Secretion and apparent activation of human hepatic lipase requires proper oligosaccharide processing in the endoplasmic reticulum

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Human hepatic lipase (HL) is a glycoprotein with four N-linked oligosaccharide side chains. The importance of glycosylation for the secretion of catalytically active HL was studied in HepG2 cells by using inhibitors of intracellular trafficking, N-glycosylation and oligosaccharide processing. Secretion of HL was inhibited by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), monensin, brefeldin A (BFA), tunicamycin, castanospermine and *N*-methyldeoxynojirimycin, but not by 1-deoxymannojirimycin. Secretion of α_1-antitrypsin, an unrelated N-glycoprotein, was also inhibited by monensin, BFA and tunicamycin, but not by CCCP, castanospermine or *N*-methyldeoxynojirimycin. Intracellular HL activity decreased with CCCP, tunicamycin, castanospermine and *N*-methyldeoxynojirimycin, but increased with monensin and BFA. In the absence of protein synthesis *de novo*, HL activity secreted into the medium was 7.8 ± 2.1-fold higher (mean $\pm$ S.D., $n = 7$) than the simultaneous fall in intracellular HL activity. In cells pretreated with monensin or BFA, this factor decreased to 1.3 ± 0.5, indicating that the apparent increase in HL activity had already occurred within these cells. After chromatography on Sepharose–heparin, the specific triacylglycerol hydrolase activity of secreted HL was only 1.7 ± 0.3-fold higher than that of intracellular HL, indicating that the secretion-coupled increase in HL activity is only partly explained by true activation. We conclude that oligosaccharide processing by glucosidases in the endoplasmic reticulum is necessary for the transport of newly synthesized human HL, but not α_1-antitrypsin, to the Golgi, where the catalytic activity of HL is unmasked.

Key words: α_1-antitrypsin, hepatic triacylglycerol lipase, HepG2, N-glycosylation, triacylglycerol hydrolase activity.

INTRODUCTION

Hepatic lipase (HL) is an extracellular enzyme that belongs to the lipase gene superfamily [1]. The enzyme is synthesized and secreted by liver parenchymal cells, and exerts its function while bound to the liver in the space of Disse [2,3]. HL hydrolyses phospholipids and triacylglycerols present in high- and intermediate-density lipoproteins, and facilitates the hepatic uptake of remnant particles [4–6] and of cholesterol (esters) carried in high-density lipoproteins [7,8]. In addition, HL might act as a ligand protein for remnant binding to the liver [9]. By contributing to reverse cholesterol transport and by decreasing the number of atherogenic remnants in the circulation, HL is thought to protect against the development of premature atherosclerosis. Indeed, a low HL activity seems to be associated with an increased atherosclerotic risk [10,11]. In line with this, the aortic accumulation of cholesterol was markedly decreased in cholesterol-fed transgenic mice that over-expressed human HL [12]. In human hypertriglyceridaemia, however, post-heparin HL activity is correlated with the production of highly atherogenic small dense low-density lipoproteins, suggesting a pro-atherogenic role for HL in these patients [13].

HL is a glycoprotein bearing two (rat) to four (human) asparagine-linked glycans [14–16]. For the synthesis and secretion of fully active HL by rat hepatocytes, N-glycosylation is a prerequisite [17,18]. This was confirmed by expression studies with HL cDNA constructs in which the glycosylation sites had been removed by site-directed mutagenesis [15,16]. These studies demonstrated that glycosylation at Asn-56/57 of human and rat HL is both necessary and sufficient for the secretion of catalytically active lipase [15]. When glycosylation is prevented, either by tunicamycin or by site-directed mutagenesis, inactive HL protein accumulates intracellularly [15,19]. When expressed in *Xenopus* oocytes, however, a small amount of non-glycosylated rat HL protein was secreted and catalytically active [20]. This observation argues against the possibility that N-glycosylation itself is necessary for catalytic activity. Rather, N-glycosylation might be required for the proper intracellular trafficking of the newly synthesized protein.

Additional studies have shown that for secretion of fully active rat HL the protein must not only be N-glycosylated but the glycan chains must subsequently undergo proper processing [18,21]. The oligosaccharides of N-glycoproteins are generally processed from high-mannose to complex-type chains successively by glucosidases in the rough endoplasmic reticulum (RER), mannosidases in the *cis*- or medial Golgi and glycosyltransferases in the medial or *trans*-Golgi [22]. Notably, trimming of the terminal glucose residues by the RER glucosidases seems to be crucial for the acquisition of catalytic activity and secretion of rat HL protein [18,21]. Once the glucose residues have been removed, activation and subsequent secretion continue independently of further oligosaccharide processing. Glucose trimming of N-glycoproteins has been implicated in the quality control system for newly synthesized proteins, which prevents badly folded proteins from leaving the RER [23,24]. Whether the presence of terminal glucose residues itself prevents the acquisition of catalytic activity of rat HL, or whether it primarily interferes with transport to the Golgi, where subsequently activation can occur, is unknown.

In the present study we examined the role of N-linked

Abbreviations used: BFA, brefeldin A; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CSP, castanospermine; dMM, 1-deoxymannojirimycin; Endo H, endo-β-*N*-acetylglucosaminidase H; HL, hepatic lipase; MdN, *N*-methyldeoxynojirimycin; RER, rough endoplasmic reticulum.

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glycosylation in secretion and activation of human HL by HepG2 cells. The four putative N-glycosylation sites identified in the HL cDNA [25] all seem to be occupied in HL secreted by these cells [15,26]. First, we determined in which intracellular compartment HL protein is apparently activated, by using inhibitors that primarily affect vesicular transport in the secretory pathway. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and monensin inhibit the transport of glycoproteins from the RER to the Golgi [27,28] and from medial to *trans*-Golgi [22] respectively. Brefeldin A (BFA) blocks the transport of proteins into post-Golgi compartments and induces the redistribution of the Golgi into the RER [29,30]. Secondly, we studied the importance of oligosaccharide processing in secretion and intracellular activation of human HL by using inhibitors of glucose trimming in the RER [castanospermine (CSP) and *N*-methyldeoxynojirimycin (MdN)] and mannose trimming in the *cis*-Golgi [1-deoxymannojirimycin (dMM)] [31,32]. The effects of the inhibitors on the expression of HL were compared with that of α_1-antitrypsin, an unrelated N-glycoprotein. The results show that proper oligosaccharide processing by RER glucosidases is essential for the secretion of fully active HL but not for the secretion of α_1-antitrypsin. Glucose trimming seems to be necessary for the translocation of HL protein to the Golgi compartment, where the triacylglycerol hydrolase activity of the protein becomes detectable.

MATERIALS AND METHODS

Materials

CSP, dMM, (+)-BFA, cycloheximide and CHAPS were from Boehringer Mannheim (Mannheim, Germany), MdN and endo-β-*N*-acetylglucosaminidase H (Endo H) from Genzyme (Boston, MA, U.S.A.), and monensin and CCCP from Calbiochem (La Jolla, CA, U.S.A.). Heparin was obtained from Leo Pharmaceuticals (Weesp, Holland); coupling to CNBr-activated Sepharose 2B (Pharmacia, Uppsala, Sweden) was performed in accordance with the manufacturer's instructions. Trasylol was from Boehringer Mannheim; other protease inhibitors and tunicamycin were from Sigma (St. Louis, MO, U.S.A.). Media, fetal bovine serum and Tran^{35}S-label (1100 Ci/mmol) were obtained from ICN (Costa Mesa, CA, U.S.A.). Glycerol tri[1-^{14}C]oleate (50–80 mCi/mmol) was from Amersham (Little Chalfont, Bucks., U.K.). Rabbit antibodies against human α_1-antitrypsin and horseradish peroxidase-conjugated rabbit anti-(goat IgG) were from Dakopatts (Glostrup, Denmark). Zysorbin was from Zymed Laboratories (San Fransisco, CA, U.S.A.). Culture plastics and ELISA code 3590 plates were from Costar (Cambridge, MA, U.S.A.).

HepG2 cell culture and incubation

Human hepatoma HepG2 cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum [26]. The cells were split 1:10 into new flasks once a week. Medium was refreshed once a week. For the experiments, cells were seeded into six-well plates. At confluence, the medium was replaced by 1 ml of fresh medium containing 25 i.u./ml heparin, and the incubations were started with the addition of inhibitors. CCCP, BFA and monensin were added from 1000-fold concentrated stock solutions in ethanol; tunicamycin was added from a 1000-fold stock solution in DMSO. Other inhibitors were added from 100-fold stock solutions in PBS. The cell cultures were incubated for the indicated durations at 37 °C in a humidified O_2/CO_2 (19:1) atmosphere. At the end of the incubation the plates were placed on ice and all subsequent handling was done at 4 °C. The medium was collected for analysis of secreted HL. After being washed twice with PBS, the cells were released from the plates by a 5 min incubation in PBS containing 2.5 mM EDTA. Cells were collected by centrifugation (15 s, 10000 ***g***), and resuspended in 250 μl of a 40 mM NH_4OH buffer, pH 8.1 [33], containing 25 i.u./ml heparin and a cocktail of protease inhibitors (1 mM EDTA, 10 units/ml Trasylol, 0.1 mM benzamidine, 2 μg/ml leupeptin, 2 μg/ml antipain, 2 μg/ml chymostatin and 2 μg/ml pepstatin). After 30 min on ice, the lysates were sonicated (15 s, at an amplitude of 14 μm with an MSE Soniprep 150) and centrifuged for 10 min at 10000 ***g***. The supernatants were used for the analysis of intracellular HL.

HL activity

HL activity was determined by a triacylglycerol hydrolase assay at pH 8.5 in 0.6 M NaCl with a gum acacia-stabilized glycerol [^{14}C]trioleate emulsion as substrate [18]. Assays were performed for 2 h at 30 °C. Activities are expressed as m-units (nmol of free fatty acids released per min). In a total assay volume of 125 μl, the release of free fatty acids was linear with time and sample volume up to 50 μl for the cell-free medium and 20 μl for the cell lysates. In immuno-inhibition assays, 40 μl of the cell-free medium or 10 μl of the cell lysates was preincubated for 3 h on ice in a total volume of 50 μl with 50 μg of goat anti-(human HL) IgG. This antibody was raised against human HL partly purified from post-heparin plasma [34]. After centrifugation (10 min, 10000 ***g***, 4 °C), 75 μl of substrate mixture was added to the supernatant and the residual immunoresistant triacylglycerol hydrolase activity was determined.

HL mass

The amount of HL protein was determined by solid-phase ELISA in which the antigen was sandwiched between rabbit and goat polyclonal antibodies. The rabbit and goat antibodies had been raised against human HL purified from post-heparin plasma by the methods of Martin et al. [25] and Persoon et al. [34] respectively. From the antisera, partly purified IgG fractions were prepared by 50 %-satd. $(NH_4)_2SO_4$ precipitation and elution through a human albumin–Sepharose column. Polystyrene 96-well ELISA plates were coated with the rabbit anti-HL IgG. After blocking with 10 mg/ml BSA in PBS, the wells were incubated successively with: (1) sample, (2) 3 μg/ml goat anti-HL IgG in PBS and (3) peroxidase-conjugated rabbit anti-(goat IgG) at 1:10000 dilution in PBS. Finally, the presence of peroxidase was detected with 3,3′,5,5′-tetramethylbenzidine (Merck, Darmstadt, Germany) as substrate. Colour development was stopped with H_2SO_4 (4 M final concentration), and A_{450} was measured in a Molecular Devices microplate reader. Absorbances were read against a standard curve prepared for each plate by serial dilutions of a human post-heparin pool plasma. The HL activity in this pool plasma amounted to 410 m-units/ml; HL protein mass was determined as 23.6 μg/ml by reading this plasma against a standard curve of purified HL. The ELISA enabled accurate measurements to be made of HL protein in the range 5–500 ng/ml.

Chromatography on Sepharose–heparin

HepG2 cells were grown in 75 cm^2 flasks. At confluence the cells were washed once with medium containing 5 i.u./ml heparin to remove any extracellularly bound HL. Subsequently the cells were incubated for 6–8 h with 7 ml of fresh medium containing

5 i.u./ml heparin. Extracellular medium and cell lysates were then prepared as described above. The medium from four flasks was pooled (28 ml) and applied to a column of Sepharose–heparin (0.5 ml wet gel) that had been equilibrated in PBS. Similarly, the cell lysates from four flasks were pooled (14 ml) and applied to Sepharose–heparin. After washing the columns with 2 ml of 0.3 M NaCl in 10 mM sodium phosphate (pH 7.0)/1 mg/ml BSA/10 % (v/v) glycerol, bound proteins were eluted with 1.5 ml of 1.5 M NaCl in the same buffer. The eluates from both columns were used in immunotitration assays in which approximately equal amounts of HL activity were incubated with serial dilutions of the goat anti-(human HL) IgG followed by determination of the residual triacylglycerol hydrolase activity, as described above.

α_1-Antitrypsin

Synthesis and secretion of α_1-antitrypsin was measured by the incorporation of [^{35}S]methionine into immunoprecipitated protein. HepG2 cells were incubated for 3 h in methionine-free medium in the presence of inhibitors as described above. Then 80 μCi of Tran^{35}S-label was added to each well and the incubation was continued for a further 3 h. Thereafter the plates were put on ice. The medium was collected into vials containing unlabelled methionine (final concentration 1 mM) and the cocktail of protease inhibitors described above. The cells were washed twice with PBS, then lysed in PBS containing 1 % (v/v) Triton X-100, 1 % (w/v) sodium deoxycholate, 0.25 % SDS, 1 mM methionine, 25 i.u./ml heparin, 10 mM Hepes, pH 7.4, and the cocktail of protease inhibitors. After 30 min on ice, the lysate was collected from the plate, the sodium deoxycholate concentration was brought to 0.3 % and the lysate was centrifuged for 10 min at 10000 ***g***. The supernant was used for further analysis.

Samples of medium and cell lysate were incubated with formaldehyde-fixed *Staphylococcus aureus* membranes (Zysorbin) in the presence of 0.2 mg/ml human serum albumin and centrifuged to remove material that bound non-specifically to Protein A. The supernatants were then incubated with rabbit antibodies against human α_1-antitrypsin at a 1:100 dilution. Antigen–antibody complexes were precipitated by incubation with Zysorbin and were collected by centrifugation. Pellets were washed twice in PBS containing 1 % (v/v) Triton X-100, 0.25 % SDS, 0.25 % sodium deoxycholate and 1 mM PMSF, then twice in PBS. The pellets were resuspended in Laemmli's sample buffer; immunoprecipitated proteins were released by being boiled for 5 min and separated by SDS/PAGE [7.5 % (w/v) gel]. The ^{35}S-labelled proteins were detected, and their radioactivity was determined, by exposure of the dried gels to a phosphor screen (GS-393 Molecular Imaging System; Bio-Rad, Hercules, CA, U.S.A.).

In some experiments immunoprecipitated proteins were released by being boiled for 5 min in 50 mM sodium phosphate buffer, pH 6.0, containing 0.25 % SDS. After removal of the bacterial membranes (5 min, 10000 ***g***, 4 °C), the eluted proteins were incubated overnight with 40 m-units/ml Endo H at 37 °C in 50 mM sodium phosphate buffer containing 0.1 % SDS [35]. The digestion was stopped by the addition of Laemmli's sample buffer, and after being boiled the samples were analysed by SDS/PAGE and phosphorimaging, as described above. Sensitivity to Endo H was indicated by an increase in electrophoretic mobility.

Protein synthesis *de novo*

Incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material was taken as a measure of overall protein synthesis *de novo*. HepG2 cells were incubated with Tran^{35}S-label, and cell-free medium and cell lysates were prepared as described above. Aliquots (5 μl) were spotted in duplicate on Whatman 3MM filters, and precipitation with trichloroacetic acid was performed as described previously [18]. The radioactivity on the filters was measured with the Molecular Imaging System.

Statistics

Statistical significances were determined by two-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test.

RESULTS

Synthesis and secretion of HL by HepG2 cells

In the presence of heparin, HepG2 cells secreted lipase activity into the extracellular medium (Figure 1A), whereas intracellular activity remained virtually constant throughout the 6–8 h incubation period (Figure 1B). Of the lipase activity present in the extracellular medium, 67 % was sensitive to immuno-inhibition with anti-(human HL) IgG (0.18 ± 0.01 compared with 0.06 ± 0.01 m-unit/ml, means $\pm$ S.D., $n = 3$, in the absence and the presence of anti-HL respectively), indicating that the extracellular activity represents mainly HL. Of the activity measured in the cell lysates, however, approx. 20 % was sensitive to immuno-inhibition (0.13 ± 0.02 compared with 0.10 ± 0.01 m-unit/ml, means $\pm$ S.D., $n = 3$), suggesting that HL represents only a small fraction of total intracellular lipase activity. When cycloheximide was added at the start of the incubation, the secretion of HL activity was hardly affected during the first 2 h (Figure 1A), although the inhibition of protein synthesis *de novo*, as determined by the incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material, occurred almost instantaneously. Between 3 and 6 h, secretion of HL activity levelled off and was completely blocked thereafter. Simultaneously, intracellular lipase activity decreased (Figure 1B). After 6–8 h, intracellular lipase activity was no longer sensitive to immuno-inhibition with anti-HL. These results indicate that, during the incubation with cycloheximide, pre-existing HL protein was secreted from the cells.

Comparison of the results in Figures 1(A) and 1(B) shows that, in the absence of protein synthesis *de novo*, much more HL activity appeared in the extracellular medium than simultaneously disappeared from the cells: whereas 0.45 m-unit was secreted per well, intracellular activity fell by only 0.05 m-unit per well. In seven independent experiments, the secretion-coupled increase in HL activity was 7.8 ± 2.1-fold (mean $\pm$ S.D.). This discrepancy was not caused by an underestimation of intracellular lipase activity: first, the triacylglycerol hydrolase assay used was linear with time and volume of cell lysate (results not shown); secondly, the triacylglycerol hydrolase activity in medium and cell lysate were additive when assayed in combination (0.18 ± 0.01, 0.13 ± 0.02 and 0.32 ± 0.02 m-unit/ml, means $\pm$ S.D., $n = 3$ respectively); and thirdly, extraction of lipase activity from the cells with the NH_4OH buffer used here was not improved by the inclusion of a detergent; the lipase activity was 0.53 ± 0.04 and 0.47 ± 0.02 m-unit/ml without and with 8 mM CHAPS in the lysis buffer respectively (means $\pm$ S.D., $n = 6$). The amount of HL protein in the cell lysates was below the detection limit of the ELISA (see below), so we could not test directly whether HL protein was extracted efficiently from the cells. We therefore tested the same extraction conditions on α_1-antitrypsin, an unrelated N-glycoprotein. Cells were first pulse-labelled for 1 h with [^{35}S]methionine, then chased in the presence of cycloheximide. The amount of ^{35}S-labelled α_1-antitrypsin that appe-

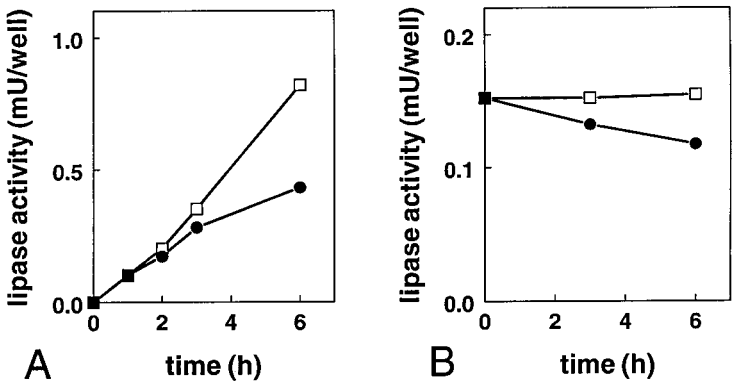

Figure 1 Synthesis and secretion of HL activity by HepG2 cells

HepG2 cells were incubated in the presence of 25 i.u./ml heparin without (□) or with (●) 20 μg/ml cycloheximide. At the times indicated, the medium and cells were harvested and triacylglycerol hydrolase activity was measured in the medium (**A**) and the cell lysates (**B**). The results are representative of three similar experiments. Abbreviation: mU, m-unit.

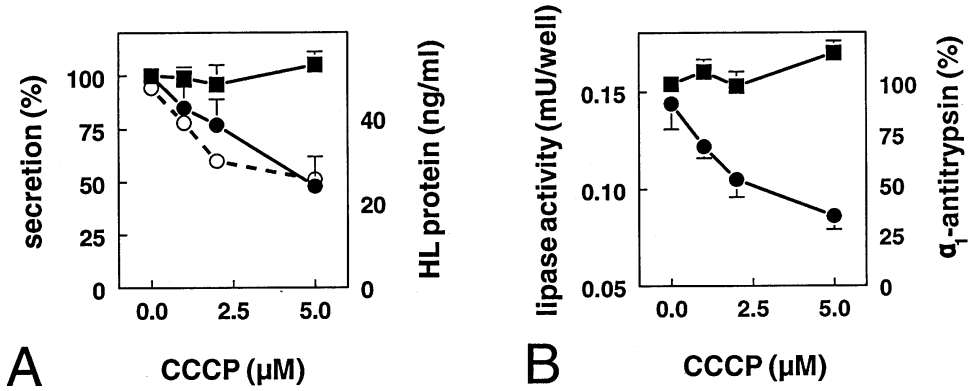

Figure 2 Effect of CCCP on expression of HL and α_1-antitrypsin

HepG2 cells were incubated for 6 h in the presence of different concentrations of CCCP. Thereafter the lipase activity (●) and the amounts of HL protein (○) and ^{35}S-labelled α_1-antitrypsin (■) were measured in the extracellular medium (**A**) and the cell lysates (**B**). Results are means ± S.E.M. for three independent experiments, except for the amount of HL protein, which was measured in one experiment. HL activities are expressed as percentages of control (0.66 ± 0.08 m-unit/ml); similarly, radioactivities in α_1-antitrypsin (expressed in arbitrary units) are given as percentages of control. Abbreviation: mU, m-unit.

Table 1 Effect of monensin and BFA on the expression of HL

HepG2 cells were incubated for 6–8 h with or without inhibitors in the presence of heparin. Then cell-free media and cell lysates were prepared for analysis of HL and lipase activity. In parallel incubations, the effect of the inhibitors on overall protein synthesis was determined by the incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material. Results are means ± S.E.M. for three to five independent experiments. *Statistically significant ($P < 0.05$) difference from controls.

Addition ...	None (control)	Cycloheximide (20 μg/ml)	Monensin (10 μM)	BFA (0.2 μg/ml)
Secretion				
HL activity (m-unit/ml)	0.92 ± 0.09	0.37 ± 0.03*	0.27 ± 0.02*	0.19 ± 0.02*
HL protein (ng/ml)	13.1	< 5	< 5	< 5
Intracellular lipase				
Activity (m-unit/ml)	1.16 ± 0.18	0.89 ± 0.11*	1.54 ± 0.15*	1.95 ± 0.12*
HL protein (ng/ml)	< 5	< 5	6.4	8.8
Overall protein synthesis (% of control)	100	11.2 ± 3.6*	97.8 ± 3.8	74.7 ± 11.3*

ared in the extracellular medium during a 6 h chase with cycloheximide was 1.6 ± 0.3-fold higher (mean ± S.D., $n = 3$) than the concomitant decrease in intracellular ^{35}S-labelled α_1-antitrypsin. These results indicate that intracellular proteins are not completely recovered from the cell layers by the protocol used, but this could not explain the observed 5–10-fold increase in HL activity associated with secretion. To determine where along the secretory pathway HL activity is acquired, several

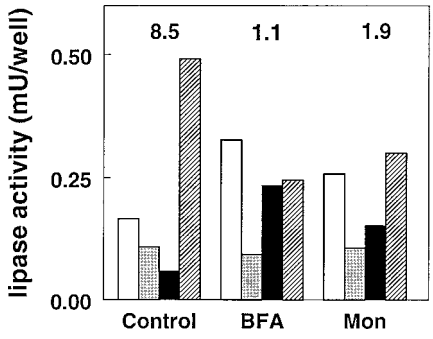

Figure 3 Apparent activation of HL during secretion in the absence of protein synthesis *de novo*

HepG2 cells were preincubated for 6 h with 25 i.u./ml heparin in the absence (Control) or presence of 0.2 μg/ml BFA or 10 μM monensin (Mon). In each treatment group, two wells were immediately harvested for measurement of intracellular lipase activity (open bars). Two other wells were washed free of inhibitor with PBS at room temperature, and incubation was continued overnight in fresh medium containing heparin and 20 μg/ml cycloheximide to prevent additional HL synthesis *de novo*. Thereafter media and cells were harvested. Lipase activity was determined in the cell lysates (grey bars) and the difference from the activity measured in the cells before the overnight incubation with cycloheximide was calculated (filled bars). This difference was compared with the HL activity that was secreted into the extracellular medium during the overnight incubation with cycloheximide (hatched bars). The results for the duplicate incubations were averaged. The numbers above the bars indicate the ratio of secreted activity to the concomitant decrease in intracellular activity. Abbreviation: mU, m-unit.

inhibitors were used that interfere with intracellular vesicle transport and N-glycosylation.

Effect of inhibitors of intracellular trafficking

With increasing concentrations of CCCP, secretion of HL activity into the extracellular medium gradually fell to approx. 40 % of control at 5 μM (Figure 2A). The amount of HL protein fell in parallel. Simultaneously, CCCP decreased the intracellular lipase activity in a concentration-dependent manner (Figure 2B). With 5 μM CCCP, intracellular lipase activity was similar to that in cycloheximide-treated cells. Under all conditions, the amount of intracellular HL protein remained below the detection limit of the ELISA. In contrast, the secretion of α_1-antitrypsin was insensitive to CCCP up to 5 μM. On SDS/PAGE, α_1-antitrypsin secreted in the presence of 5 μM CCCP migrated as an Endo H-resistant 54 kDa band that was indistinguishable from the protein secreted by control cells (results not shown). The amount of intracellular α_1-antitrypsin was not affected by CCCP up to 5 μM (Figure 2B). Intracellular α_1-antitrypsin consisted of the Endo H-sensitive 49 kDa and the Endo H-resistant 54 kDa protein bands also seen in control cells (see Figure 4C).

When HepG2 cells were incubated with 10 μM monensin or 0.2 μg/ml BFA, the secretion of HL activity decreased to levels well below that observed with cycloheximide (Table 1). The amount of HL protein in the extracellular medium decreased in parallel. Simultaneously, the intracellular lipase activity increased markedly (Table 1). Immuno-inhibition with anti-HL IgG showed that this increase occurred exclusively in the immuno-sensitive fraction (results not shown). In addition, the amount of HL protein was also elevated to levels well above the detection limit of the ELISA (Table 1). The overall protein synthesis *de novo* was not affected by monensin, whereas it was diminished approx. 25 % by BFA. The secretion of newly synthesized α_1-antitrypsin was blocked almost completely by monensin and BFA (results not shown). With both agents, α_1-antitrypsin accumulated intracellularly as an Endo H-sensitive 49 kDa protein.

Apparent activation of HL during incubation with BFA and monensin

The inhibition of secretion by BFA and monensin and the concomitant increase in intracellular HL activity was at least partly reversible. On their removal from the medium, the secretion of HL activity resumed even in the presence of cycloheximide. This enabled us to measure the secretion-linked increase in HL activity in these cells. Cells were first incubated for 6–8 h with or without BFA or monensin in the presence of heparin. After removing the extracellular medium, the cells were re-incubated overnight but in the presence of cycloheximide. As shown in Figure 3, the intracellular lipase activity of the control cells decreased by approx. 0.06 m-unit per well, whereas the

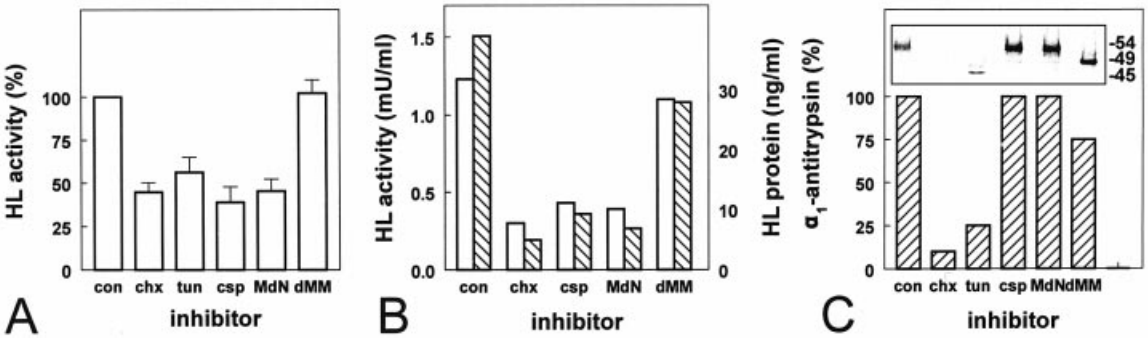

Figure 4 Effect of glycosylation inhibitors on the secretion of HL and α_1-antitrypsin

HepG2 cells were incubated for 6–8 h in the absence (con) or presence of 20 μg/ml cycloheximide (chx), 10 μg/ml tunicamycin (tun), 100 μg/ml CSP (csp), 1 mM MdN or 1 mM dMM. The extracellular appearance of HL (**A**, **B**) and α_1-antitrypsin (**C**) was measured as outlined in the Materials and methods section. (**A**) HL activities in the extracellular medium (means $\pm$ S.E.M.; $n = 6$ or 7) are expressed as percentages of control, which was 1.02 $\pm$ 0.10 m-units per well. Except for dMM, the HL activity in the medium containing glycosylation inhibitors was significantly different ($P < 0.05$) from controls. (**B**) HL activity and amount of HL protein, as determined by ELISA, both measured in the same extracellular medium. (**C**) α_1-Antitrypsin was immunoprecipitated from medium from 6 h incubations also containing Tran^{35}S-label, and the radiolabelled proteins were analysed by SDS/PAGE and phosphorimaging. Part of the image is shown at the top; the molecular mass of the radioactive bands is indicated (in kDa) at the right. The radioactivity in each band (in arbitrary units) is expressed relative to the control incubation. The results in (**B**) and (**C**) are representative of two similar experiments. Abbreviation: mU, m-unit.

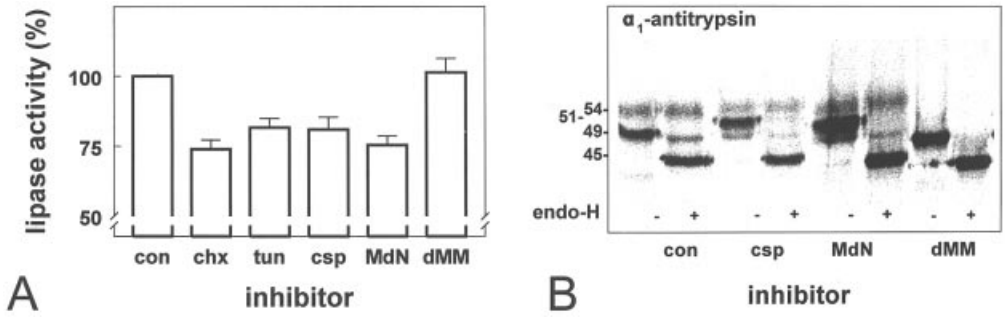

Figure 5 Effect of glycosylation inhibitors on intracellular HL and α_1-antitrypsin

Experiments were performed as described in the legend to Figure 4, and lipase activity (**A**) and radiolabelled α_1-antitrypsin (**B**) were determined in cell lysates. Results for intracellular lipase activity are means $\pm$ S.E.M. ($n = 5$ or 6) and are expressed as percentages of control (0.24 ± 0.05 m-unit per well). Except for dMM, the HL activity in the medium containing glycosylation inhibitors was significantly different ($P < 0.05$) from controls. In (**B**) the immunoprecipitates prepared from the cell lysates were treated without ($-$) or with ($+$) Endo H before electrophoretic separation; the molecular masses of the radioactive proteins are indicated (in kDa) at the left. Results are representative of two similar experiments.

extracellular HL activity increased to 0.50 m-unit per well. This 8.5-fold increase in HL activity is in agreement with the results presented above. In contrast, the HL activity secreted by BFA-pretreated cells in the presence of cycloheximide was similar to the concomitant decrease in intracellular lipase activity. In six similar experiments the secretion-coupled increase in HL activity was only 1.3 ± 0.5-fold (mean $\pm$ S.D.) for BFA-treated cells, whereas a value of 1.9 was found in an experiment with monensin-treated cells. Hence the apparent activation of HL activity that is associated with the secretory process has already taken place in BFA- and monensin-treated cells.

Effect of N-glycosylation inhibitors

After 6–8 h of incubation with tunicamycin, CSP or MdN, the secretion of HL activity into the medium was inhibited to a similar extent to that with cycloheximide (Figure 4A). The secretion of HL protein decreased in parallel with HL activity (Figure 4B). With tunicamycin, overall protein synthesis *de novo* decreased to $86 \pm 17\%$ of control as compared to with $11 \pm 7\%$ with cycloheximide (means $\pm$ S.D., $n = 4$). With CSP and MdN, protein synthesis *de novo* decreased only slightly to $93 \pm 10\%$ and $97 \pm 5\%$ of control respectively (means $\pm$ S.D., $n = 3$). Despite normal protein synthesis, the decreased secretion of HL activity was not accompanied by an increase in intracellular lipase activity; instead, intracellular lipase activity decreased to approx. 75–80 % of control cells (Figure 5A). Immuno-inhibition assays with anti-(human HL) IgG showed that the decrease in intracellular lipase activity was due to a decrease in the immunosensitive part (results not shown). In contrast with the other inhibitors, the secretion of both HL activity (Figure 4A) and HL protein (Figure 4B) continued almost unaffected in the presence of the mannosidase inhibitor dMM, whereas intracellular lipase activity was maintained at control levels (Figure 5A). Under all conditions tested, the amount of HL protein in the cell lysates remained below the detection limit of the ELISA.

Tunicamycin markedly decreased the secretion of α_1-antitrypsin (Figure 4C); the small amount of α_1-antitrypsin in the extracellular medium migrated at an apparent molecular mass of 45 kDa, which corresponds to the non-glycosylated protein. The secretion of α_1-antitrypsin was not affected by CSP or MdN. On SDS/PAGE, the secreted protein was indistinguishable from the Endo H-resistant 54 kDa protein present in control medium (Figure 4C). Intracellular α_1-antitrypsin migrated predominantly as an Endo H-sensitive 51 kDa band, with the 49 kDa and 54 kDa bands also seen in control cells as minor components (Figure 5B). Intracellular and extracellular levels of α_1-antitrypsin were hardly affected by incubation of the cells with dMM but the proteins migrated as Endo H-sensitive 49 kDa bands. Hence the secretion of HL by HepG2 cells was highly sensitive to the RER glucosidase inhibitors CSP and MdN, whereas that of α_1-antitrypsin was not.

Table 2 Effect of co-incubation of glycosylation inhibitors and BFA on HL expression

HepG2 cells were incubated for 6–8 h in the presence of heparin with or without BFA (0.2 μg/ml) and glycosylation inhibitors. At the end of the incubation, cell lysates and cell-free media were prepared for analysis of triglyceridase activity. Results are means $\pm$ S.E.M. for three to five independent experiments. *Statistically significant ($P < 0.05$) difference from the controls incubated without glycosylation inhibitor.

	Intracellular HL (m-unit per well)		Extracellular HL (m-unit per well)	
Addition	$-$ BFA	$+$ BFA	$-$ BFA	$+$ BFA
None (control)	0.23 ± 0.05	0.36 ± 0.08	1.28 ± 0.15	0.26 ± 0.06
Tunicamycin (10 μg/ml)	0.18 ± 0.07*	0.33 ± 0.10	0.60 ± 0.18*	0.26 ± 0.09
Castanospermine (100 μg/ml)	0.17 ± 0.06*	0.32 ± 0.09	0.47 ± 0.07*	0.20 ± 0.10
MdN (1 mM)	0.17 ± 0.06*	0.36 ± 0.09	0.65 ± 0.18*	0.22 ± 0.08

Combined effect of BFA and glycosylation inhibitors

When co-incubated with 0.2 μg/ml BFA, CSP (100 μg/ml) and MdN (1 mM) were no longer able to decrease intracellular lipase activity in HepG2 cells (Table 2). Instead, intracellular lipase activity increased on incubation with BFA, whether or not CSP or MdN was present. By ELISA, similar results were found for intracellular HL protein (results not shown). The BFA-induced inhibition of HL secretion (Table 2) and α_1-antitrypsin secretion were not affected by co-incubation with the glycosylation inhibitors. With CSP or MdN, however, the intracellular appearance of the additional 51 kDa form of α_1-antitrypsin was observed in both the absence and the presence of BFA (results not shown). This observation suggests that the glucosidase

Table 3 Chromatography on Sepharose–heparin

Secretion media and cell lysates from four 75 cm^2 culture flasks were pooled and HL was isolated by chromatography on a Sepharose–heparin column. The column was washed with 0.3 M NaCl, and bound proteins were eluted with 1 ml of 1.5 M NaCl. Triacylglycerol hydrolase activity was determined in the elution fractions, and also in the combined breakthrough and wash fractions, for the calculation of total recovery. The bound fraction of lipase activity was calculated as a percentage of recovered activity. Results are means ± S.D. for four independent experiments.

Parameter	Medium	Cell lysate
Lipase activity applied (m-units)	12.9 ± 2.8	6.0 ± 1.5
Total recovery (%)	79.3 ± 9.5	77.3 ± 7.5
Bound fraction (%)	62.7 ± 9.9	34.3 ± 9.7
Eluted activity (m-units)	6.4 ± 0.6	1.6 ± 0.3
Immunosensitive fraction (%)	84.5 ± 7.5	72.0 ± 8.8

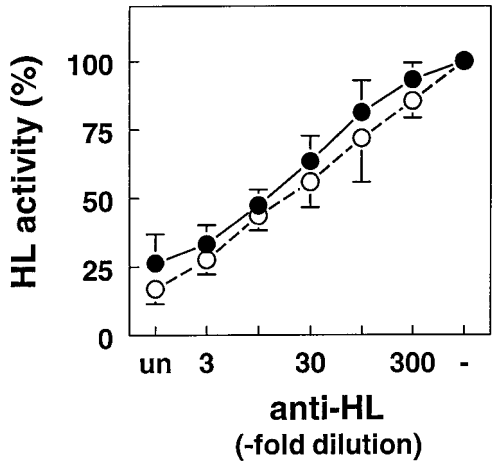

Figure 6 Immunotitration of secreted and intracellular HL after isolation on Sepharose–heparin

HL from secretion medium (○) and cell lysate (●) was bound to Sepharose–heparin. After the column had been washed with 0.3 M NaCl, bound proteins were eluted in a single batch with 1.5 M NaCl. The activity of secreted HL was diluted with elution buffer to the same activity as in the intracellular HL fraction (approx. 1.2 m-units/ml). Both preparations were incubated with goat anti-(human HL) IgG at different dilutions, and the residual triacylglycerol hydrolase activity was measured. Results (means ± S.D., $n = 4$) are expressed as percentages of the activity measured after parallel incubations in the absence of anti-HL. Abbreviation: un, undiluted.

inhibitors were still effective in the presence of BFA. Nevertheless their inhibitory effect on intracellular HL activity was overcome by co-incubation with BFA.

Immunotitration of intracellular and secreted HL

In a large-scale experiment, HL was isolated from 6–8 h medium and cell lysates by binding to Sepharose–heparin followed by batchwise elution with 1.5 M NaCl. For both sources, total recovery of lipase activity was approx. 80 %, but only 35 % of the lipase activity in the cell lysate bound to the column compared with 65 % of the activity from medium (Table 3). In contrast with the cell lysate, the lipase activity in the single elution fraction was largely sensitive to immuno-inhibition with anti-(human HL) IgG, as with the medium fraction. After adjusting the medium fraction with elution buffer to the same HL activity as the lysate fraction, both fractions were subjected to immuno-inhibition with serially diluted anti-(human HL) IgG (Figure 6). Between 300-fold and 3-fold dilution of the antibody preparation, the HL activity decreased gradually in both fractions. In four independent experiments, we found a small but significant difference ($P = 0.04$) between the two immunotitration curves. Compared with the curve for intracellular HL, that for secreted HL was shifted towards higher antibody dilutions and thus towards lower antibody titres. This suggests that after chromatography on Sepharose–heparin, the specific enzyme activity of secreted HL was 1.7 ± 0.3-fold higher (mean ± S.D., $n = 4$) than that of intracellular HL. Using the same approach, we did not detect inactive HL in the column breakthrough plus wash fractions (results not shown).

DISCUSSION

We show here that HL activity increases 5–10-fold during maturation in human HepG2 cells. After chromatography on Sepharose–heparin, the specific enzyme activity of secreted HL was less than 2-fold greater than that of intracellular HL, which suggests that the increase in enzyme activity is only partly explained by true activation. Apparently the previously hidden enzyme activity of HL protein becomes unmasked during maturation. Several lines of evidence suggest that this increase in enzyme activity occurs in the *cis*- to medial Golgi region of the secretory pathway. First, this apparent activation has already occurred in cells treated with monensin, which inhibits intra-Golgi transport (Figure 3). Secondly, when HL protein is retained within the RER, by treating the cells either with CCCP (Figure 2) or with the glucosidase inhibitors CSP or MdN (Figure 5), intracellular HL activity does not increase. Thirdly, when the contents of the Golgi stacks are transported back into the RER by treating the cells with BFA, the effect of CSP and MdN on intracellular HL activity is reversed (Table 2). The mechanism by which the catalytic activity of HL is unmasked in the *cis*- to medial Golgi remains unknown. It is unlikely that the trimming of mannose residues by Golgi mannosidase I itself is responsible for the apparent activation, because inhibition of this process *in situ* by dMM has no effect on the intrcellular and secreted HL activity (compare Figures 4 and 5).

Previous studies with Chinese hamster ovary cells transfected with HL cDNA constructs have demonstrated that N-linked glycosylation of rat and human HL is essential for the secretion of a catalytically active protein by these cells [15,16]. N-glycosylation of human HL was shown to be necessary for the proper intracellular trafficking of newly synthesized protein [15,20]. Here we show that the secretion of catalytically active human HL by HepG2 cells requires not only N-glycosylation but also subsequent glucose trimming by RER glucosidases I and II. In this respect human HL behaves similarly to rat HL, the secretion and apparent activation of which are also highly sensitive to inhibition by RER glucosidase inhibitors [18,21], but differently from a number of other N-glycoproteins including α_1-antitrypsin (Figure 4) [35,36]. Glucose trimming by the RER glucosidases has been implicated in the quality control system of the secretory pathway, which prevents badly folded or unassembled glycoproteins from leaving the RER for the Golgi [23,24]. In this model, glycoproteins bearing terminal glucose residues associate with the molecular chaperone calnexin, which assists in the folding process [23,37,38]. Our observations suggest that glucose trimming of newly synthesized HL protein is necessary for folding into a transport-competent form. In the presence of the glucosidase inhibitors CSP and MdN, or in the absence of N-glycosylation, only a small amount of newly

synthesized HL, if any, is properly folded and subsequently transported to the Golgi and beyond.

Why HL trafficking depends so much more on glucose trimming than α_1-antitrypsin remains unclear. Unlike that of HL, the secretion of α_1-antitrypsin was unaffected by CSP or MdN. In the presence of these inhibitors, the maturation of α_1-antitrypsin proceeded through a distinct intracellular intermediate of 51 kDa (Figure 5C) [35]. Despite the inhibition of the RER glucosidases, α_1-antitrypsin seemed to be normally deglucosylated and further processed to the Endo H-resistant form. The Golgi system of HepG2 cells is shown to contain a endo-α-D-mannosidase that removes the mannose residue with the attached glucoses, thereby providing an alternative processing route for α_1-antitrypsin and several other N-glycoproteins [36,39]. Utilization of this alternate route requires that these glycoproteins are first transported from the RER to the Golgi. Apparently the Golgi endo-mannosidase is not accessible to HL protein in CSP- and MdN-treated HepG2 cells, probably because the presence of the terminal glucose residues prevents HL protein, but not α_1-antitrypsin, from leaving the RER. Compared with that of α_1-antitrypsin, the maturation of HL was also much more sensitive to inhibition by CCCP (Figure 2), an H^+-ionophore and mitochondrial uncoupler. This observation might indicate that the folding of HL into a transport-competent structure requires some additional pH- or ATP-dependent steps such as the release from chaperones in the RER. Perhaps α_1-antitrypsin folds into a transport-competent form without the help of such ATP-dependent chaperones. Alternatively, as human HL and rat HL seem to be oligomeric proteins [40–43], the proper folding of HL might require assembly into oligomers, in contrast with the monomeric α_1-antitrypsin.

The effects of the glucosidase and trafficking inhibitors reported here are best explained by a model in which glucose trimming of HL in the RER is necessary for transport to the Golgi, where its triacylglycerol hydrolase activity becomes detectable. Glucose trimming in the RER is necessary for newly synthesized and properly folded HL protein to detach from calnexin. Other RER chaperones can then bind to HL, which prevents activation or masks its catalytic activity. These chaperones can escort HL to the Golgi compartment, where HL is liberated and its catalytic activity becomes apparent.

These studies were supported by the Dutch Heart Foundation (grant no. 91.075).

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Received 10 August 1998; accepted 21 October 1998