Glycosylation and Secretion of Lipoprotein Lipase by 3T3-L1 Adipocytes: Effects of Brefeldin A

Hiroshi Masuno and Hiromichi Okuda

Time courses of synthesis and secretion of lipoprotein lipase (LPL) were examined in 3T3-L1 adipocytes. LPL was glycosylated in the endoplasmic reticulum (ER) within 10 min after synthesis, and was transported after 20-30 min to the trans Golgi where it was converted to the mature form with M_r=55,000-58,000, which was resistant to endoglycosidase H (endo H). LPL subunits with M_r=55,000-58,000 appeared in the medium within 30 min after synthesis. The effects of brefeldin A (BFA), which inhibits transport of glycoproteins in various types of cells, on secretion and glycosylation of LPL were also examined. BFA completely blocked release of LPL activity into the medium, causing accumulation of the activity in cells. The suppressive effect of BFA on release of LPL activity was reversible. BFA-treated cells synthesized LPL with M_r=53,000-55,000 consisting of 2 types of subunits, the main type being totally endo H-sensitive and the other partially endo H-sensitive. No LPL subunits were secreted into the medium by BFA-treated cells.

Kew words: Lipoprotein lipase, Brefeldin A, Glycosylation, Secretion

Lipoprotein lipase (LPL) is synthesized by parenchymal cells in extrahepatic tissues, secreted from the cells, and located on the membrane-bound heparan sulfate of the luminal surface of vascular endothelial cells where it hydrolyzes triacylglycerol in chylomicron and very low density lipoprotein (1-4). This enzyme is a glycoprotein with two N-linked glycosylation sites (5). Human LPL has one complex type and one high-mannose type oligosaccharide chain (6), while mouse LPL has two complex type chains (7-10).

In general, proteins exported to the cell surface or secreted from the cells are glycosylated in the endoplasmic reticulum (ER) and then sequentially transported through the cis, medial, and trans Golgi, where the oligosaccharide chains are processed. Such processed glycoproteins are ultimately located on the cell surface and/or secreted.

There have been some reports on the effects of inhibitors of movement of protein from the ER to the Golgi on the activation of LPL (9, 11). Carbonyl cyanide m-chlorophenylhydrazone, which blocks movement of glycoproteins from the ER to the Golgi (12, 13), caused production of inactive LPL in cultured Ob17 adipocytes (11). Monensin, an inhibitor of transport of glycoproteins from the medial to the trans Golgi (14, 15), caused accumulation of LPL activity in cultured Ob17 adipocytes (11), and cultured mouse brown adipocytes (9). These studies showed that transport of LPL from the ER to the Golgi is necessary for expression of LPL activity.

Brefeldin A (BFA), a macrolide antibiotic that strongly inhibits virus multiplication (16), was also shown to impair transport of glycoproteins in various types of cells (17-22). BFA causes intracellular accumulation of high-mannose type G protein and inhibition of its expression on the cell surface in vesicular stomatitis virus-infected baby hamster kidney cells (20). Lippincott-Schwartz et al. (21) observed a rapid and dramatic redistribution of components of the cis/medial Golgi to the ER in response to
BFA. Doms et al. (22) also reported that BFA induces retrograde transport of both resident and itinerant Golgi proteins to the ER in a fully reversible manner.

In this study, we showed that LPL is synthesized and glycosylated in the ER, and transported from the ER to the Golgi, becoming the mature form of LPL in the trans Golgi. The effects of BFA on the processing, activity, and secretion of LPL in 3T3-L1 adipocytes were also examined.

Materials and Methods

Materials

The following materials were used: L-[³⁵S] methionine and ENHANCE® (Du Pont-New England Nuclear); tri[9, 10(n)-³H] oleoylglycerol (Amersham); Dulbecco’s modified Eagle’s medium containing 5.5 mM glucose (DMEM; Nissui Pharmaceutical Co., Tokyo); methionine-deficient DMEM (GIBCO); fetal bovine serum (M.A. Bioproducts, Nissui Pharmaceutical Co., Tokyo); methionine-deficient Eagle’s medium containing 5.5 mM glucose (DMEM; 10(n)-³H] oleoylglycerol (Amersham); Dulbecco’s modified Eagle’s medium containing 5.5 mM glucose (DMEM; Nissui Pharmaceutical Co., Tokyo); methionine-deficient DMEM (GIBCO); fetal bovine serum (M.A. Bioproducts, MD); brefeldin A (Evotec Technologies, WI); endo-glycosidase H (endo H; Genzyme), and rabbit antichicken IgG (Pel-Freez). Chicken antiserum to bovine LPL was a gift from Dr. Thomas Olivecrona (Departments of Medicine, Biochemistry and Biophysics, University of Umeå, Umeå, Sweden). All other chemicals were of the highest quality commercially available.

Cell culture

3T3-L1 cells were induced to differentiate into adipocytes in 60-mm plates as described previously (10). The culture medium contained 5 μg/ml insulin, 10% fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B in DMEM. Cultures were used for experiments within 6-12 days after reaching confluence.

Assay of LPL activity

LPL activity associated with cells was measured in aqueous extracts of acetone/ether powders of cells with tri[9, 10(n)-³H] oleoylglycerol as a substrate in the presence of heat-inactivated (56°C, 10 min) serum from starved rats (8). LPL activity in the culture medium was measured in aliquots of medium filtered through 0.22 μm filters and assays were begun within 3 min after obtaining samples. One milliunit of lipolytic activity was defined as that releasing 1 nmole of fatty acid/min at 37°C.

Pulse-chase experiment

Cells were incubated for 30 min at 37°C in 1.5 ml of culture medium containing 0 or 2.5 μg/ml BFA. Cells were then washed with phosphate-buffered saline (PBS) and 1.5 ml of methionine-deficient DMEM containing the appropriate additions was added to each plate. After 30 min, 160 μCi of [³⁵S] methionine was added, and the cells were incubated (pulsed) for 10 min at 37°C. The medium was then removed, and the plates were washed quickly with PBS. Culture medium (1.5 ml) containing appropriate additions was added, and the plates were incubated (chased) for 0-90 min. The cells were then harvested, sonicated briefly at 0°C, and centrifuged, and the infranatant was stored at -80°C for immunoprecipitation (10).

³S-Labeled proteins in the medium were precipitated with 10% trichloroacetic acid, solubilized, and stored at -80°C for immunoprecipitation (10).

³S-Labeled LPL was immunoprecipitated with chicken antiserum to LPL (10) and resolved by SDS-PAGE in a Laemmli-type system (23) using 10% acrylamide resolving gel and 3% acrylamide stacking gel. Autoradiographs were obtained by exposure of the gels to Kodak X-Omat film at -80°C.

Enzymatic deglycosylation of ³S-labeled LPL

Cells preincubated in media containing 0 or 2.5 μg/ml BFA as described above were incubated for 1-2 h with 160 μCi of [³⁵S] methionine. Then the cells were harvested, sonicated briefly at 0°C, and centrifuged, and the infranatant was stored at -80°C for immunoprecipitation. ³S-Labeled LPL immunoprecipitated with chicken antiserum to LPL was digested with endo H as described previously (10). The digestion products were resolved by SDS-PAGE.

Chemical analysis

DNA content was determined fluorometrically as described by Hinegardner (24) with calf thymus DNA as a standard.

Results

Synthesis and secretion of LPL in 3T3-L1 adipocytes

Time courses of synthesis and secretion of LPL in 3T3-L1 adipocytes were examined (Figs.1 and 2A). Cells were pulsed for 10 min with [³⁵S] methionine and chased for 0-90 min in culture medium without [³⁵S] methionine. At the beginning of the chase (0 min) and 10 min later, LPL subunits with Mr = 53,000-55,000 were observed (Fig. 1). At 20 min, the subunits with Mr = 55,000-58,000 appeared. After that, the subunits with Mr = 53,000-55,000 decreased and those with Mr = 55,000-58,000 increased. 3T3-L1 adipocytes spontaneously released 7% of the cell-associated LPL activity in 1 h (Table 1). Pulse-chase experiments showed that the subunits with Mr = 55,000-58,000 appeared in the medium after 20 min of chase (Fig. 2A). However, the subunits with Mr = 53,000-55,000 were not released into the medium (Figs. 2A and 2B).

Effects of BFA on activity, synthesis and secretion of LPL

BFA at 0.1 μg/ml suppressed release of LPL activity by cells by 69%, and at higher concentrations blocked the release of activity almost completely (Table 1). At the same time, it caused accumulation of LPL activity in cells, concentrations of 1 and 10 μg/ml increasing the intracel-
The complete blockage of release of LPL activity by BFA at 2.5 μg/ml persisted for at least 3 h (Fig. 3A). To determine whether the suppressive effect of BFA on release of LPL activity was reversible, we incubated cells for 3 h with or without BFA, washed them with PBS, followed by incubation in BFA-free medium, and then measured LPL activity released into the medium at the indicated times (Fig. 3B). BFA-treated cells released about 30% as much LPL activity as that released by untreated cells in 1.5 h.

Pulse-chase experiments showed a single form of LPL subunits with Mr = 53,000–55,000 in BFA-treated cells at the beginning of the chase, but the formation of subunits with Mr = 55,000–58,000 was completely blocked throughout the 90-min chase (Fig. 1).
Treatment of cells with BFA caused complete blockage of release of LPL subunits into the medium throughout the 90-min chase (Fig. 2A). BFA-treated cells incubated for 1 or 2 h with [35S] methionine also did not release any subunits of LPL (Fig. 2B).

### Effects of BFA on incorporation of [35S]methionine into total protein and LPL

Cells were incubated for 1 or 2 h with [35S]methionine in the absence or presence of BFA, and then [35S]-labeled LPL in the infranatants of cell extracts was immunoprecipitated with antiserum to bovine LPL and resolved by SDS-PAGE. The amounts of [35S]methionine incorporated into total protein in BFA-treated cells increased linearly for at least 2 h, the incorporation being 88 and 82% of that into untreated cells at 1 and 2 h, respectively (Fig. 4 upper panel). The amount of radioactivity in LPL increased with incubation time, but the increase was not linear and the radioactivity in LPL in BFA-treated cells at 1 and 2 h were 52 and 54%, respectively, of that in untreated cells at the same time point (Fig. 4 lower panel).

### Determination of the types of oligosaccharide chains of LPL

To determine the type of oligosaccharide chains of LPL, [35S]-labeled LPL was digested with endo H, which cleaves high-mannose type chains (25) but not complex type chains, and the digested products were resolved by SDS-PAGE. [35S]-Labeled LPL in untreated cells consisted of

<table>
<thead>
<tr>
<th>BFA (μg/ml)</th>
<th>LPL Activity (milliunits/mg DNA)</th>
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<tbody>
<tr>
<td></td>
<td>Cells</td>
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<tr>
<td>0</td>
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<tr>
<td>0.1</td>
<td>680±46</td>
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<tr>
<td>1.0</td>
<td>893±126</td>
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<tr>
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<tr>
<td>10.0</td>
<td>960±102</td>
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* not determined.

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**Figure 3. Effects of BFA on release of LPL activity by 3T3-L1 adipocytes.**

**A.** The medium was removed from cells on day 7 after reaching confluence, and the plates were washed with PBS. The cells were incubated with 2 ml of culture medium containing 0 or 2.5 μg/ml BFA at 37°C for 1 h. The medium was then replaced by 2.5 ml of fresh culture medium containing an appropriate additive, and the cells were incubated at 37°C for 1-3 h. Aliquots of the media were taken at the indicated times and LPL activity was assayed.

**B.** The medium was removed, and the plates were washed with PBS, the cells were incubated with 2 ml of culture medium containing 0 and 2.5 μg/ml of BFA at 37°C for 3 h. The cells were then washed with PBS, and incubated with 2 ml of fresh BFA-free culture medium at 37°C. Aliquots of the media were taken at the indicated times and LPL activity was assayed.
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Discussion

N-linked glycosylation is initiated by transfer of the common precursor oligosaccharide Glc$_3$Man$_9$GlcNAc$_2$ to the sequence Asn-X-Ser/Thr (X≠Pro) of newly synthesized proteins in the ER. Following removal of three glucose residues and one mannose residue in the ER, some mannose residues are trimmed in the cis/medial Golgi, and the oligosaccharide chains are finally processed to the complex type in the trans Golgi.

Our previous study using castanospermine, an inhibitor of glucosidase I in the ER (26, 27), suggested a minimal Mr value of 57,000 for the murine LPL subunit with two fully glycosylated chains (Glc$_3$Man$_9$GlcNAc$_2$) (10). The results of the present and previous studies suggested the following time course of processing of LPL in 3T3-L1 adipocytes. LPL was glycosylated, and three glucose residues and one mannose residue were removed in the ER within 10 min after synthesis, yielding a form with Mr = 53,000-55,000. Within 30 min, heavier subunits with Mr = 55,000-58,000 appeared in cells, and these were resistant to endo H (Fig. 5), indicating that all three glucose residues and six of nine mannose residues were removed in the ER and the cis/medial Golgi, and addition of other sugars, such as N-acetylglucosamine, galactose, sialic acid, and fucose, occurred in the trans Golgi during this period. Approximately 30-40 min after synthesis, LPL subunits with Mr = 55,000-58,000 began appearing in the medium (Fig. 2A).

Figure 1 shows the presence of a form of LPL subunits with Mr = 53,000-55,000 in both untreated and BFA-treated cells at the beginning of the chase (0 min). The subunits with two fully glycosylated chains (Mr = 57,000) were not observed (Fig. 1). These results indicate that BFA did not affect removal of three glucose residues and one mannose residue from oligosaccharide chains of LPL in the ER during the first 10 min after glycosylation.

In untreated cells, oligosaccharide chains of the LPL subunit were processed to endo H-resistant (complex) type within 30 min after synthesis (Fig. 1), which occurs in the trans Golgi. However, in BFA-treated cells, no such processing of oligosaccharide chains of the LPL subunit was observed, resulting in the production of only subunits with Mr = 53,000-55,000. These results indicate that BFA blocked transport of LPL from the medial to the trans Golgi.

Some of the subunits synthesized during treatment of cells with BFA were processed to partially endo H-sensitive LPL (Fig. 5). The ratio of radioactivity in totally endo
Effects of Brefeldin A on Lipoprotein Lipase

Untreated cells

<table>
<thead>
<tr>
<th>Incubation Time (h)</th>
<th>Endo H-digestion</th>
<th>53-58 k</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2</td>
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</tr>
<tr>
<td>4</td>
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BFA-treated cells

<table>
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<th>Incubation Time (h)</th>
<th>Endo H-digestion</th>
<th>53-55 k</th>
</tr>
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<tr>
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<td>-</td>
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<tr>
<td>2</td>
<td>+</td>
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<td>4</td>
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Fig. 5. Digestion with endo H of $^3$S-labeled LPL extracted from untreated and BFA-treated 3T3-L1 adipocytes. Cells on day 12 after reaching confluence were incubated for 1 or 2 h with or without 2.5 μg/ml BFA with addition of 160 μCi of $[^3]$S-methionine. The cells treated for 4 h with or without BFA were labeled with $[^3]$S-methionine during the last 2 h of treatment. Cells were harvested, sonicated briefly at 0°C, and centrifuged. $^3$S-Labeled LPL in the infranatants was immunoprecipitated with antiserum to LPL, incubated with or without endo H, resolved by SDS-PAGE and autoradiographed.

H-sensitive subunits to that in partially endo H-sensitive subunits did not change during longer (4 h) treatment with BFA (Fig. 5). BFA at a higher concentration (10 μg/ml) also had a similar effect on processing of oligosaccharide chains of subunits (data not shown). The rate of hydrolysis of the oligosaccharide Asn-(GlcNAc)2-(Man)$_3$ by endo H is very low (28, 29). Lippincott-Schwartz et al. (21) reported that the formation of endo H-resistant types of T cell antigen receptor subunits in BFA-treated 2B4 cells is due to removal of mannose residues by mannosidase II of the medial Golgi relocated to ER. These findings suggest that some of LPL subunits could be processed by relocated mannosidase II in the medial Golgi of BFA-treated cells.

The amount of newly synthesized LPL was lower in BFA-treated than in untreated cells (Fig. 4). The ratios of radioactivity incorporated into LPL to that incorporated into total protein in 1 h were 0.061% in untreated cells and 0.033% in BFA-treated cells. These results suggest that accumulation of LPL activity in BFA-treated cells did not result from increased synthesis of enzyme protein, and was probably secondary to its inhibition of secretion of active LPL from the cells to the medium.

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References

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