Secretion of rat hepatic lipase is blocked by inhibition of oligosaccharide processing at the stage of glucosidase I

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Abstract Rat hepatic lipase is a glycoprotein bearing two N-linked oligosaccharide chains. The importance of glycosylation in the secretion of hepatic lipase was studied using freshly isolated rat hepatocytes. Various inhibitors of oligosaccharide synthesis and processing were used at concentrations that selectively interfere with protein glycosylation. Secretion of hepatic lipase activity was abolished by tunicamycin, castanospermine, and N-methyldeoxynojirimycin. No evidence was found by ELISA or Western blotting for secretion of inactive protein. Inhibition of secretion became apparent after a 30-min lag, corresponding to the time of intracellular transport of pre-existing protein. Simultaneously, intracellular hepatic lipase activity was depleted. Secretion of hepatic lipase protein and activity was not affected by deoxymannojirimycin and swainsonine. Upon SDS-polyacrylamide gel electrophoresis, hepatic lipase secretion by deoxymannojirimycin- or swainsonine-treated cells showed an apparent M_r of 53 kDa and 55 kDa, respectively, which was distinct from hepatic lipase secreted by untreated cells (M_r = 58 kDa). We conclude that glycosylation and subsequent oligosaccharide processing play a permissive role in the secretion of hepatic lipase. As secretion is prevented by the glucosidase inhibitors castanospermine and N-methyldeoxynojirimycin, but not by inhibitors of subsequent oligosaccharide trimming, the removal of glucose residues from the high-mannose oligosaccharide intermediate in the rough endoplasmic reticulum appears the determining step. —**Verhoeven, A. J. M., and H. Jansen.** Secretion of rat hepatic lipase is blocked by inhibition of oligosaccharide processing at the stage of glucosidase I. *J. Lipid Res.* 1990. **31:** 1883–1893.

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Hepatic lipase (HL) and lipoprotein lipase (EC 3.1.1.34; LPL) are two lipolytic enzymes that function in the metabolism of circulating lipoproteins (1). Both enzymes are synthesized and secreted by parenchymal cells and are bound to the surface of endothelial cells from which they can be released by administration of heparin. HL may hydrolyze phospholipid and triacylglycerol in high and intermediate density lipoproteins (1–3). It is present in the liver of many vertebrates, but it has also been detected in adrenal glands and ovaries (4–7). LPL is present in most extrahepatic tissues and mobilizes fatty acids from triacylglycerol in chylomicrons and very low density lipoproteins (1, 8).

The structure of both lipases from various species has been deduced from partial amino acid sequences of proteolytic fragments (9, 10), and more recently, from cloned cDNA sequences (11–17). The high degree of homology between the sequences of the two lipases led to the suggestion that both proteins are members of the same gene family (9, 14–16). Both enzymes are glycoproteins bearing at least two N-linked oligosaccharide chains of the complex type (18–21). The importance of this glycosylation in maturation and secretion has been extensively studied for LPL. Prevention of glycosylation by treatment of adipocytes (22, 23) and rat heart cells (24) with tunicamycin revealed that the unglycosylated form of LPL is not secreted. In COS cells, the presence of only one of the two carbohydrate chains on human LPL was required for secretion (25). In one study with Ob17 adipocytes, however, the inactive unglycosylated form was detected in the extracellular medium (26). Once glycosylated, the protein is transported from the endoplasmic reticulum to the Golgi, and becomes secretable. In guinea pig adipocytes, this process is not affected by inhibitors of subsequent oligosaccharide processing (21), indicating that processing of the oligosaccharide chains from the high-mannose to the complex-type is not necessary for secretion of LPL activity in these cells.

Abbreviations: BSA, bovine serum albumin; dMM, deoxymannojirimycin; DMSO, dimethyl sulfoxide; EDTA, ethylenediamine tetraacetate; HL, hepatic lipase; IgG, immunoglobulin G; LPL, lipoprotein lipase; MdN, N-methyldeoxynojirimycin; PBS, Dulbecco's phosphate-buffered saline; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay.

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In contrast, little is known about the role of glycosylation in secretion of HL. In primary cultures of rat hepatocytes, tunicamycin was found to prevent the secretion of enzyme activity (27), whereas in another study, using freshly isolated hepatocytes, no HL protein could be detected extracellularly under similar conditions (20). These observations indicate that glycosylation might also be essential for secretion of HL.

The purpose of the present study was to characterize the relationship between asparagine-linked glycosylation and secretion of HL in greater detail. Inhibitors were used that interfere with different stages of oligosaccharide synthesis and processing [reviewed in (28, 29)]. Tunicamycin is known to inhibit the biosynthesis of the dolichol-linked high-mannose oligosaccharide precursor, thus preventing the formation of glycoproteins with N-linked oligosaccharides. The other inhibitors do allow the addition of the oligosaccharide precursor but interfere with the subsequent conversion of high-mannose sugar chains to complex-type ones. Castanospermine and N-methyldeoxynojirimycin are inhibitors of glucosidase I (and II) present in the endoplasmic reticulum, and cause the accumulation of glycoproteins bearing high-mannose sugar chains containing three glucose residues. Deoxymannojirimycin is a specific inhibitor of Golgi mannosidase I, resulting in glycoproteins bearing high-mannose structures without glucose. Swainsonine acts at a later stage of oligosaccharide processing at the level of mannosidase II; in its presence glycoproteins are produced bearing hybrid oligosaccharides. We report here on the differential effects of these agents on HL secretion by freshly isolated rat hepatocytes. The data unambiguously show that proper oligosaccharide processing is essential to secretion of HL.

MATERIALS AND METHODS

Materials

Castanospermine, swainsonine, N-methyldeoxynojirimycin (MdN) and (+)-1-deoxymannojirimycin (dMM) were purchased from Genzyme, Boston, MA; tunicamycin was from Sigma. Cycloheximide was from Boehringer Mannheim, FRG. Glycerol tri[9,10(n)-^{3}H]oleate, L(4,5-^{3}H)leucine, and D-(6-^{3}H)glucosamine were obtained from Amersham International, UK, at specific radioactivities of 1, 40, and 32 Ci/mmol, respectively. Heparin (Thromboliquine) was a product of Organon Oss, The Netherlands, and CNBr-activated Sepharose 4B was from Pharmacia, Uppsala, Sweden. Ham F10 medium was obtained from Flow Lab, UK, and bovine serum was from BioTrading, Wilnis, The Netherlands. Alkaline phosphatase-conjugated goat anti-mouse IgG and swine anti-goat IgG were purchased from Tago, Burlingame CA, and *p*-nitrophenol phosphate was from Merck, Darmstadt, FRG. Gelatin, Tween-20, acrylamide, and bisacrylamide were obtained from Bio-Rad, Richmond, CA. Prestained protein molecular weight markers were from Diversified Biotech, Newton Centre MA. All other chemicals were from Sigma. Nitrocellulose paper was from Schleicher & Schuell, Dassel, FRG. Polystyrene 96-well EIA plates (code 3590) were from Costar, Cambridge MA.

Antibodies

HL was purified to homogeneity from postheparin rat liver perfusates by the method of Jensen and Bensadoun (30) and used to immunize a goat as described elsewhere (31). The IgG fraction was purified from plasma by two successive precipitations each with 50% $(NH_4)_2SO_4$ and 17% Na_2SO_4, followed by extensive dialysis against Dulbecco's phosphate-buffered saline (PBS). Final protein concentration was 20 mg/ml. The titer of this preparation was determined by immunoinhibition of HL activity from a liver perfusate (see below), and amounted to ca. 3500 mU/ml. Nonspecific IgGs, isolated similarly from the serum of a nonimmunized goat, failed to inhibit HL activity. In a Western blot of a liver perfusate, a single band with M_r = 58,000 Da corresponding to HL was recognized by the specific IgG preparation, but not by the control IgGs (data not shown).

The monoclonal anti-HL preparation used here was a mixture of five different hybridoma supernatants that have been described in detail previously (32). The titer of this preparation was ca. 20 mU/ml.

Hepatocyte isolation and incubation

Hepatocytes were isolated from male Wistar rats (200–250 g body weight) by collagenase perfusion; nonparenchymal cells were removed by differential centrifugation as described previously (33). Cell viability was determined by Trypan blue exclusion and ranged from 85 to 95%. The cells were suspended at ca. 5 mg/ml of cell protein in Ham F10 medium containing 50 U/ml of heparin and 20% of heat-inactivated serum (34), either rat or bovine; both sera are equally potent in supporting secretion of HL activity by these cells (35). Cell suspensions were incubated at 37°C under an atmosphere of 5% CO_2/95% O_2 in a shaking water bath. Inhibitors were present from the start of the incubation. Tunicamycin was dissolved in DMSO at 40 mg/ml; immediately before use, this stock solution was diluted in PBS to give the proper final concentration. Preliminary experiments showed that secretion of HL activity was not affected by these low DMSO concentrations. The other inhibitors were added to the incubation from stock solutions prepared in PBS. To control suspensions, only PBS was added. In some experiments the cells were re-isolated after 2 h of preincubation by centrifugation (50 *g*, 2 min, room temp.), washed once by resuspension in PBS, and then incubated further with freshly prepared medium containing the

same concentration of inhibitor. Cell viability remained well above 85% throughout incubation in the presence or absence of inhibitors.

At the times indicated, samples of cell suspension were collected. The cells were separated from the medium by centrifugation (50 *g*, 2 min, room temp.), and the cell-free medium was used for analysis of secreted HL. For analysis of intracellular HL activity, the cells were washed once in PBS and finally resuspended at 10% of the original volume in PBS containing heparin (50 U/ml), phenylmethylsulfonyl fluoride (2 mM), leupeptin (2 μg/ml), and EDTA (4 mM). This suspension was rapidly frozen to −80°C. After slow thawing on ice, the cells were sonified for 5–10 sec (MSE 150 W ultrasonic disintegrator, amplitude 7 μ, on ice). The homogenate was centrifuged (10 min, 10,000 *g*, 4°C); the supernatant was collected on ice and assayed for HL activity on the same day. Immediately before use in the assay, the cell homogenates were diluted 10-fold in PBS to obtain a final volume comparable to the initial cell suspension. Control experiments showed that addition of either 0.2% Triton X-100 or 4 mM CHAPS to the freeze-thawed and sonified cells failed to extract any additional lipase activity (data not shown).

Hepatic lipase activity

HL activity was determined by a triglyceridase assay at pH 8.5 in 0.6 M NaCl using a gum acacia-stabilized [^{3}H]triolein emulsion as substrate (34). Activities were expressed as mU (nmoles of free fatty acids released per min). Since cell homogenates prepared from rat liver also contain other lipase activities (36), HL activity was detected by its sensitivity to inhibition by polyclonal anti-HL. In immunoinhibition assays, 40 μl of the (diluted) cell homogenates were mixed with 10 μl of either nonimmune IgGs or the undiluted antibody preparation described above. After incubation for 1 h at 0°C, substrate was added and residual lipase activity was determined. HL activity was taken as the difference in residual activity after incubation with nonspecific IgG versus anti-HL. Preliminary studies showed that under these conditions HL activity in the cell-free media was not affected by nonimmune IgGs, whereas it was completely suppressed by anti-HL. Therefore, immunoinhibition assays were not routinely performed on cell-free media.

ELISA for rat hepatic lipase

A solid-phase ELISA was developed for rat HL using the goat polyclonal and the mixture of monoclonal anti-HL described above as the primary and secondary antibody, respectively (A. J. M. Verhoeven and H. Jansen, unpublished results). In short, microtiter plate wells were coated with goat anti-HL IgG. After blocking with gelatin, the wells were incubated successively with *i*) sample, *ii*) the monoclonal antibodies at a 1:5 dilution, and *iii*) alkaline phosphatase-conjugated goat anti-mouse IgG at a 1:500 dilution. To reduce nonspecific binding, incubations were performed in the presence of 1 M NaCl, 0.1% Tween-20, and 0.5% BSA. Finally, the presence of alkaline phosphatase was detected using *p*-nitrophenol phosphate as substrate. Color development was stopped with NaOH (1 N, final concentration), and the absorbance was read at 405 nm in a Titertek EIA analyzer. Absorbances were read against a standard curve prepared for each plate by serial dilutions of partially purified HL.

Immunopurification of hepatic lipase

HL was purified from the cell-free media by immunoadsorption to immobilized goat anti-HL IgG. Twenty mg of the goat antibody preparation described above was coupled per gram of CNBr-activated Sepharose 4B according to the manufacturers instructions; coupling efficiency was greater than 75%. The gel was stored for up to 3 months in 5 ml of PBS at 4°C until use. A small column was made in a pipet tip using 1 ml of this gel. The column was pretreated with 2 ml of 1% BSA in PBS, and then equilbrated in 20% glycerol/10 mM NaP_i; pH 7.0 (buffer A). Three ml of cell-free medium was loaded onto the column. Thereafter, the column was washed with 2 ml each of *i*) buffer A, *ii*) 2 M NaCl in buffer A, *iii*) 0.2% Triton X-100 and 0.5 M NaCl in buffer A, and *iv*) 150 mM NaCl in buffer A. The entire procedure was performed at 4°C and a flow of ca. 5 ml/h. Finally, the bound material was eluted with 1 ml of 3% SDS in 10 mM NaP_i (pH 7.0) at room temperature. BSA (30 μg) was added to the eluate as a carrier, and the protein was precipitated overnight at −20°C with 10 ml of acetone. The precipitates were pelleted by centrifugation, the supernatants were aspirated, and the precipitates were dried. The protein was then dissolved in 200 μl of Laemmli's sample buffer by heating for 5 min at 100°C, and subjected to electrophoresis in an SDS-containing 7.5%-polyacrylamide gel (37). After electrophoretic transfer to nitrocellulose, immunoblotting was performed using the goat anti-HL IgG followed by alkaline phosphatase-conjugated swine anti-goat IgG. The blots were developed using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as color reagents.

Other methods

For the preparation of postheparin liver perfusates, rat livers were perfused in situ in a noncirculating system at 37°C and a flow of 30 ml/min (33). After a 10-min washout, heparin was added to the perfusion buffer at 5 U/ml, and perfusate was collected on ice during the subsequent minute. For the partial purification of HL, perfusates prepared from two rats were applied to a Sepharose-heparin column. After washing the column with 3 volumes of 0.4 M NaCl in buffer A, the HL activity was eluted with 1 M NaCl in the same buffer. The peak fraction was collected and kept at −80°C until use.

For the study of [^{3}H]leucine and [^{3}H]glucosamine incorporation into total protein, the radiolabeled tracers were added to parallel suspensions of hepatocytes at 10 μCi/ml. After 1 h of incubation, cell-free media and cell homogenates were prepared as outlined above. Fifty μl thereof were applied onto Whatman 3M filter. After drying, the filters were boiled for 10 min in 5% (w/v) TCA. The solution was discarded, and the filters were washed twice with cold TCA (5%), once with ether–ethanol 1:1 (by vol) and finally, with ether. The filters were air-dried and the radioactivity was determined by liquid scintillation counting.

Protein was determined by the method of Bradford (38) using BSA as standard.

RESULTS

Selective inhibition of protein glycosylation

To verify that the inhibitors specifically affected protein glycosylation at the concentrations used in the present study, their effect on [^{3}H]glucosamine and [^{3}H]leucine incorporation into total TCA-precipitable material was determined. Cells preincubated for 2 h with or without inhibitors were incubated for 1 h in the presence of either radiolabeled precursor, and incorporation into total protein was determined in cell-free media and cell homogenates. As shown in **Table 1**, all four inhibitors significantly reduced [^{3}H]glucosamine incorporation into secreted protein, whereas incorporation of [^{3}H]leucine remained unaffected. As a consequence, the ratio of [^{3}H]glucosamine to [^{3}H]leucine incorporation was well below 1.0, indicating the selective inhibition of protein glycosylation over protein synthesis. Castanospermine, dMM, and swainsonine also showed a specific effect on [^{3}H]glucosamine incorporation into total cellular protein. With tunicamycin, [^{3}H]leucine incorporation into total cellular protein was inhibited to ca. 60% of control values despite the absence of an effect on [^{3}H]leucine in secreted protein, an observation that was left unexplored here. Incorporation of [^{3}H]glucosamine into total cellular protein was reduced even more than that of [^{3}H]leucine, resulting in a ratio well below 1.0. Hence, all four inhibitors specifically inhibited protein glycosylation at the concentrations used.

Secretion of hepatic lipase activity

Initially, we examined the effect of tunicamycin, castanospermine, and swainsonine on secretion of HL activity. Cells were incubated for 2 h with the inhibitors at concentration ranges that were reported to be effective in various cells including hepatocytes (28, 29). As heparin was present throughout all incubations, inactivation of secreted HL was negligible (34, 39). With tunicamycin, secretion of enzyme activity was reduced at all concentrations tested (**Fig. 1A**). Half-maximal inhibition was obtained at less than 0.2 μg/ml. Under the conditions used, secretion of HL activity was inhibited by 55% with respect to the untreated controls at tunicamycin concentrations of 1 μg/ml and higher. A concentration-dependent inhibition was also obtained with castanospermine; half-maximal effects were observed at ca. 20 μg/ml, whereas maximal inhibition was obtained at 100 μg/ml and higher. In contrast, swainsonine failed to inhibit the secretion of HL activity at all concentrations tested.

TABLE 1. Effect of inhibitors on protein synthesis and glycosylation

	Incorporation of ^{3}H into Protein (% of control)			
	Tunicamycin	Castanospermine	dMM	Swainsonine
Cell-free media				
[^{3}H]Glucosamine	35 ± 1*	48 ± 9*	20 ± 5*	63 ± 3*
[^{3}H]Leucine	103 ± 5	87 ± 14	113 ± 21	107 ± 5
Ratio	0.34	0.55	0.18	0.61
Cell homogenates				
[^{3}H]Glucosamine	23 ± 3*	81 ± 8*	65 ± 2*	66 ± 6*
[^{3}H]Leucine	57 ± 7*	98 ± 19	101 ± 10	98 ± 2
Ratio	0.40	0.83	0.65	0.67

Cells were incubated for 2 h in the absence of inhibitors (control), or in the presence of tunicamycin (2 μg/ml), castanospermine (100 μg/ml), dMM (1 mM), or swainsonine (2 μg/ml). After washing the cells with PBS, the incubation was continued for an additional hour with the same amount of inhibitor but in the presence of either [^{3}H]glucosamine or [^{3}H]leucine (see Methods). Incorporation of radio-label into TCA-precipitable material was determined in cell-free media and in cell homogenates. Data are expressed as percentages of the radioactivity found in control incubations, and are given as means ± SD for three to five experiments. The ratio of [^{3}H]glucosamine to [^{3}H]leucine incorporation into total protein, which is a measure for the selectivity of the inhibitors towards protein glycosylation, was calculated from these percentages. In the controls, [^{3}H]leucine radioactivities measured in TCA precipitates of cell-free media and cell homogenates were 3000 ± 1300 dpm and 7100 ± 2900 dpm, respectively. Corresponding figures for [^{3}H]glucosamine radioactivities were 12400 ± 5800 dpm and 24500 ± 9700 dpm, respectively (means ± SD, n = 5).

*Significantly different from controls ($P<0.05$, by paired t-test).

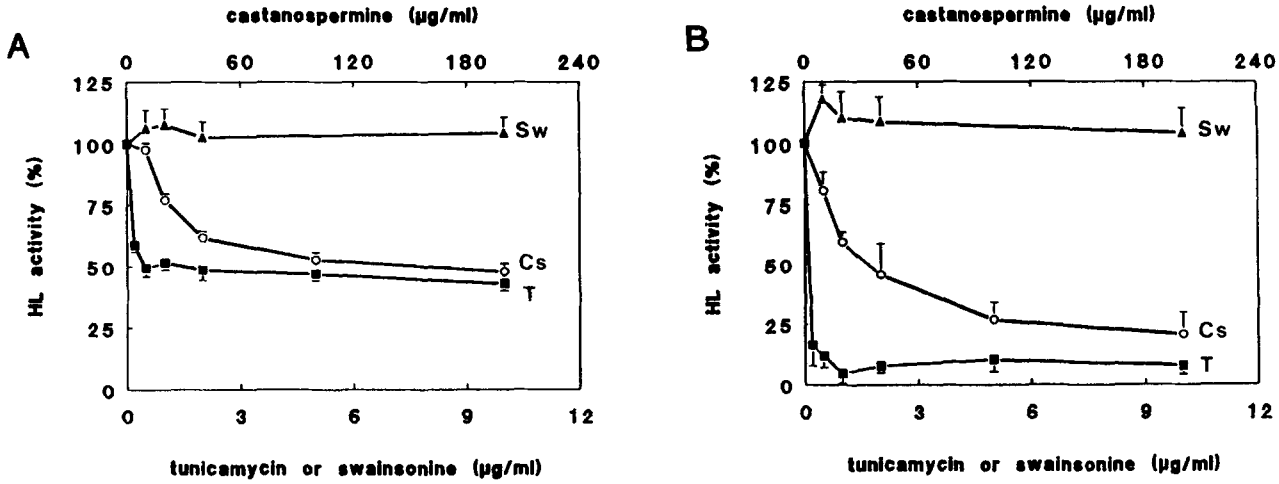

Fig. 1. Effect of tunicamycin, castanospermine, and swainsonine on secretion of HL activity. Hepatocytes were incubated for 2 h with different doses of either tunicamycin (■; T), castanospermine (○; Cs), or swainsonine (▲; Sw), and HL activity was measured in cell-free media (A). The cells were washed once with PBS, incubated for an additional hour in the presence of the same dose of inhibitor, and HL activity in the cell-free media was determined (B). Data are expressed as percentages of enzyme activity found in cell-free medium of suspensions incubated without any inhibitor (9.2 ± 1.6 mU/ml and 3.1 ± 0.8 mU/ml in A and B, respectively; means ± SD, n = 9), and are given as means ± SD for three independent experiments.

Similar dose–response curves were obtained for tunicamycin and castanospermine when cells that had been preincubated for 2 h with inhibitors were incubated for an additional hour in fresh medium containing the same dose of inhibitor (Fig. 1B). The inhibitory effects of both agents were much more pronounced as secretion of enzyme activity was reduced to 10–15% of control values. Even under these conditions, no inhibitory effect of swainsonine was observed on the secretion of HL activity.

The effect of tunicamycin and castanospermine on the time course of HL secretion was studied (**Fig. 2**). With tunicamycin present at 2 μg/ml from the start of the incubation, secretion initially proceeded at control rates for ca. 30 min. Thereafter, secretion rates gradually decreased; secretion was completely abolished from 60 min. A similar time course was observed with castanospermine present at 100 μg/ml. This effect of tunicamycin and castanospermine on the progression of HL secretion was compared with that of cycloheximide, which primarily affects protein synthesis instead of glycosylation. When cycloheximide was added to the incubation at 10 μg/ml, protein de novo synthesis was instantaneously blocked, as illustrated by the abrupt inhibition of [^{3}H]leucine incorporation into total cellular protein (Fig. 2, insert). Despite the complete absence of protein synthesis, secretion of HL activity continued for up to 60 min and followed a similar

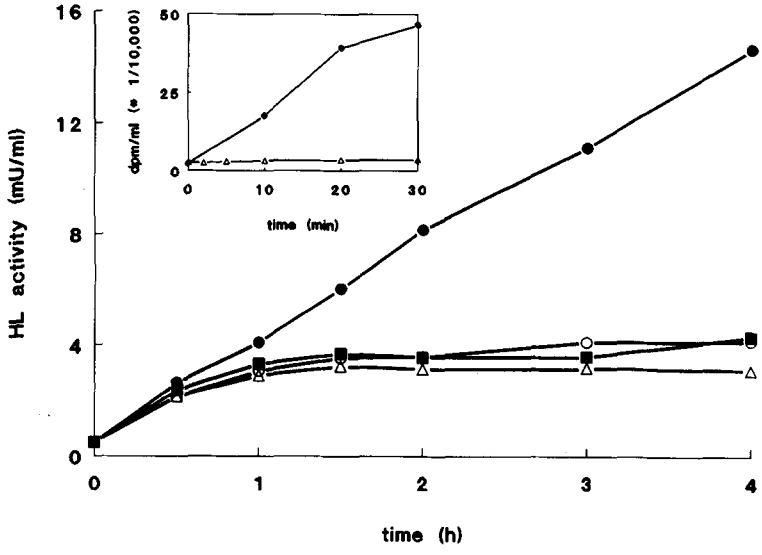

Fig. 2. Time-dependent inhibition of HL secretion by tunicamycin, castanospermine, and cycloheximide. Hepatocytes were incubated without any inhibitor (●), or with 10 μg/ml cycloheximide (△), 2 μg/ml tunicamycin (■), or 100 μg/ml castanospermine (○). The inhibitors were present from the start of the incubation. At the times indicated, samples were withdrawn for analysis of HL activity in the cell-free media. Data are representative of six similar experiments. Control cells secreted enzyme activity at a constant rate of 0.65 ± 0.04 mU/h per mg cell protein (mean ± SD). The inset shows the initial effect of cycloheximide on protein de novo synthesis. To one set of incubations, [^{3}H]leucine (10 μCi/ml) was added simultaneously with cycloheximide (△); to another set only [^{3}H]leucine was added (●). During the first 30 min, samples were collected on ice, and incorporation of ^{3}H into TCA-precipitable material was determined in triplicate.

time course as observed with tunicamycin and castanospermine (Fig. 2). Hence, this HL must already have been present intracellularly at the onset of inhibition, and the time that secretion continued apparently corresponds to the time of intracellular transport. As the time course of secretion observed with tunicamycin and castanospermine was superimposable on that with cycloheximide, these agents probably also interfered almost instantaneously with maturation of newly formed HL but left secretion of HL from a pre-existing pool intact.

Effects on intracellular HL activity

Cells were incubated for 2 h in the absence or presence of inhibitors, and cell homogenates were assayed for intracellular HL activity. In control cells that had been incubated without any inhibitor, 0.53 ± 0.09 mU (mean ± SD, n = 7) of total lipase activity was found per mg cell protein. Approximately 40% of this activity was sensitive to immunoinhibition by polyclonal anti-HL, indicating the presence of about 0.2 mU of HL activity per mg cell protein (**Fig. 3**). In swainsonine-treated cells similar amounts of total lipase activity were detected, 40% of which could be inhibited with anti-HL. Hence, intracellular HL activity was not affected by swainsonine. In contrast, cells treated with tunicamycin, castanospermine, or cycloheximide all contained a total lipase activity that was approximately 60% of that in the control or swainsonine-treated cells, and that was no longer sensitive to immunoinhibition by anti-HL. These data strongly suggest that of all intracellular lipases only HL activity was affected. Apparently, intracellular HL activity was completely abolished upon incubation with these inhibitors. The finding that intracellular HL activity was depleted while secretion of HL activity continued initially after addition of tunicamycin, castanospermine, and cycloheximide (Fig. 2) is in accord with the hypothesis that these agents interfere with the maturation of HL but leave secretion from pre-existing, mature HL intact.

Restoration of HL secretion

In order to investigate whether inhibition of HL secretion by tunicamycin or castanospermine could be restored, cells that had been pre-incubated for 2 h with inhibitor, were incubated for up to 2 h in fresh medium without any inhibitor (**Fig. 4**). At the end of the incubation, hardly any HL activity could be detected in the extracellular medium of tunicamycin-pretreated cells. Hence, the tunicamycin-induced inhibition of HL secretion was not readily reversible under the conditions used. In contrast, considerable HL activity was found extracellularly with castanospermine-pretreated cells. The final amount of HL activity observed in these suspensions was similar to that of control cells that had been pre-incubated in the absence of any inhibitor. However, the time course of HL secretion differed markedly. Control cells secreted HL activity at a constant rate throughout incubation, while the appearance of HL activity in the extracellular medium of castanospermine-pretreated cells was delayed by ca. 30 min (Fig. 4). After this lag-time, secretion proceeded at a rate that was even higher than that found for control cells. The lag-time observed here is in accordance with that observed in Fig. 2 for the inhibition of HL secretion by cycloheximide, tunicamycin, and castanospermine. Therefore, this lag probably corresponds to the time of intracellular transport of HL protein.

Effects of MdN and dMM

Apparently, secretion of enzyme activity not only required the mere presence of N-linked sugar chains on HL protein but also depended on subsequent oligosaccharide

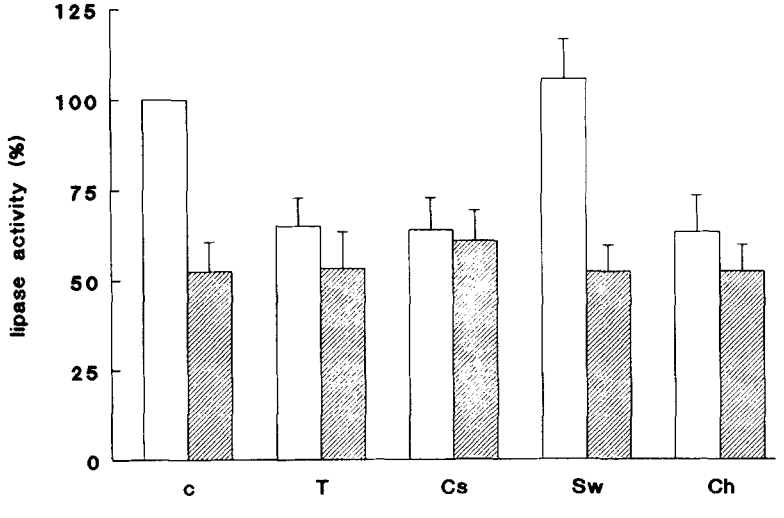

Fig. 3. Effect of inhibitors on intracellular lipase activities. Hepatocytes were incubated for 2 h in the presence of tunicamycin (2 μg/ml; T), castanospermine (100 μg/ml; Cs), swainsonine (2 μg/ml; Sw), or cycloheximide (10 μg/ml; Ch), or in the absence of any inhibitor (c). At the end of the incubation, cell homogenates were prepared and analyzed for lipase activity. Aliquots of cell homogenates were incubated for 1 h at 0°C with either nonimmune goat IgG (open bars) or goat anti-HL IgG (hatched bars) prior to lipase assay. Data are expressed as percentages of total lipase activity present in the control cells (0.53 ± 0.09 mU/mg cell protein, mean ± SD, n = 7), and are given as means ± SD from four to five independent experiments. Total lipase activity in the control cells was not affected by incubation with nonimmune IgGs (98 ± 4%, n = 4). Total lipase activities in T-, Cs-, and CH-treated cells were significantly different from controls and SW-treated cells, whereas the effect of anti-HL was statistically different only for controls and SW-treated cells ($P < 0.05$; by paired Student t-test). Other mutual differences were nonsignificant.

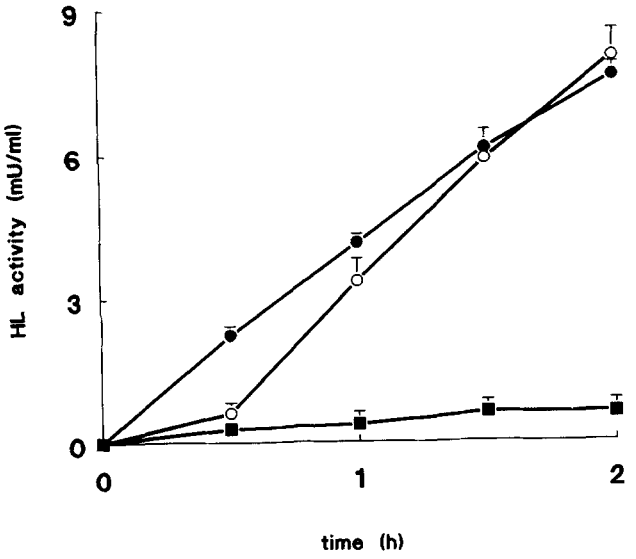

Fig. 4. Recovery from castanospermine inhibition. Hepatocytes were pre-incubated for 90 min in the absence (●) or presence of 100 μg/ml castanospermine (○) or 2 μg/ml tunicamycin (■). Thereafter, the cells were washed once with PBS and then incubated again in the absence of any inhibitor. At the times indicated, samples were withdrawn for analysis of secreted HL activity. Data are given as means ± SD from three to four independent experiments.

processing, as is illustrated by the inhibitory effects observed with castanospermine. To verify this, the effects on secretion of HL activity were also examined for MdN, an alternative glucosidase I (and II) inhibitor with a structure quite distinct from that of castanospermine (28). As shown in **Fig. 5A**, secretion of HL activity was also inhibited by this sugar analogue. In the presence of 1 mM MdN, the extracellular appearance of enzyme activity followed a time course similar to that with cycloheximide present. Moreover, intracellular HL activity was not detectable in MdN-treated cells (data not shown). In contrast, secretion of HL activity was not affected by dMM, which is known to inhibit mannosidase I, a subsequent step in oligosaccharide processing. Fig. 5B shows that after 2 h of pre-incubation, extracellular HL activity was similar whether or not dMM was present at concentrations up to 2 mM. When, after this pre-treatment, the cells were incubated for an additional hour with fresh medium containing the same dose of inhibitor, still no effect of dMM could be observed, despite its pronounced effect on [^{3}H]glucosamine incorporation into total cellular and secreted protein (Table 1). In agreement with this, total lipase activity in cells treated for 2 h with 1 mM dMM was similar to that in control cells, and ca. 40% of this activity was sensitive to inhibition by anti-HL (data not shown). Hence, the structural requirements that permit secretion of active enzyme are already met after the oligosaccharide chains have been trimmed by the glucosidases present in the rough endoplasmic reticulum.

Secretion of HL protein

Secretion of HL protein was studied using an ELISA developed for rat HL. Cells were allowed to secrete preexisting HL during a 2-h pre-incubation with the inhibitors (Fig. 2), and then incubated with fresh medium containing the same dose of inhibitor. Cell-free media were prepared after 2 h of incubation and analyzed for the presence of HL protein. **Fig. 6 (left)** shows that the amount of HL protein secreted by dMM- and swainsonine-treated cells was similar to that secreted by control cells. As HL activities measured in these media were also in the same range, the specific enzyme activity of HL secreted by these cells was very similar. In contrast, only little protein was detectable in suspensions treated with

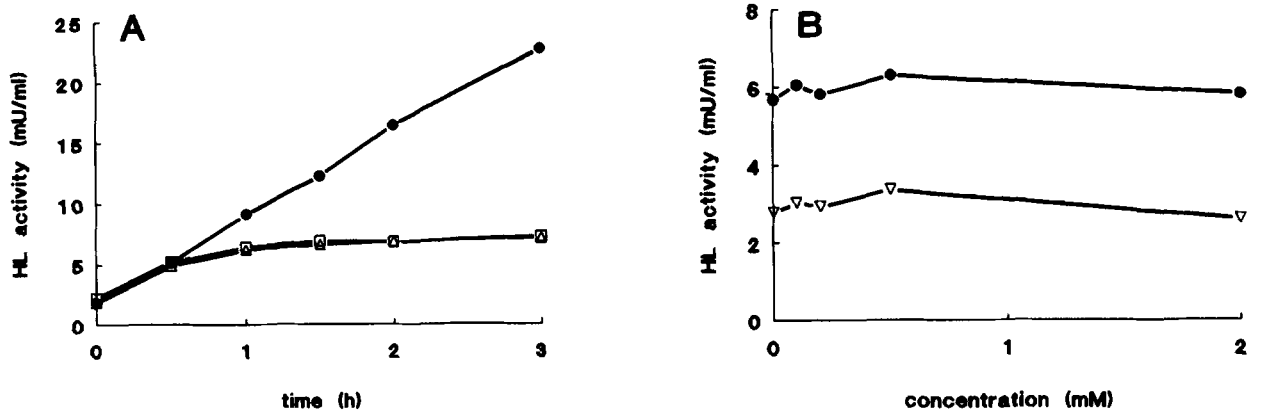

Fig. 5. Effect of MdN (A) and dMM (B) on secretion of HL activity. A: Hepatocytes were incubated without any inhibitor (●), or with 1 mM MdN (□) or 10 μg/ml of cycloheximide (△) present from the start of the incubation. At the times indicated, samples were collected from the incubations, and HL activity was measured in the cell-free media. B: Cells were incubated for 2 h in the presence of different concentrations of dMM, and HL activity was measured in the extracellular medium (●). After this pre-incubation, the cells were washed once in PBS, and incubation was continued for an additional hour in fresh medium containing the same dose of dMM. At the end of this incubation, cell-free media were prepared and analyzed for HL activity (▽). Data in A and B are representative of two similar experiments.

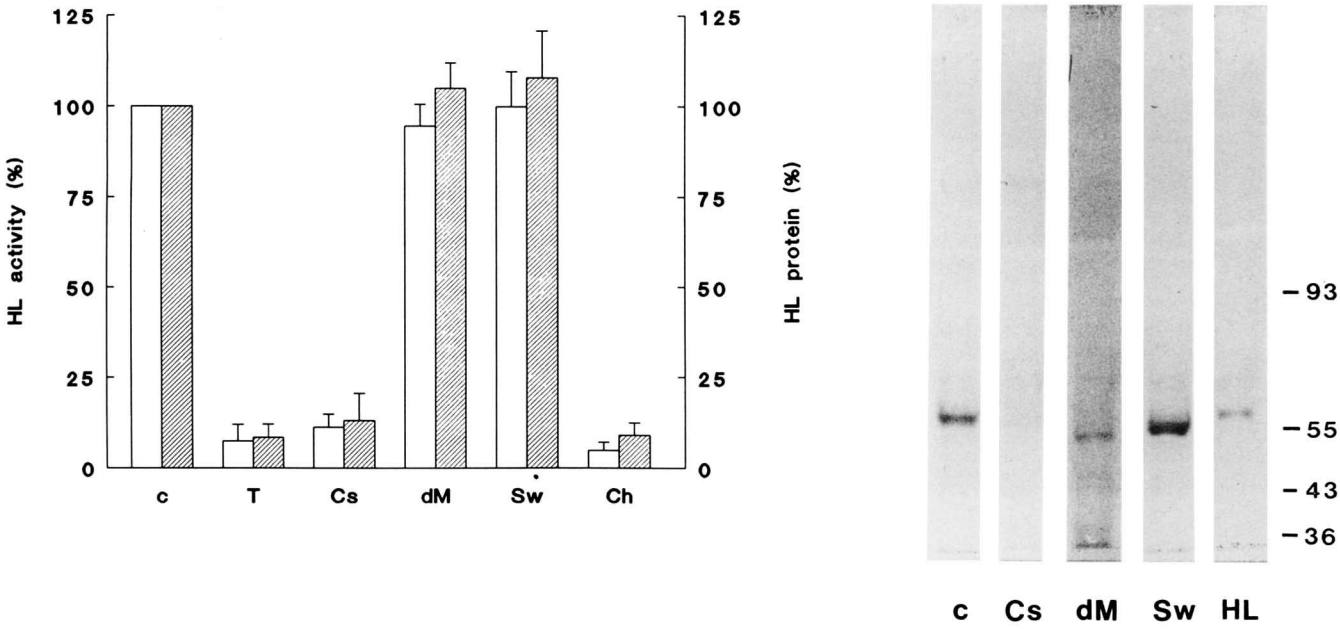

Fig. 6. Effect of inhibitors on secretion of HL protein. Hepatocytes were incubated as detailed for Fig. 1B in the absence (c) or presence of 10 μg/ml cycloheximide (CH), 2 μg/ml tunicamycin (T), 100 μg/ml castanospermine (CS), 1 mM deoxymannojirimycin (dM), or 2 μg/ml swainsonine (Sw). At the end of the incubations, cell-free media were analyzed for HL activity (open bars, left panel) and HL protein was analyzed either by ELISA (hatched bars, left panel) or by Western blotting after immunopurification (right panel). Right panel: HL stands for hepatic lipase partly purified from postheparin liver perfusates by affinity-chromatography on Sepharose-heparin (30). The position of the molecular weight markers is indicated on the right ($\times 10^{-3}$ kDa). Data in the left panel are given as means ± SD (n = 3); data in the right panel are representative of at least three independent experiments.

tunicamycin, castanospermine, or cycloheximide, in agreement with the low enzyme activities found. Hence, secretion of HL protein and enzyme activity were inhibited in parallel by these agents. No evidence was found for secretion of inactive HL in the presence of tunicamycin and castanospermine. Similar results were obtained with MdN (data not shown).

HL protein was also determined in the cell-free media by Western blotting after immunopurification on immobilized goat anti-HL. In agreement with the results obtained in the ELISA, hardly any immunoreactive protein was detectable by this technique in cell-free media prepared from suspensions treated with either castanospermine (Fig. 6 (right), lane 2), cycloheximide, tunicamycin, or MdN (data not shown). In cell-free media prepared from either dMM- or swainsonine-treated cells, however, a single protein was found to cross-react with polyclonal anti-HL (Fig. 6 (right), lanes 3 and 4, respectively). The protein found in the dMM-treated suspensions showed a slightly higher mobility than the protein secreted by the swainsonine-treated cells, having apparent molecular weights of about 53 kDa and 55 kDa, respectively. Both proteins moved slightly faster than the one secreted by control cells (M_r = 58 kDa; Fig. 6 (right), lane 1), whereas the latter co-migrated with HL from postheparin liver perfusates (Fig. 6 (right), lane 5).

DISCUSSION

The present study demonstrates the crucial role of glycosylation and subsequent oligosaccharide processing in the secretion of HL by rat hepatocytes. When glycosylation was entirely prevented by tunicamycin, the secretion of both HL activity and protein was abolished, thus confirming earlier observations (20, 27). In addition, we show here that secretion of HL is also abolished by inhibition of early but not of late oligosaccharide processing. This can be deduced from the fact that secretion was inhibited by castanospermine and MdN, but not by dMM and swainsonine. Assuming similar specificities for these agents in rat hepatocytes as in all other cell models (28, 29), these findings show that HL is secreted only when the protein is glycosylated and its sugar chains have been processed beyond the point of glucosidase action. Hence, the crucial step in oligosaccharide processing that determines whether HL is secreted appears to be the removal of the distal glucose residues from the precursor sugar chain. These structural requirements of HL secretion resemble those for certain other liver-derived, but otherwise unrelated glycoproteins, such as α_1-antitrypsin and α_1-antichymotrypsin (40, 41). Lodish and Kong (40) demonstrated that for this class of glycoproteins the removal of the terminal glucose residues from their oligosaccharides

is required for the efficient movement from the rough endoplasmic reticulum to the Golgi. They postulated that the de-glucosylated oligosaccharide moieties are recognized by a putative receptor that mediates the selective transport of such glycoproteins. Alternatively, removal of the terminal glucoses may allow proper folding of the protein or the formation of aggregates, which may affect its transfer to the Golgi (42). It is noteworthy, that native HL may be a tetramer (30, 43). It is unknown when and where such an HL tetramer is assembled, but oligomeric proteins are usually formed in the endoplasmic reticulum prior to transfer to the Golgi (42, 44).

The data presented here show that the structural requirements that permit secretion of HL are distinct from those reported for guinea pig LPL (21), despite the high degree of homology among their primary structures. Particularly, the complete conservation of the N-glycosylation sites among both lipases derived from various species is highly conspicuous, and suggests an important role for these oligosaccharide chains in the functioning of these proteins (16). Nevertheless, the impact of these sugar chains on secretion differs markedly as LPL secretion by guinea pig adipocytes is not affected by MdN-induced inhibition of trimming glucosidases (21), and unglycosylated LPL is secreted by tunicamycin-treated *ob/ob* adipoblasts (26). These different requirements for the maturation of HL and LPL may be encoded in as yet unrecognized parts of their primary structure, or may be determined by species- or tissue-specific differences. Human LPL bears two N-linked glycans, of which only the one at Asn-43 appears essential for secretion in COS cells (25). Interestingly, the oligosaccharide chain present at this position in guinea pig LPL is of the high-mannose type, which might explain the relative insensitivity to inhibition of oligosaccharide processing (21). In contrast to guinea pig adipocytes, high-mannose LPL is not secreted by brown fat adipocytes from *cld/cld* mice (45), suggesting that processing of the N-linked glycans is essential to secretion of LPL in these cells.

Besides its importance in secretion of HL, protein glycosylation and subsequent oligosaccharide processing may also be necessary for HL to become catalytically active. As no evidence was found for the secretion of any inactive HL protein, this issue could not be addressed directly in the present study. The observation that, initially after addition of tunicamycin or castanospermine, HL activity continued to appear extracellularly while intracellular HL activity was depleted, may indicate that these agents prevented newly synthesized HL protein from becoming catalytically active. Alternatively, newly formed HL bearing no or nonprocessed oligosaccharide side chains may be rapidly degraded. In addition, it cannot be excluded that de novo synthesis of HL protein is suppressed when oligosaccharide coupling and subsequent processing are impaired. Obviously, additional experiments are required to settle this issue. Whether activation of HL protein is directly or indirectly linked to glycosylation and oligosaccharide processing is currently under investigation in our laboratory.

HL secreted by control cells and by cells treated with dMM or swainsonine showed markedly different mobilities upon SDS-polyacrylamide electrophoresis (Fig. 6B). A similar reduction in apparent molecular weight has also been observed with many other glycoproteins upon relatively minor changes in oligosaccharide composition [e.g., (21, 23, 40, 41)]. For HL, the existence of an intracellular precursor form has been reported with an apparent molecular mass of 55.4 kDa (19) or even 53 kDa (20), substantially lower than for secreted, mature HL (57.6 kDa and 59 kDa, respectively). The low-M_r form of HL was fully endo H-sensitive (19), suggesting that its oligosaccharides were of the high-mannose type, which may also be expected of the HL species secreted by dMM-treated cells. The observed differences in apparent M_r between HL secreted by control cells and cells treated with dMM or swainsonine are, therefore, compatible with these HL species differing only in their oligosaccharide structures. Apparently, such a variation in oligosaccharide structure does not affect its secretion nor its catalytic activity. Whether the different oligosaccharide structures affect other properties of HL such as its phospholipase A_1 activity or its interaction with liver endothelial cells remains to be established.

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