

# Glycosylation, activity and secretion of lipoprotein lipase in cultured brown adipocytes of newborn mice

## Effect of tunicamycin, monensin, 1-deoxymannojirimycin and swainsonine

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The effect of inhibitors on the glycosylation, activity and secretion of lipoprotein lipase was studied in brown adipocytes cultured from newborn mice. Such cells synthesized and secreted active lipoprotein lipase. It is generally accepted that active lipoprotein lipase is a homodimer. Glycosylation of lipoprotein lipase was analysed by PAGE of endoglycosidase H (endo H)-digested subunits of lipoprotein lipase immunoprecipitated from cells incubated for 1–2 h with [<sup>35</sup>S]methionine. The most prevalent <sup>35</sup>S-labelled lipase subunit (*M<sub>r</sub>* 57 000–58 000) in these cells contained endo H-resistant oligosaccharide chains, the next most prevalent contained totally endo H-sensitive chains, and the least prevalent subunit contained partially endo H-sensitive chains. Complete blocking of the glycosylation of lipoprotein lipase with tunicamycin (1 µg/ml) for 24 h resulted in synthesis of an inactive non-secretable form of lipase with a smaller subunit (*M<sub>r</sub>* 51 000–52 000). Immunofluorescent studies showed that unglycosylated lipase in tunicamycin-treated cells was retained in the endoplasmic reticulum. Cells treated with 1 µM-monensin, an intra-Golgi transport inhibitor, synthesized an active form of lipase which was not secreted, but was retained in the Golgi. The lipase in monensin-treated cells contained only partially or totally endo H-sensitive chains. Blocking either Golgi mannosidase I with 4 mM-1-deoxymannojirimycin or Golgi mannosidase II with 10 µM-swainsonine resulted in production of a form of lipoprotein lipase which was active and secreted, and which contained only endo H-sensitive chains. Our findings demonstrate that core glycosylation of lipoprotein lipase in the endoplasmic reticulum is required for lipase activity and transport from the reticulum, whereas processing of the oligosaccharide chains to endo H-resistant (complex) type chains in the Golgi is not required for either the activity or the secretion of lipoprotein lipase.

## INTRODUCTION

Lipoprotein lipase is required for the uptake of plasma triacylglycerol by extrahepatic tissues [1–3]. It is synthesized by parenchymal cells and transferred to capillaries, where it acts [4]. Active lipoprotein lipase is generally considered to be a non-covalent dimer of identical subunits (*M<sub>r</sub>* 55 000) [3,5–7]. Studies in cultured and isolated adipocytes indicate that mouse lipase has two *N*-linked oligosaccharide chains per subunit [8,9] and guinea pig lipase has three [10]. The oligosaccharide chains transferred to the subunits in endoplasmic reticulum are the high-mannose [endoglycosidase H (endo H)-sensitive] type, and two chains of each subunit are processed in normal adipocytes to give complex (endo H-resistant)-type chains in the Golgi apparatus before the lipase is secreted [8–10].

Glycosylation of lipoprotein lipase is required for synthesis of active secretable lipase [11–13]. Ob17 mouse adipocytes treated with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) synthesized inactive lipase which was retained in the endoplasmic reticulum [14]. Whether CCCP produced these effects by blocking transport of protein from the endoplasmic reticulum [15,16] or by impairing glycosylation [17–19] was not determined. Brown adipocytes from *cld/cld* mice synthesize lipoprotein lipase which is glycosylated, but the lipase is inactive and is retained in the endoplasmic reticulum [9]. Monensin, an inhibitor of protein transport between the medial and trans Golgi compartments

[20,21], blocked secretion but not synthesis of active lipoprotein lipase in mouse [14] and rat [22] adipocytes. A subnormal ratio of [<sup>3</sup>H]galactose to [<sup>14</sup>C]mannose in lipoprotein lipase of monensin-treated cells [22] suggested that processing of oligosaccharide chains of the lipase in the Golgi might be impaired by monensin. Guinea pig adipocytes treated with 1-deoxymannojirimycin, an inhibitor of Golgi mannosidase I [23–25], synthesized lipoprotein lipase which had endo H-sensitive chains and was active and secreted [10], suggesting that the lipase was active before it reached the site of action of mannosidase I in cis Golgi. Findings in 3T3.F244A mouse adipocytes, however, suggest that lipoprotein lipase becomes active after the oligosaccharide chains are processed to endo H-resistant types in medial/trans Golgi [8,26].

We used inhibitors of synthesis, transport and processing of glycoproteins to probe in more detail the relationship of glycosylation to the activity and secretion of lipoprotein lipase. This paper reports the effects of tunicamycin, monensin, 1-deoxymannojirimycin and swainsonine (an inhibitor of Golgi mannosidase II [27–29]) on lipoprotein lipase in cultured brown adipocytes from newborn mice.

A preliminary report of this study was presented at the Joint Meeting of the American Society for Biochemistry and Molecular Biology and the American Society for Cell Biology, San Francisco, California, January–February 1989 [29a].

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium containing 25 mM-glucose; PBS, Dulbecco's phosphate-buffered saline; PMSF, phenylmethanesulphonyl fluoride; endo H, endoglycosidase H.

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## MATERIALS AND METHODS

### Materials

L-[<sup>35</sup>S]Methionine and ENHANCE were from Du Pont–New England Nuclear (Wilmington, DE, U.S.A.). Tri[9,10(n)-<sup>3</sup>H]-oleoylglycerol and [<sup>14</sup>C]methylated proteins for  $M_r$  standards were from Amersham (Arlington Heights, IL, U.S.A.).  $M_r$  standards for SDS/PAGE were from Bio-Rad (Richmond, CA, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) and methionine-deficient DMEM were from Biofluids (Rockville, MD, U.S.A.). Fetal bovine serum was from Gibco (Gaithersburg, MD, U.S.A.). 1-Deoxymannojirimycin, swainsonine, leupeptin and pepstatin were from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Tunicamycin B complex, monensin, insulin, tri-iodothyronine, antipain, benzamidin, aprotinin and octanoic acid were from Sigma (St. Louis, MO, U.S.A.). Heparin was from Hynson, Wescott and Dunning (Baltimore, MD, U.S.A.). Collagenase (type I) was from Worthington (Freehold, NJ, U.S.A.). Endo- $\beta$ -N-acetylglucosaminidase H and crystalline BSA were from United States Biochemical Co. (Cleveland, OH, U.S.A.). Rabbit anti-chicken IgG was from Pel-Freez (Rogers, AR, U.S.A.). Chicken antiserum to bovine lipoprotein lipase was kindly given by Dr. Thomas Olivecrona, University of Umeå, Umeå, Sweden. All other chemicals were of the highest quality commercially available.

Solution A, used for aqueous extraction of acetone/ether powders, contained 50 mM-NH<sub>3</sub>/NH<sub>4</sub>Cl and 20  $\mu$ g of heparin/ml, pH 8.1. Solution B, used when acetone/ether powders were prepared from cells, contained 2% BSA in solution A. Solution C, used to dissolve immunoprecipitates for SDS/PAGE, contained 0.0625 M-Tris, 2% SDS, 5% 2-mercapto-ethanol, 10% glycerol and 0.001% Bromophenol Blue, pH 6.8. Solution D, used when cells were harvested for immunoprecipitation, contained 0.2 M-Tris, 3% Triton X-100, 1% N-lauroylsarcosine, 0.15 M-NaCl, and 1 mM-phenylmethanesulphonyl fluoride (PMSF), pH 7.5. Standard culture medium contained 10% fetal bovine serum, 17  $\mu$ M-D-pantothenic acid, 33  $\mu$ M-D-biotin, 100  $\mu$ M-ascorbic acid, 100 units of penicillin/ml, 0.1 mg of streptomycin/ml and 0.25  $\mu$ g of amphotericin B/ml in DMEM. Differentiation medium contained 0.5 nM-insulin, 0.5 nM-tri-iodothyronine and 1 mM-octanoic acid in standard culture medium.

### Animals

The mice used in this study were unaffected +/*cld* heterozygotes and homozygous wild types derived from a colony of mice bearing the combined lipase deficiency (*cld*) mutation [30]. The *cld* mutation is an autosomal recessive mutation that was extracted from a chromosome bearing mutations at the T/t complex of mouse chromosome 17. Mice homozygous for this defect (*cld/cld*) have < 5% normal amounts of lipoprotein lipase and hepatic lipase activities [30–32], and 50× and 125× normal plasma triacylglycerol concentration at 6–8 h and 12–30 h respectively [30]. The plasma triacylglycerol levels of wild-type and +/*cld* heterozygote weanling littermates were virtually identical, at about 80 mg/dl. It was not feasible to measure plasma triacylglycerol concentrations of individual tissue donors before processing the adipose tissue because of the large numbers of mice needed for each experiment and the very small amount of plasma (< 20  $\mu$ l) that could be obtained from each mouse. The very high plasma triacylglycerol concentration in suckled > 12-h-old *cld/cld* mice (> 10000 mg/dl) gave the blood of such animals a creamy pink colour, in contrast with the non-creamy red colour of blood of unaffected normolipemic mice [9]. Because of the close association of the *cld* mutation and mutations affecting tail length, about 95% of the mice born with

combined lipase deficiency have no tail, and the other 5% have a normal or subnormal length tail [30]. Thus suckled mice that were tail-less and had creamy pink blood were classified as defective (*cld/cld*), whereas suckled mice that had either a normal or a subnormal length tail and non-creamy red blood were classified as unaffected. Both +/*cld* heterozygotes and homozygous wild types were included in the unaffected group because it was not possible to identify homozygous wild-type mice in litters containing *cld/cld* mice and, in addition, there was no gene dosage effect of the *cld* mutation on lipoprotein lipase [30]. The mice were raised in the 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.

### Culture of brown adipocytes

Primary cultures of brown adipocytes were grown from the stromal-vascular fraction of brown adipose tissue taken from suckled 12–24-h-old unaffected mice. Although white adipose tissue normally develops around interscapular brown fat pads in mice, it was difficult to detect this tissue in 1-day-old unaffected mice, even with a dissecting microscope at 10× magnification. About 13 mg of brown adipose tissue could be obtained from each mouse, and tissue from three mice usually provided sufficient cells to seed one 60 mm-diam. plate. The stromal-vascular fraction was prepared, using collagenase digestion, by a modification of the method of Forest *et al.* [33], and the cells were grown in differentiation medium [9]. The cells became confluent 4–5 days after seeding and were used for experiments on days 2–4 of confluence.

Our earlier studies showed that the increase in lipoprotein lipase activity in cultured mouse brown adipocytes was 13 times greater on days 3 and 4 than on days 1 and 2 of confluence, whereas the increase in DNA content was steady during the same period of time [9]. The untreated cultures used in experiments in Tables 3 and 4 had lower DNA contents and lower lipoprotein lipase activities on day 4 of confluence than those used in the other experiments. Because these experiments were carried out at different times and by different investigators, the differences in DNA content and lipase activity probably reflect differences in judgement of when cultures became confluent.

### Assay of lipoprotein lipase activity

Lipoprotein lipase activity associated with cells was measured in aqueous extracts of dried-defatted (acetone/ether) powders of cells [11]. The cells were harvested into 0.5 ml of solution B, sonicated briefly at 0 °C and centrifuged for 20 min at 4 °C and 12000 g. A portion of the infranatant was used for preparing an acetone/ether powder. The extract was made by adding the powder to ice-cold solution A, letting the mixture stand at 0 °C for 60 min, sonicating briefly at 0 °C, centrifuging for 10 min at 4 °C and 1200 g and decanting the supernatant for assay. Lipoprotein lipase activity in the culture medium was measured in portions of the medium filtered through 0.22  $\mu$ m Millex-GS filter units. Assays of media were begun within 3 min of taking the samples.

A stock emulsion containing 5 mCi of tri[9,10(n)-<sup>3</sup>H]oleoylglycerol, 1.13 mmol of trioleoylglycerol, 60 mg of 1- $\alpha$ -phosphatidylcholine (bovine liver) and 9 ml of glycerol was prepared according to the method of Nilsson-Ehle & Schotz [34]. Before assay, 1 vol. of the stock emulsion, 19 vol. of 3% BSA in 0.2 M-Tris/HCl buffer (pH 8.1) and 5 vol. of heat-inactivated fasted-rat serum (heated at 60 °C for 10 min) were mixed and incubated at 37 °C for 15–30 min. For assay, 100  $\mu$ l of this activated substrate mixture, containing 2.0  $\mu$ Ci of tri[9,10(n)-<sup>3</sup>H]oleoylglycerol, 450 nmol of trioleoylglycerol and 34 nmol of albumin,

was added to 100  $\mu$ l of diluted tissue extract or medium and incubated at 37 °C for 60 min [9]. Fatty acids produced by lipolysis were extracted and measured as described previously [9]. 1 munit of lipolytic activity represents release of 1 nmol of fatty acid/min.

#### [<sup>35</sup>S]Methionine incorporation studies

Incorporation of [<sup>35</sup>S]methionine into lipoprotein lipase was studied in cultured brown adipocytes incubated with methionine-deficient DMEM containing 0.5 nM-insulin, 0.5 nM-triiodothyronine, 17  $\mu$ M-D-pantothenic acid, 33  $\mu$ M-D-biotin, 100  $\mu$ M-ascorbic acid, 1 mM-octanoic acid and 2% fetal bovine serum which had been dialysed overnight against methionine-deficient DMEM. In some experiments, cultured brown adipocytes were incubated for 21.5 h with or without inhibitors in 5 ml of differentiation medium in an atmosphere of 5% CO<sub>2</sub> in air at 37 °C. Then the medium was removed, the plate was rinsed quickly twice with Dulbecco's phosphate-buffered saline (PBS), 2 ml of methionine-deficient DMEM with or without inhibitors was added to each plate and the plates were returned to the incubator. After 30 min, 180  $\mu$ Ci of [<sup>35</sup>S]methionine was added to each plate and the cells were incubated for another 2 h. In other experiments, the culture medium was removed on the day of the experiment, the plates were rinsed twice with PBS, 2 ml of methionine-deficient DMEM was added to each plate, and the plates were returned to the incubator. After 30 min, 180  $\mu$ Ci of [<sup>35</sup>S]methionine and inhibitors were added to each plate, and the cells were incubated for another 2 h. At the end of each experiment, the medium was removed and the plate was rinsed twice with ice-cold PBS. The cells were immediately harvested with a plastic cell scraper into 0.5 ml of solution D and sonicated briefly at 0 °C; the suspension was then centrifuged for 20 min at 4 °C and 12000 g, and the infranatant, below the fatty layer, was removed and stored at -70 °C.

A sample of the infranatant was taken for measurement of the amount of [<sup>35</sup>S]methionine incorporated into total protein, which was precipitated with 10% trichloroacetic acid. The precipitate containing <sup>35</sup>S-labelled proteins was washed four times by mixing the precipitate with 10 vol. of 10% trichloroacetic acid and centrifuging the mixture for 10 min at 4 °C and 2750 g. <sup>35</sup>S-labelled lipoprotein lipase was immunoprecipitated with chicken antiserum to bovine lipoprotein lipase as described previously [9] and resolved by SDS/PAGE in a Laemmli-type system [35] with

a 10% acrylamide resolving gel and a 3% acrylamide stacking gel.

#### Enzymic deglycosylation of lipoprotein lipase

Lipoprotein lipase immunoprecipitated from <sup>35</sup>S-labelled protein with antiserum was dissolved in 30  $\mu$ l of 1% SDS in 0.1 M-sodium citrate at pH 5.5 with heating for 6 min, and then centrifuged for 3 min at 25 °C and 2750 g. A 10  $\mu$ l sample of the supernatant was mixed with 4  $\mu$ l of proteinase inhibitor mixture (1 mg of leupeptin/ml, 1 mg of antipain/ml, 10 mg of benzamidine/ml and 3.5  $\mu$ g of aprotinin/ml) and 110  $\mu$ l of 0.22% 2-mercaptoethanol in 0.1 M-sodium citrate at pH 5.5. A 6  $\mu$ l portion of endo H (46 units/ml) in 0.1 M-sodium citrate at pH 5.5 was then added and the mixture, with a total volume of 130  $\mu$ l, was incubated for 20–24 h at 30 °C. The reaction was terminated by addition of 130  $\mu$ l of double-strength solution C and heating at 95 °C for 10 min.

#### Chemical analysis

DNA was measured fluorimetrically by the method of Hinegardner [36], using calf thymus DNA as standard.

#### RESULTS

Primary cultures of adipocytes derived from brown adipose tissue of 1-day-old mice were used to study the effect of various inhibitors on the glycosylation, activity and secretion of lipoprotein lipase. Brown adipocytes harvested on day 3 or 4 of confluence contained lipoprotein lipase activity (see Tables 1–5), released lipase activity spontaneously into the culture medium (see Table 2), and incorporated [<sup>35</sup>S]methionine into total protein (see Tables 1, 3 and 5).

There was variation in lipase activity, ranging from 25 to 525 munits/mg of DNA, between groups of untreated cells, but the standard deviation within individual groups was usually < 12% of the mean. The variations were probably due to differences in the degree of maturation of the cells, resulting from variability in preparation of primary cultures of brown adipocytes from neonatal tissue (see the Materials and methods section).

Glycosylation of lipoprotein lipase was analysed by PAGE of endo H-digested subunits of lipase immunoprecipitated from cells incubated for 1–2 h with [<sup>35</sup>S]methionine. Undigested subunits migrated on SDS/PAGE as a broad band of  $M_r$

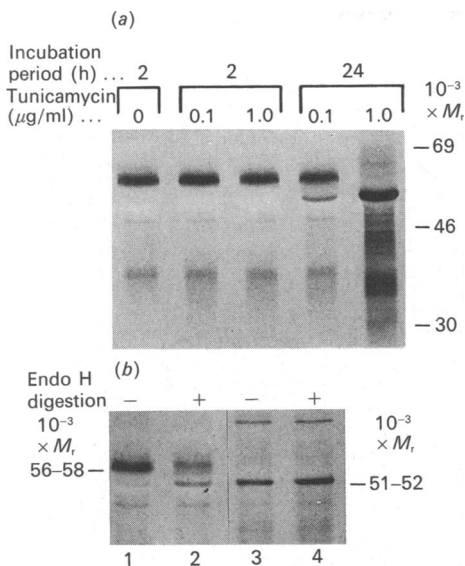
**Table 1. Effect of tunicamycin on lipoprotein lipase activity and total protein synthesis in cells cultured from brown adipose tissue**

Lipoprotein lipase activity was measured in aqueous extracts of acetone/ether powders of cells treated for 2 or 24 h with 0, 0.1 or 1.0  $\mu$ g of tunicamycin/ml. Incorporation of [<sup>35</sup>S]methionine into total protein was measured during the last 2 h of treatment. The amount of DNA in untreated cell cultures harvested on day 3 of confluence was  $47 \pm 0.3$   $\mu$ g/plate, and that on day 4 was  $56 \pm 0.7$   $\mu$ g/plate. Values given are means  $\pm$  S.D. The values in parentheses indicate the numbers of plates per group. Significant differences from untreated cells: \*  $P < 0.05$ , \*\*  $P < 0.01$ .

Duration of treatment (h)	Concentration of tunicamycin in medium ( $\mu$ g/ml)	Lipoprotein lipase activity in cells (munits/mg of DNA)	$10^{-7} \times$ <sup>35</sup> S in total cell protein (c.p.m./plate)
0	0	$490 \pm 24$ (3)†	$9.1 \pm 0.4$ (2)†
2	0.1	$451 \pm 30$ (3)†	$10.0 \pm 0.2$ (2)†
2	1.0	$361 \pm 18$ (3)†**	$10.3 \pm 0.4$ (2)†
24	0	$525 \pm 50$ (4)‡	$9.1 \pm 0.4$ (2)†
24	0.1	$307 \pm 28$ (3)‡**	$9.0 \pm 0.3$ (2)†
24	1.0	$4 \pm 4$ (3)‡**	$7.5 \pm 0.3$ (2)†*

† Cells harvested on day 3 of confluence.

‡ Cells harvested on day 4 of confluence.



**Fig. 1.** Effect of tunicamycin on the glycosylation of lipoprotein lipase in brown adipocytes

Cells on days 3 and 2 of confluence were treated for 2 or 24 h respectively with 0, 0.1 or 1.0 µg of tunicamycin/ml and incubated with 180 µCi of [<sup>35</sup>S]methionine during the last 2 h of treatment. The cells were harvested into solution D, sonicated briefly at 0 °C and centrifuged. <sup>35</sup>S-labelled lipoprotein lipase was immunoprecipitated from the infranatants, digested with or without endo H and resolved by SDS/PAGE. (a) Radioautographs of lipoprotein lipase from treated and untreated cells. (b) Radioautographs of endo H-digested lipoprotein lipase from cells treated for 24 h without (lanes 1 and 2) or with (lanes 3 and 4) 1.0 µg of tunicamycin/ml.

56 000–58 000 (see Figs. 1a and 4–8). The most prevalent subunit was endo H-resistant with an *M<sub>r</sub>* of 57 000–58 000; the next most prevalent was endo H-sensitive, yielding a product with *M<sub>r</sub>* 51 000–52 000, and the least prevalent subunit was partially endo

**Table 2.** Effect of monensin on lipoprotein lipase activity in cultured brown adipocytes and released into the medium

Lipoprotein lipase activity was measured in medium and in aqueous extracts of acetone/ether powders of cells treated on day 3 of confluence with or without monensin. The amount of DNA in the untreated cell cultures was 51 ± 3 µg/plate. Values given are means ± s.d. for three plates. \* Different from untreated, *P* < 0.05. n.d., not determined.

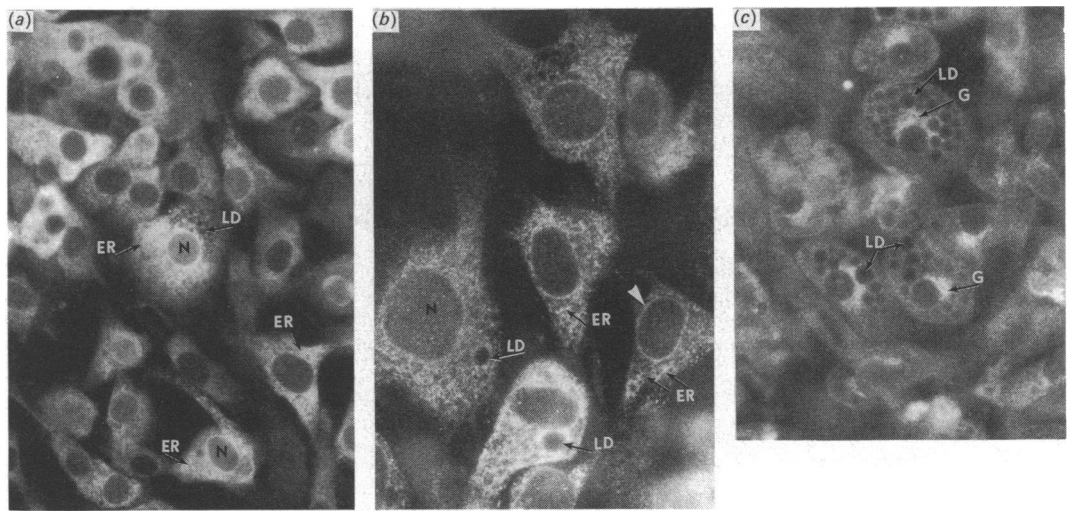
Duration of treatment (h)	Concentration of monensin in medium (µM)	Lipoprotein lipase activity (munits/mg of DNA)	
		Cells	Released into medium in 2 h
1	0	267 ± 22	n.d.
1	1.0	856 ± 14*	n.d.
2	0.1	465 ± 32*	n.d.
2	1.0	573 ± 18*	n.d.
2	10.0	825 ± 78*	n.d.
3	1.0	406 ± 21*	n.d.
4	0	301 ± 11	n.d.
4	1.0	637 ± 22*	n.d.
2	0	278 ± 4	5.1 ± 0.5
2	1.0	615 ± 11*	0.8 ± 0.4*

H-sensitive, yielding a digestion product of *M<sub>r</sub>* 53 000–54 000 (see Figs. 1b and 5–7).

**Tunicamycin**

Tunicamycin [37,38] at 0.1 µg/ml had no effect on lipoprotein lipase activity in 2 h, and decreased activity by 42 % in 24 h, whereas at 1.0 µg/ml it decreased lipoprotein lipase activity by 26 % in 2 h and completely suppressed activity in 24 h (Table 1). Tunicamycin at 1.0 µg/ml decreased total protein synthesis by 18 % during the last 2 h of a 24 h treatment (Table 1).

Tunicamycin had no effect in 2 h on synthesis of <sup>35</sup>S-labelled



**Fig. 2.** Light micrographs showing immunolocalization of lipoprotein lipase in tunicamycin-treated and monensin-treated brown adipocytes

Lipoprotein lipase was localized in cells with a fluorescent double antibody technique, using chicken antiserum to bovine lipoprotein lipase and rhodamine-labelled affinity-purified rabbit antibodies against chicken IgG. (a) and (b) Immunofluorescence of lipoprotein lipase in cells treated for 24 h with tunicamycin, starting on day 2 of confluence. The reticular pattern of distribution of immunofluorescence indicates that lipoprotein lipase was located in the endoplasmic reticulum (ER) in these cells. A thin line of immunofluorescence surrounding a nucleus in (b), marked by an arrowhead, indicates that lipase was present in the nuclear envelope. (c) Immunofluorescence of lipoprotein lipase in the Golgi region of cells treated with monensin. Cells on day 2 of confluence were incubated for 2 h with 15 µM-cycloheximide and 3 µg of heparin/ml, and then for 90 min with 1 µM-monensin alone before they were taken for morphological study. Immunofluorescence of lipoprotein lipase is present in the perinuclear Golgi region (G) of adipocytes. LD, lipid droplets; N, nucleus. Magnification: (a) × 200, (b) × 400; (c) × 200.

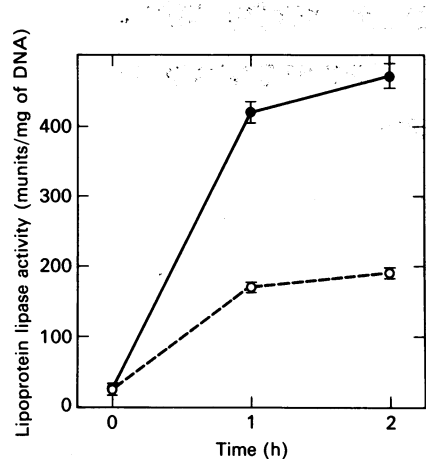


Fig. 3. Effect of monensin on the accumulation of lipoprotein lipase activity in brown adipocytes pretreated with cycloheximide and heparin

Cells at day 3 of confluence were incubated for 2 h with 15 μM-cycloheximide and 3 μg of heparin/ml, and were then incubated for 1–2 h without (○) or with (●) 1 μM-monensin. Lipoprotein lipase activity was measured in aqueous extracts of acetone/ether powders of the cells. The lipoprotein lipase activity in cells prior to treatment with cycloheximide and heparin was 411 ± 6 munits/mg of DNA. Values given are means ± S.D. of three plates.

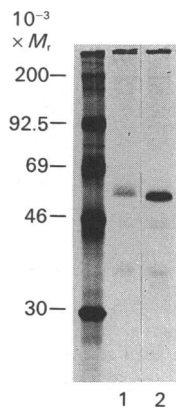


Fig. 4. Effect of monensin on the incorporation of [<sup>35</sup>S]methionine into lipoprotein lipase in brown adipocytes

Cells on day 3 of confluence were treated for 2.5 h without (lane 1) or with (lane 2) 1 μM-monensin, and incubated with 180 μCi of [<sup>35</sup>S]methionine during the last 2 h of treatment. The cells were harvested into solution D, sonicated briefly at 0 °C and centrifuged. <sup>35</sup>S-labelled lipoprotein lipase immunoprecipitated from the infranatants was resolved by SDS/PAGE and radioautographed. Radioactive zones corresponding to lipoprotein lipase were cut from the gels and dissolved in 0.75 ml of 35 % H<sub>2</sub>O<sub>2</sub> at 65 °C overnight, and radioactivity in the zones was determined in a liquid scintillation counter. <sup>35</sup>S-labelled lipoprotein lipase from untreated cells contained 10000 ± 1600 c.p.m./plate, and that from monensin-treated cells contained 23000 ± 1600 c.p.m./plate (values are means ± S.D. of 2 plates).

lipase subunits, whereas it caused synthesis of a smaller subunit during the last 2 h of a 24 h treatment (Fig. 1a). Cells treated with tunicamycin at 0.1 μg/ml for 24 h contained mostly normal-sized subunits and a small amount of the smaller subunit (*M<sub>r</sub>* 51000–52000), whereas cells treated with 1.0 μg of tunicamycin/ml for 24 h contained only the smaller subunit. The smaller subunit in tunicamycin-treated cells was resistant to endo H digestion (Fig. 1b) and had the same *M<sub>r</sub>* as endo F-sensitive lipase subunits from untreated brown adipocytes [9], indicating

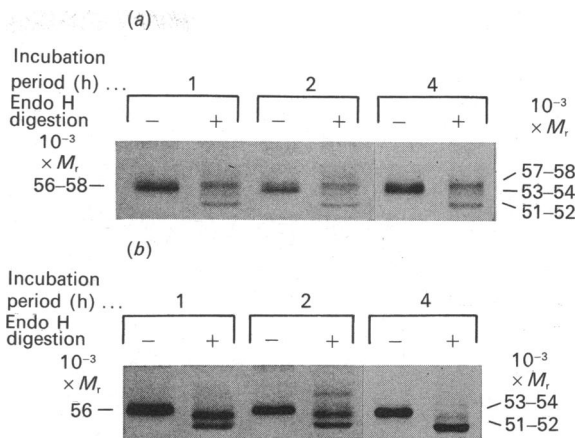


Fig. 5. Effect of monensin on the glycosylation of lipoprotein lipase in brown adipocytes

Cells at day 3 of confluence were treated for 1, 2, or 4 h without (a) or with (b) 1 μM-monensin and incubated with 180 μCi of [<sup>35</sup>S]methionine during the last 2 h of treatment. Cells were harvested into solution D, sonicated briefly at 0 °C and centrifuged. <sup>35</sup>S-labelled lipoprotein lipase in the infranatants was immunoprecipitated with antiserum, digested with or without endo H, resolved by SDS/PAGE and radioautographed.

Table 3. Effect of 1-deoxymannojirimycin on lipoprotein lipase activity and total protein synthesis in cells cultured from brown adipose tissue

Lipoprotein lipase activity was measured in aqueous extracts of acetone/ether powders of cells treated for 24 h with 0, 1 or 4 mM-1-deoxymannojirimycin. Incorporation of [<sup>35</sup>S]methionine into total protein was measured during the last 2 h of treatment in a second set of cells. The cells were harvested on day 4 of confluence. The amount of DNA in untreated cell cultures was 42 ± 8 μg/plate. Values given are the means ± S.D. The values in parentheses indicate the numbers of plates per group.

Concentration of 1-deoxymannojirimycin in medium (mM)	Lipoprotein lipase activity in cells (munits/mg of DNA)	10 <sup>-7</sup> × <sup>35</sup> S in total cell protein (c.p.m./plate)
0	33 ± 7 (5)	6.2 ± 2.0 (3)
1	29 ± 3 (5)	8.2 ± 0.2 (2)
4	25 ± 3 (5)	5.7 ± 2.0 (3)

that tunicamycin at 1.0 μg/ml blocked glycosylation of lipoprotein lipase completely.

Localization of lipoprotein lipase in untreated brown adipocytes by immunofluorescence was difficult because of the rapid turnover of the lipase in such cells [9]. However, immunofluorescent lipase was readily found in cells treated with 1.0 μg of tunicamycin/ml for 24 h. The lipase was distributed in a reticular pattern throughout the cell (Fig. 2a) and in narrow areas surrounding the nucleus (Fig. 2b), demonstrating for the first time that lipoprotein lipase in tunicamycin-blocked cells is retained in the endoplasmic reticulum.

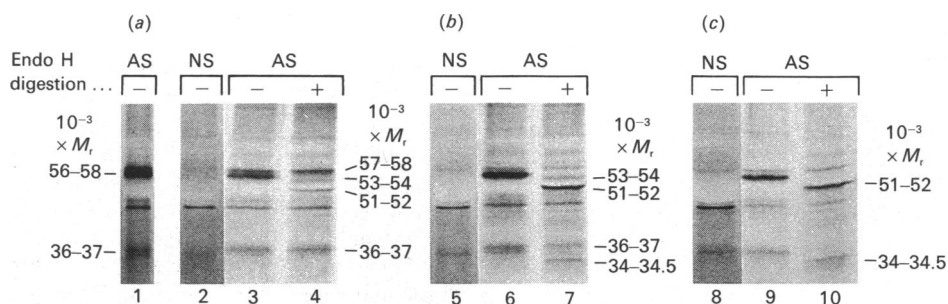
Monensin

Monensin at 1 μM increased lipoprotein lipase activity in brown adipocytes by 220 % in 1 h, and decreased the release of lipase activity into the medium by 84 % in 2 h (Table 2). A time-course study showed that the maximal effect of 1 μM-monensin on lipase activity in cells occurred at 1 h, and a dose-response

**Table 4.** Effect of 1-deoxymannojirimycin on the release into the medium of lipoprotein lipase activity by heparin from cultured brown adipocytes

Cells on day 3 of confluence were treated for 23.5 h with 0 or 4 mM-1-deoxymannojirimycin. The medium was removed from each plate and replaced with 3 ml of differentiation medium containing the appropriate concentration of 1-deoxymannojirimycin, and the plates were returned to the incubator for 30 min. The plates were then removed from the incubator and placed on crushed ice. After 2 min, 1 ml of medium was taken from each plate for assay of initial lipoprotein lipase activity, and heparin was added to the medium, as indicated, to give a final concentration of 20  $\mu$ g/ml. At 10 min after addition of heparin, another sample of medium was taken for the assay of lipoprotein lipase activity. The samples of medium were immediately passed through a 0.22  $\mu$ m filter and assayed for lipoprotein lipase activity. The cells were harvested into solution B, sonicated briefly at 0 °C, processed into acetone/ether powder and assayed for lipoprotein lipase activity. The amount of DNA in the untreated cell cultures was  $34 \pm 6$   $\mu$ g/plate. The values given are the means  $\pm$  s.d. for four plates. \* Different from untreated control,  $P < 0.01$ ; † different from untreated and 1-deoxymannojirimycin-treated controls,  $P < 0.01$ ; ‡ different from heparin-treated control,  $P < 0.01$ .

1-Deoxymannojirimycin in medium (mM)	Heparin added to medium ( $\mu$ g/ml)	Lipoprotein lipase activity			
		In medium ( $\mu$ units/ml)		Remaining in cells (munits/mg of DNA)	Released into medium in 10 min (munits/mg of DNA)
		Initial	10 min after addition of heparin		
0	0	$29 \pm 24$	$70 \pm 33$	$45 \pm 1$	$1 \pm 1$
0	20	$62 \pm 31$	$237 \pm 61$	$44 \pm 6$	$7 \pm 1^*$
4.0	0	$53 \pm 36$	$88 \pm 34$	$50 \pm 10$	$2 \pm 2$
4.0	20	$8 \pm 9$	$389 \pm 26$	$35 \pm 8$	$16 \pm 4^{\dagger\dagger}$

**Fig. 6.** Effect of 1-deoxymannojirimycin on the glycosylation of lipoprotein lipase in brown adipocytes

Cells at day 3 of confluence were treated for 24 h with (a) 0, (b) 1 or (c) 4 mM-1-deoxymannojirimycin and incubated with 360  $\mu$ Ci of [ $^{35}$ S]methionine during the last 2 h of treatment. The cells were harvested into solution D, sonicated briefly at 0 °C and centrifuged.  $^{35}$ S-labelled lipoprotein lipase in the infranatants was immunoprecipitated with antiserum (AS), digested with or without endo H, resolved by SDS/PAGE, and radioautographed.  $^{35}$ S-labelled protein precipitated from infranatants with non-immune serum (NS) was also resolved by SDS/PAGE and radioautographed.

study showed that the effect at 2 h increased with concentration, producing a maximal 2-fold increase at 10  $\mu$ M (Table 2).

Brown adipocytes treated with 15  $\mu$ M-cycloheximide (to block protein synthesis) and 3  $\mu$ g of heparin/ml (to remove lipase from the surface of the cells) [14] lost 80 % of their lipase activity in 0.5 h and 98 % in 2 h. The half-life of lipase activity in such cells was about 20 min, similar to that observed in other cells [8,10,13,14]. When treatment with cycloheximide and heparin was stopped, cells treated with 1  $\mu$ M-monensin accumulated the equivalent of 100 % of their original lipase activity within 1 h, whereas untreated cells accumulated only 40 % during the same time (Fig. 3). Immunofluorescent studies showed that lipoprotein lipase accumulated in the Golgi of cells treated with monensin (Fig. 2c).

Brown adipocytes treated for 2.5 h with 1  $\mu$ M-monensin and incubated for the last 2 h with [ $^{35}$ S]methionine contained  $^{35}$ S-labelled subunits which migrated on SDS/PAGE slightly faster than those in untreated cells (Fig. 4). The amount of  $^{35}$ S-labelled lipase in monensin-treated cells was 140 % more than that in untreated cells, and the proportion of radioactivity in the lipase to that in total protein was greater in treated cells (0.041 % versus 0.017 %), reflecting the blocking effect of monensin on the secretion of newly synthesized lipoprotein lipase.

Monensin (1  $\mu$ M) impaired the processing of lipase subunits within 1 h, and this effect progressed during the next 3 h (Fig. 5). Untreated cells, as noted above, contained mostly endo H-resistant and small amounts of totally and partially endo H-sensitive subunits. Cells treated for 1–2 h with monensin contained no endo H-resistant subunits and about equal amounts of partially and totally endo H-sensitive forms, whereas cells treated for 4 h and labelled during the last 2 h of treatment contained mostly totally endo H-sensitive subunits (Fig. 5). The progressive impairment of processing of lipase oligosaccharide chains in the Golgi of monensin-treated cells was probably secondary to the blockage of protein transport from the medial to the trans Golgi by monensin [20,21].

#### 1-Deoxymannojirimycin

Brown adipocytes treated with 1 or 4 mM-1-deoxymannojirimycin for 24 h contained the same amount of lipoprotein lipase activity as untreated cells (Table 3). Cells treated with 4 mM-1-deoxymannojirimycin released 30 % of their lipase activity into the medium in 10 min when exposed to 20  $\mu$ g of heparin/ml in ice-cold medium, whereas untreated cells released 14 % (Table 4), as observed earlier [9].

1-Deoxymannojirimycin had no significant effect on the



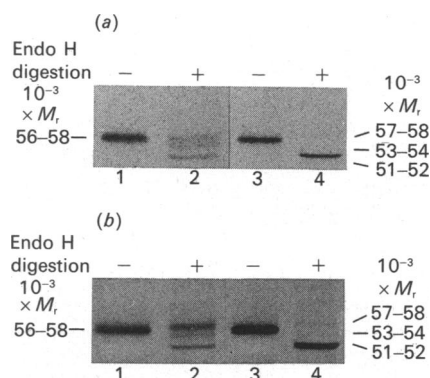
**Table 5. Effect of swainsonine on lipoprotein lipase activity and total protein synthesis in cells cultured from brown adipose tissue**

Lipoprotein lipase activity was measured in aqueous extracts of acetone/ether powders of cells treated for 2 or 24 h with 0, 10 or 100  $\mu\text{M}$ -swainsonine, and in the medium at the end of 24 h of treatment. Incorporation of [ $^{35}\text{S}$ ]methionine into total protein was measured during the last 2 h of treatment. The amount of DNA in the untreated cell cultures harvested on day 3 of confluence was  $65 \pm 2 \mu\text{g}/\text{plate}$ , and that on day 4 was  $63 \pm 5 \mu\text{g}/\text{plate}$ . Values in parentheses indicate the numbers of plates per group. Significant differences from untreated cells: \*  $P < 0.05$ , \*\*  $P < 0.01$ . n.d., not determined.

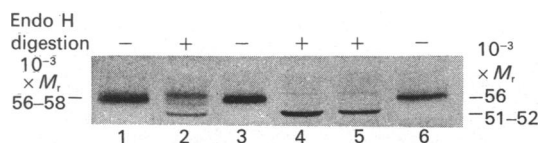
Duration of treatment (h)	Concentration of swainsonine in medium ( $\mu\text{M}$ )	Lipoprotein lipase activity (munits/mg of DNA)		$10^{-7} \times ^{35}\text{S}$ in total cell protein (c.p.m./plate)
		Cells	Medium at end of 24 h	
2	0	$170 \pm 5$ (3)†	n.d.	$8.6 \pm 0.1$ (2)†
2	10	$169 \pm 4$ (3)†	n.d.	$8.4 \pm 0.2$ (2)†
2	100	$160 \pm 5$ (3)†	n.d.	n.d.
24	0	$352 \pm 43$ (3)‡	$55 \pm 1$	$8.6 \pm 0.1$ (2)†
24	10	$293 \pm 33$ (3)‡	$45 \pm 1$ *	$7.4 \pm 0.1$ (2)†**
24	0	$282 \pm 27$ (3)‡	$50 \pm 15$	n.d.
24	100	$193 \pm 17$ (3)‡**	$24 \pm 3$ *	n.d.

† Cells harvested on day 3 of confluence.

‡ Cells harvested on day 4 of confluence.

**Fig. 7. Effect of swainsonine on the glycosylation of lipoprotein lipase in brown adipocytes**

Cells at day 3 or 2 of confluence were treated for (a) 2 or (b) 24 h respectively without (lanes 1 and 2) or with (lanes 3 and 4) 10  $\mu\text{M}$ -swainsonine, and incubated with 180  $\mu\text{Ci}$  of [ $^{35}\text{S}$ ]methionine during the last 2 h of treatment. The cells were harvested into solution D, sonicated briefly at 0  $^{\circ}\text{C}$  and centrifuged.  $^{35}\text{S}$ -labelled lipoprotein lipase was immunoprecipitated from the infranats, digested with or without endo H, resolved by SDS/PAGE and radioautographed.

**Fig. 8. Effect of swainsonine on the glycosylation of lipoprotein lipase in brown adipocytes pretreated with cycloheximide and heparin**

Cells at day 3 of confluence were pretreated for 2 h with 15  $\mu\text{M}$ -cycloheximide and 3  $\mu\text{g}$  of heparin/ml, and then incubated for 1 h (lanes 1–4) or 2 h (lanes 5 and 6) without (lanes 1 and 2) or with (lanes 3–6) 10  $\mu\text{M}$ -swainsonine in the presence of 180  $\mu\text{Ci}$  of [ $^{35}\text{S}$ ]methionine. The cells were harvested in solution D, sonicated briefly at 0  $^{\circ}\text{C}$  and centrifuged.  $^{35}\text{S}$ -labelled lipoprotein lipase was immunoprecipitated from the infranats, digested with or without endo H, resolved by SDS/PAGE and radioautographed.

amount of [ $^{35}\text{S}$ ]methionine incorporated into total protein (Table 3). Cells treated with 1 mM-1-deoxymannojirimycin for 24 h and incubated with [ $^{35}\text{S}$ ]methionine during the last 2 h contained totally endo H-sensitive  $^{35}\text{S}$ -labelled lipase subunits, traces of partially endo H-sensitive subunits, and no endo H-resistant subunits, whereas cells treated with the larger dose, 4 mM, contained only totally endo H-sensitive subunits (Fig. 6). The immunoprecipitated component of  $M_r$  36000–37000, a product of proteolytic cleavage of the lipase [9], was endo H-resistant in untreated cells and totally endo H-sensitive in cells treated with 4 mM-1-deoxymannojirimycin (Fig. 6). The above findings indicate that the action of mannosidase I in the Golgi is not required for the synthesis and secretion of active lipoprotein lipase, confirming a similar observation in isolated guinea pig adipocytes [10].

### Swainsonine

Swainsonine decreased lipoprotein lipase activity in cells and medium by only 18 % at 10  $\mu\text{M}$  and by about 32 % at 100  $\mu\text{M}$  in 24 h, whereas it had no effect at either dosage in 2 h (Table 5). Swainsonine at 10  $\mu\text{M}$  for 24 h decreased by < 15 % the amounts of  $^{35}\text{S}$ -labelled total protein (Table 5) and  $^{35}\text{S}$ -labelled lipoprotein lipase in cells incubated with [ $^{35}\text{S}$ ]methionine during the last 2 h of a 24 h treatment. Regardless of the duration of treatment, cells treated with 10  $\mu\text{M}$ -swainsonine contained mostly endo H-sensitive  $^{35}\text{S}$ -labelled lipase subunits and no endo H-resistant subunits (Fig. 7).

Swainsonine at 10  $\mu\text{M}$  had no effect on the accumulation of lipase activity in cells pretreated for 2 h with cycloheximide and heparin, even though it blocked the synthesis of endo H-resistant and partially endo H-sensitive subunits within 1 h (Fig. 8). These findings indicate that the action of mannosidase II in the Golgi is not required for the synthesis and secretion of active lipoprotein lipase in brown adipocytes.

### DISCUSSION

We used inhibitors of synthesis, processing and transport of glycoproteins to study the importance of glycosylation to the activity and secretion of lipoprotein lipase in brown adipocytes

of newborn mice. Active lipoprotein lipase is usually considered to be dimeric [3,5–7]. The procedure used here to evaluate the effects of inhibitors on glycosylation was based on analyses of subunits of the lipase. The most prevalent subunit ( $M_r$  57 000–58 000) in brown adipocytes contained endo H-resistant oligosaccharide chains, the next most prevalent contained totally endo H-sensitive chains, and the least prevalent subunit contained partially endo H-sensitive chains. The subunit with partially endo H-sensitive chains is in an intermediate between the other two subunits, having one endo H-sensitive and one endo H-resistant chain [9].

Mouse brown adipocytes treated with 1.0  $\mu$ g of tunicamycin/ml for 24 h contained an inactive form of lipoprotein lipase which had a subunit ( $M_r$  51 000–52 000) that is smaller than those in normal lipase. The subunit was resistant to digestion by endo H, as in mouse [8] and rat [12] adipocytes, and had the same  $M_r$  as endo F-digested subunits of lipoprotein lipase from untreated brown adipocytes [9]. Thus glycosylation of the lipase was completely blocked in brown adipocytes treated with the above dosage of tunicamycin. Our immunofluorescent localization studies (Figs. 2a and 2b) show for the first time that lipoprotein lipase is retained in the endoplasmic reticulum in tunicamycin-blocked cells. Earlier studies in 3T3-L1 mouse [13], 3T3-F442A mouse [8] and rat [12] adipocytes also showed that lipoprotein lipase was not secreted by tunicamycin-blocked cells. Findings in tunicamycin-treated Ob17 mouse white adipocytes [39] have been cited as evidence that glycosylation in the endoplasmic reticulum is not necessary for the intracellular migration or secretion of (inactive) lipoprotein lipase [8,17,26]. However, it was not demonstrated in that study that glycosylation had been blocked [39]. Our findings indicate that core glycosylation is required for transport of lipoprotein lipase from the endoplasmic reticulum.

Monensin, a carboxylic ionophore, causes immediate swelling of medial and cis Golgi [21] and blocks transport of glycoproteins from medial to trans Golgi [21,40]. Monensin caused accumulation of lipoprotein lipase in the Golgi of brown adipocytes (Fig. 2c), increased lipase activity several-fold in the cells, and stopped secretion of the lipase, confirming earlier observations in Ob17 [14] and rat adipocytes [22]. Monensin did not impair synthesis of active lipase. Our studies show that monensin initially blocked synthesis of endo H-resistant lipase subunits and allowed synthesis of totally and partially endo H-sensitive subunits, and later blocked synthesis of partially but not totally endo H-sensitive subunits. Thus processing of endo H-sensitive chains of lipoprotein lipase to endo H-resistant chains is not required for activity of the lipase.

The effect of monensin on the processing of lipoprotein lipase is probably secondary to its inhibition of protein transport in the Golgi [21,40–42]. Monensin would be expected to block immediately movement of lipase molecules to the trans Golgi, where oligosaccharide chains are normally transformed to endo H-resistant (complex) types. Some of the subunits synthesized during the first part of the blockade were processed to partially endo H-sensitive forms, indicating that one chain of each subunit had been processed, in medial Golgi, to an endo H-resistant type. As protein molecules accumulated in medial and cis Golgi, they probably prevented lipase molecules from reaching sites of action of mannosidase II and I, thereby preventing processing of oligosaccharide chains to confer endo H resistance.

When the action of mannosidase I in cis/medial Golgi [43] is blocked by 1-deoxymannojirimycin, endo H-sensitive glycoproteins are produced which contain only high-mannose type oligosaccharides [23–25]. Lipoprotein lipase in brown adipocytes treated with 4 mM-1-deoxymannojirimycin was totally endo H-sensitive, indicating that the action of mannosidase I on oligo-

saccharides of the lipase had been inhibited. Nonetheless, lipoprotein lipase in these cells was active and secreted. Guinea pig adipocytes treated with deoxymannojirimycin also synthesized and secreted active endo H-sensitive lipase [10]. These results indicate that trimming of high-mannose type chains by mannosidase I is not required for the activity or secretion of lipoprotein lipase.

Blocking the action of mannosidase II in medial Golgi [43] with swainsonine causes production of endo H-sensitive glycoproteins, but the oligosaccharide chains formed are sometimes hybrids [27–29], intermediates between high-mannose and complex type chains [43,44]. Glycoproteins produced in the presence of swainsonine often have normal activity and are secreted [17,27,28]. Brown adipocytes treated with 10  $\mu$ M-swainsonine synthesized and secreted active lipoprotein lipase. The lipase contained only endo H-sensitive subunits, indicating that swainsonine had blocked the action of mannosidase II on oligosaccharide chains of lipoprotein lipase. It was not determined whether the chains were of a high-mannose or hybrid type, or a mixture. These findings show, for the first time, that trimming of oligosaccharide chains by mannosidase II, the first committed step towards synthesis of complex type oligosaccharides [17], is not necessary for lipoprotein lipase to be active and secreted.

Our findings in the mouse and those in guinea pig cells [10] clearly indicate that processing of oligosaccharide chains of lipoprotein lipase to an endo H-resistant complex type is not required for activity of the lipase. In contrast, findings in 3T3-F442A mouse adipocytes [8] suggested that activation of the lipase occurs after oligosaccharide chains are converted in the Golgi to endo H-resistant chains [26]. However, only two forms of lipase were examined in that study [8], an inactive short-lived precursor form composed of endo H-sensitive subunits, and an active mature form composed of endo H-resistant subunits; the catalytic activity of intermediate forms was not examined.

The active form of lipoprotein lipase in bovine milk [5,7], rat adipose tissue and heart [6] and cultured mouse adipocytes [8] is a dimer of identical glycopeptide subunits [5,7], and conversion of the dimeric form to the monomeric form renders the lipase inactive [5–7]. However, recent studies of lipoprotein lipase isolated from human post-heparin plasma suggest that this lipase is active in a monomeric form [45].

The intracellular site of dimerization of lipoprotein lipase is not known. Findings elsewhere indicate that most proteins are oligomerized in the endoplasmic reticulum [46]. Guinea pig adipocytes, as noted earlier, retained for several hours an active form of endo H-sensitive lipase [10]. This form of lipase required the same high salt concentration for elution from heparin-Sepharose as did mature active guinea pig lipase, suggesting that the active endo H-sensitive lipase in these cells was dimerized. Based on the assumption that endo H sensitivity of glycoproteins indicates that such proteins have not reached the Golgi [10], it was concluded that active endo H-sensitive lipase retained in guinea pig cells was located in the endoplasmic reticulum, and hence dimerization had occurred in this organelle. However, this interpretation is tentative because endo H-sensitive glycoproteins are normally transported to the Golgi [43], and such proteins can accumulate in Golgi when processing or transport of glycoproteins is disrupted [16,21,24–26,40–42]. Recent findings in 3T3-F442A adipocytes [8] were presented as evidence that dimerization of lipoprotein lipase occurs in the trans Golgi, when endo H-sensitive oligosaccharide chains are converted into endo H-resistant chains. However, this interpretation is questionable because only two forms of lipase were examined, a short-lived monomeric precursor and a mature dimeric form; intermediate forms, such as active endo H-sensitive lipase, were not examined.



More specific studies are needed to determine the site of dimerization of lipoprotein lipase in cells.

That incompletely processed lipoprotein lipase can be active and secreted in cells treated with mannosidase inhibitors challenges the importance of terminal glycosylation of the lipase. Endo H-resistant chains formed during terminal processing may be necessary for intracellular trafficking [46] or for stability of normally secreted active lipase [47]. Another possibility is that processing to complex type chains is required for the interaction of the lipase with substances, such as proteoglycan sulphate [4,5,48,49] or phosphatidylinositol glycan [50,51], which may be involved in the transport of lipoprotein lipase *in vivo* from parenchymal cells to the luminal surface of capillary endothelial cells, where the lipase normally acts [4].

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