated with or without 10 μ g/ml BFA during the last 2 h of treatment. LPL activity was measured in aqueous extracts of acetone/ether powders of cells and in media taken at end of 2 h. Each value is the average of results obtained with three plates of cells. Similar results were obtained for cellular LPL activity in all groups of cells and for medium LPL activity in normal cells in 2–4 separate experiments performed with different cell preparations. LPL activities in media of *cld/cld* and CST-treated cells were measured in only one experiment.

Cells	LPL activity in cells (m-units/mg of cellular DNA)		LPL activity released into medium in 2 h (m-units/mg of cellular DNA)	
	Control	BFA	Control	BFA
Normal	3040	6970	76	0.8
<i>cld/cld</i>	122	2100	0.8	0.8
CST-treated	430	2590	6.0	4.5

Figure 1 Effect of BFA on cellular and medium LPL activity in normal adipocytes

Cells were incubated on day 3 of confluence with or without 10 μ g/ml BFA for 0.5–4 h. LPL activity was measured in aqueous extracts of acetone/ether powders of cells and in media at designated times. Each value is the average of results obtained with three plates of cells.

released no LPL activity to the medium (Table 1). BFA at 10 μ g/ml increased in 2 h LPL activity in *cld/cld* adipocytes to 2100 m-units/mg of DNA, 69% of that in untreated normal cells, and had no effect on LPL secretion (Table 1).

Normal adipocytes treated with 200 μ g/ml CST for 18 h contained 430 m-units of LPL activity/mg of DNA and released 3.0 m-units of LPL activity/mg of DNA per h to the medium (Table 1). BFA at 10 μ g/ml increased in 2 h LPL activity in CST-treated cells to 2600 m-units/mg of DNA, 86% of that in

Figure 2 Effect of BFA on LPL subunits in normal, *cld/cld* and CST-treated adipocytes

Normal and *cld/cld* cells were incubated with or without 10 μ g/ml BFA for 2 h on day 4 of confluence. CST-treated cells were normal cells treated on day 3 of confluence for 18 h and then incubated with or without 10 μ g/ml BFA during the last 2 h of treatment. LPL in lysates of cells was digested with endo H, and LPL subunits were separated by SDS/PAGE and visualized by immunoblotting. The findings are representative of 3–4 independent experiments.

untreated normal cells, and had no effect on secretion of LPL activity (Table 1).

Effect of BFA on glycosylation of LPL in normal, *cld/cld* and CST-treated adipocytes

Processing of LPL oligosaccharides was analysed by digesting LPL in cell lysates with endo H, separating LPL subunits by SDS/PAGE, and visualizing the subunits by immunoblotting.

Cultured normal brown adipocytes contained LPL composed of subunits with $M_r = 56\,000$ – $57\,000$ (Figure 2). About one-third of the subunits were totally resistant to endo H, with $M_r = 57\,000$; about one-third were partially endo H-resistant, yielding a digestion product with $M_r = 54\,000$; and about one-third were totally endo H-sensitive, yielding a product with $M_r = 51\,000$. Slightly different results, in brown adipocytes, were obtained with LPL labelled with [35 S]methionine: more than one-half of the subunits were totally endo H-resistant, about one-third were endo H-sensitive, and less than one-third were partially endo H-resistant [8,17]. BFA treatment decreased to zero the proportion of LPL units in normal adipocytes which were totally endo H-resistant, increased markedly the proportion which were partially endo H-resistant, and had very little effect on those which were totally endo H-sensitive (Figure 2).

cld/cld adipocytes contained LPL composed of subunits with $M_r = 56\,000$ (Figure 2). Most of the subunits in these cells were totally endo H-sensitive, a very small proportion were partially endo H-resistant, and none were totally endo H-resistant, confirming earlier findings with [35 S]-labelled LPL [8,17]. BFA treatment changed markedly the composition of LPL subunits in *cld/cld* cells: BFA increased the proportion of subunits which were partially endo H-resistant and decreased the proportion which were totally endo H-sensitive. Totally endo H-resistant subunits were not found in BFA-treated *cld/cld* cells.

Normal brown adipocytes treated with CST contained LPL composed of subunits with $M_r = 57\,000$ (Figure 2). A large proportion of the subunits were totally endo H-sensitive, a small proportion were partially resistant, and none were totally endo H-resistant. BFA treatment lowered the M_r of LPL subunits to 56000, as it increased the proportion which were partially endo

H-resistant and decreased the proportion which were totally endo H-sensitive. Cells treated with CST + BFA did not contain totally endo H-resistant subunits.

Effect of BFA on sedimentation coefficient of LPL in normal, *cld/cld* and CST-treated adipocytes

The sedimentation coefficient of LPL was determined by zonal (sucrose gradient) centrifugation, SDS/PAGE and radio-immunoblotting of LPL in post-nuclear membrane lysates of cells.

LPL from normal control cells had a sedimentation coefficient of 5.9 S and M_r of 99 500, while denatured mouse LPL (boiled with 2% SDS) had a coefficient of 3.1 S and M_r of 48 500 (Figure 3a). The values obtained for M_r of native and denatured LPL are 1.8 and 0.9 times, respectively, the value for M_r of LPL subunits determined by SDS/PAGE and immunoblotting (56 000–57 000) (Figure 2). Thus, most of the LPL in normal cells was dimeric and the denatured LPL was monomeric. Similar values were observed for dimeric and monomeric bovine LPL [46,47].

BFA increased by 30% the proportion of LPL which was dimeric. It also increased greatly LPL activity in the fractions containing dimeric LPL (Figure 3b), indicating that BFA-treated cells contained more active LPL dimers than control cells. This dramatic effect of BFA on LPL activity, compared with its effect on LPL activity in whole cell extracts (Figure 1), probably reflects the total retention of LPL activity in ER by BFA.

About 40% of LPL in *cld/cld* cells (Figure 3c) and a similar proportion in CST-treated cells (Figure 3e) sedimented as inactive dimers and the rest sedimented as aggregates, or perhaps as higher oligomers [47]. BFA increased the proportion of LPL present as dimers in both groups of cells. It also increased LPL activity in the dimer fractions (Figures 3d and 3f), indicating that BFA caused synthesis of active dimers or, possibly, activation of LPL dimers.

Effect of CST on synthesis of [35 S]-labelled LPL in normal and *cld/cld* adipocytes

A recent study showed that CST-treated 3T3-L1 adipocytes pulsed 10 min with [35 S]methionine had [35 S]-labelled LPL subunits with an M_r 2000–4000 larger than that in untreated normal cells (57 000), indicating that CST blocked trimming of LPL oligosaccharide chains [18]. The findings also indicated that considerable trimming of oligosaccharides occurred during the first 10 min of LPL synthesis in untreated cells. The M_r of LPL subunits in CST-treated cells suggested a minimal value of 57 000 for M_r of murine LPL subunits with two fully core-glycosylated chains ($\text{Glu}_3\text{Man}_9\text{GluNAc}_2$) [18].

A similar study was made in *cld/cld* brown adipocytes to determine if the *cld* mutation affected either core-glycosylation or trimming of oligosaccharides of LPL in ER. Normal and *cld/cld* adipocytes were treated with 0 or 200 μ g/ml CST for 17.5 h and incubated with [35 S]methionine during the last 10 min of treatment. M_r of [35 S]-labelled LPL subunits in normal and *cld/cld* cells treated with CST was 57 000, whereas that in untreated cells was 55 000 (Figure 4). The endo H-digested subunits in all groups of cells had the same M_r , 51 000. The findings indicate that core-glycosylation of LPL and trimming of LPL oligosaccharides in ER were normal in *cld/cld* cells.

Effect of BFA on distribution of LPL in normal adipocytes

A double-antibody technique was used for localization of LPL, Golgi and lysosomes in cells by immunofluorescence.

Figure 3 Effect of BFA on sedimentation coefficient of LPL in normal (a, b), *cld/cld* (c, d) and CST-treated (e, f) adipocytes

Normal and CST-treated cells were incubated for 2 h and *cld/cld* cells incubated for 4 h with (○) or without (●) 10 μ g/ml BFA (see Figure 2). LPL in post-nuclear membrane lysates prepared from each group of cells and denatured LPL (Δ) (LPL of normal cells boiled with 2% SDS) were resolved by zonal centrifugation using a 5–20% (w/v) sucrose gradient as described in the Experimental section. Fractions were collected from the top of centrifuge tubes and analysed for LPL protein (a,c,e) and LPL activity (b,d,f). Arrows indicate fractions which contained sedimentation markers, BSA ($s = 4.3$ S, M_r 65000) and alcohol dehydrogenase ($s = 7.6$ S, M_r 150000); markers were centrifuged with each sample. Data for each experimental group were obtained with a single post-nuclear membrane lysate prepared from a pool of cells grown in three 25 cm^2 culture flasks.

Figure 4 Effect of CST on synthesis of ^{35}S -labelled LPL in normal and *cld/cld* adipocytes

Normal and *cld/cld* cells were incubated with or without 200 μ g/ml CST for 17.5 h. The medium was then replaced with methionine-deficient DMEM containing the appropriate concentration of CST. After 30 min, [^{35}S]methionine was added to the medium and the cells were incubated for 10 min, immediately chilled to 0 $^{\circ}\text{C}$, and lysed with solution B. ^{35}S -labelled LPL was immunoprecipitated from cell lysates, incubated with or without endo H, resolved by SDS/PAGE, and radioautographed.

Immunolabelled LPL was not detectable in cultured normal brown adipocytes (Figure 5A), confirming earlier results [8]. LPL was readily found in normal brown adipocytes treated with BFA for 2 h (Figure 5B). LPL in these cells was present in large tubular and spherical structures and in a reticular pattern, suggesting it was present also in ER. Golgi compartments in BFA-treated cells were displaced from the paranuclear regions (results not shown). LPL did not co-localize with either immunolabelled Golgi or immunolabelled lysosomes in BFA-treated cells (results not shown).

BFA was withdrawn and protein synthesis was blocked by incubating BFA-treated adipocytes in BFA-free medium containing 15 μM cycloheximide. Within 30 min after withdrawal of

BFA, Golgi reformed and immunofluorescent LPL disappeared from large vesicles and appeared in paranuclear regions (Figure 5C) where it co-localized with Golgi (Figure 5E). LPL did not co-localize with lysosomes. Immunofluorescent LPL was not present in normal cells at 60 min after withdrawal of BFA (Figure 5D). The findings indicate that LPL was converted into partially endo H-resistance, processed to active lipase, and retained in ER during the BFA blockade. When the blockade was lifted, LPL produced during the blockade was transported from ER to Golgi and then disappeared within 60 min.

Effect of BFA on distribution of LPL in *cld/cld* adipocytes

Immunolabelled LPL was found in ER of untreated *cld/cld* cells (Figure 6A), as reported earlier [8]. LPL was also found in *cld/cld* cells treated with BFA for 2 h, but it was localized primarily in large dilated tubular and spherical structures (Figure 6B), similar to that in BFA-treated normal cells (Figure 5B). In addition, Golgi compartments were dispersed throughout the cytoplasm. LPL did not co-localize with Golgi in BFA-treated *cld/cld* cells (results not shown).

When BFA was withdrawn and protein synthesis blocked in *cld/cld* cells, LPL which had been synthesized during the BFA-blockade was retained in the large tubular and vesicular structures and in ER for at least 60 min (Figures 6C and 6D). Although Golgi reformed in the perinuclear regions during the washout, LPL did not co-localize with immunolabelled Golgi or with immunolabelled lysosomes (results not shown). The findings indicate that LPL in *cld/cld* cells was converted into partial endo H-resistance, processed to active lipase, and retained in ER

Figure 5 Effect of BFA on distribution of LPL in normal brown adipocytes

Normal cells were incubated 2 h with 3 $\mu\text{g/ml}$ heparin (to remove extracellular LPL from cells), 2 h with 10 $\mu\text{g/ml}$ BFA in heparin-free medium, and then 60 min without BFA in medium containing 15 μM cycloheximide (to block protein synthesis during withdrawal of BFA). Cells were taken for microscopy at the end of incubation with heparin (**A**), at the end of incubation with BFA (**B**), and at 30 (**C**, **E**) and 60 min (**D**) after withdrawal of BFA. Cells were processed for immunofluorescence microscopy with antibodies to LPL (**A–D**) and with antibodies to Golgi membranes (**E**). Immunolabelled LPL, which was not detectable in control cells (**A**), accumulated in enlarged tubular and spherical structures (arrows) and in endoplasmic reticulum (ER) in cells treated with BFA (**B**). After withdrawal of BFA, LPL in normal cells moved within 30 min to paranuclear regions (arrows) (**C**) where it co-localized with immunolabelled Golgi membranes (**E**), and then disappeared during the next 30 min (**D**). Abbreviation: N, nucleus. Magnification: **A** and **B**, $\times 850$; **C–E**, $\times 530$.

Figure 6 Effect of BFA on distribution of LPL in *cld/cld* brown adipocytes

cld/cld cells were treated as described in Figure 1. Cells were taken for microscopy at the end of incubation with heparin (A), at the end of incubation with BFA (B), and at 30 (C) and 60 min (D) after withdrawal of BFA and blockade of protein synthesis. Immunolabelled LPL was present in ER in *cld/cld* cells incubated with heparin (A), and present in enlarged tubular and spherical structures (arrows), as well as ER, in cells incubated with BFA (B), similar to that in BFA-treated normal cells (Figure 5B). However, withdrawal of BFA for 30 (C) and 60 min (D) did not cause relocation or disappearance of LPL in *cld/cld* cells. Abbreviation: N, nucleus. Magnification: A and B, $\times 850$; C and D, $\times 530$.

during the BFA blockade. However, the LPL produced in *cld/cld* cells during the blockade could not be transferred from ER when the blockade was withdrawn.

DISCUSSION

cld/cld cells synthesize LPL which is inactive, has endo H-sensitive oligosaccharide chains, and is retained in ER [8,28]. BFA treatment, in the present study, enabled synthesis of active LPL and processing of one oligosaccharide per subunit to endo H-resistance in *cld/cld* cells. BFA also blocked transport of LPL to Golgi, resulting in accumulation of LPL in large tubular and spherical structures and in ER. That LPL subunits were processed to partial endo H-resistance in BFA-treated cells indicates that Golgi mannosidases I and II [21,48,49] were translocated to ER by BFA. The results in CST-treated *cld/cld* cells showed that core glycosylation of LPL and trimming of LPL oligosaccharide chains in ER were normal in *cld/cld* adipocytes (Figure 3). These

findings demonstrate that LPL subunits synthesized in *cld/cld* cells are capable of being processed to active lipase, and the enzymes needed for activation of LPL are present in Golgi of such cells, but *cld/cld* cells are unable to transport LPL to Golgi. The fact that other proteins are not affected by the *cld* mutation [28] indicates that the defect is LPL-specific.

The inability of *cld/cld* cells to transport LPL was also reflected by retention of partially endo H-resistant LPL in ER after withdrawal of BFA. Various findings suggest that LPL may be anchored to cellular membranes by heparan sulphate proteoglycan [50–52], or glycosylphosphatidylinositol [53,54]. That inactive LPL in *cld/cld* cells has high affinity for heparin [8] suggests that binding of LPL to heparan sulphate may not be impaired in these cells. Retention of LPL in ER in *cld/cld* cells may result from a defective LPL-transport system, inability of LPL to interact with the LPL-transport system, or some other defect.

In CST-treated adipocytes, BFA enabled synthesis of active

LPL and processing of LPL subunits to partial endo H-resistance. This required removal of glucose residues from oligosaccharide chains in cells in which glucosidase I was inhibited. Several types of cells have endo- α -D-mannosidase in Golgi which can trim en bloc glucose and mannose residues from oligosaccharides, and thereby provide a glucosidase-independent pathway for formation of endo H-resistant oligosaccharides [55–57]. That BFA promoted oligosaccharide processing in CST-treated brown adipocytes indicates that endo- α -D-mannosidase was present in brown adipocytes and it was translocated, with mannosidases I and II, to ER by BFA.

BFA blocked in normal brown adipocytes transport of LPL from ER and, thereby, blocked processing of LPL oligosaccharide chains within Golgi. However, BFA promoted processing of one oligosaccharide chain per subunit to endo H-resistance by translocating *cis/medial* Golgi mannosidases I and II to ER. Similar observations were made in 3T3-L1 mouse adipocytes [58]. In contrast, LPL synthesized in BFA-treated pro-5 Chinese hamster ovary cells was totally endo H-resistant [19]. That the second oligosaccharide chain was not processed in mouse cells suggests that enzymes in *trans* Golgi may be required in mouse cells for processing the second chain to endo H-resistance. The oligosaccharide chains of LPL are located in different domains, one linked to Asn⁴⁴ in the N-terminal domain and the other linked to Asn³⁶¹ in the C-terminal domain [59,60]. One possibility is that LPL must undergo conformational changes in *trans* Golgi before the second oligosaccharide chain can be accessible to trimming/elongating enzymes in Golgi.

It is generally thought that normal cells have two forms of LPL, monomers, which are inactive, and dimers, which are active [5–9,15,52]. Dimerization of most proteins occurs in ER, usually soon after nascent subunits enter the ER lumen [61–63]. There are several reports suggesting that LPL becomes dimerized and active in ER [6,9,19], while others [5,7] suggests that both events occur in Golgi when LPL subunits are processed to endo H-resistance. The present study shows that *cld/cld* and CST-treated cells contained LPL dimers which were inactive. That LPL in such cells is present primarily in ER (Figure 6A) [8,18] suggests that dimerization of LPL can occur in ER independently of activation of the lipase.

Our findings shows that LPL became active and oligosaccharide chains became endo H-resistant when exposed to *cis/medial* Golgi components, either *in situ* in normal cells, or in ER of BFA-treated cells. Processing of oligosaccharide chains to endo H-resistance, however, is not required for synthesis and secretion of active LPL (in cells treated with 1-deoxymannojirimycin) [6,17,19]. The present results indicate that activation of LPL may require some modification in Golgi other than oligoglycan processing, such as sulphation [64], phosphorylation, proteolysis, or acylation [48,65].

The above findings suggest the following scheme for synthesis of active LPL in normal cells: LPL subunits are glycosylated during their transport across the ER membrane, and three glucose and one mannose residues are removed from each oligosaccharide chain in ER. LPL subunits are dimerized in ER and then transferred to the Golgi where LPL becomes active through some modification other than oligoglycan processing. Nonetheless, one oligosaccharide of each subunit is processed to endo H-resistance in *cis/medial* Golgi and the other in *trans* Golgi. Processing of oligosaccharides in Golgi may be necessary *in vivo* for stability of secreted active lipase [66], or for transport of LPL from parenchymal cells to the luminal surface of capillary endothelial cells where the lipase normally acts [12].

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