Hepatic Lipase:
Regulation at the post-transcriptional level

Lever lipase:
Regulatie op het post-transcriptionele niveau

Proefschrift

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Aan mijn ouders
Aan Gino

CONTENTS

ABBREVIATIONS

AF-1	apolipoprotein factor 1
AP-2	activator protein 2
ALLN	N-acetyl-leucyl-leucyl-norleucinal
ANOVA	analysis of variance
ATP	adenosine triphosphate
BFA	brefeldin A
BiP	immunoglobulin binding protein
8-Br-cAMP	8-bromo-cAMP
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CSP	castanospermine
CCCP	carbonyl cyanide *m*-chlorophenyl hydrazone
C/EBP	CAAT-enhancer binding protein
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate
cld	combined lipase deficiency
dMM	1-deoxymannojirimycin
MdN	N-methyldeoxynojirimycin
mRNA	messenger ribonucleic acid
EDTA	ethylene diamine tetra acetate
EGF	epidermal growth factor
EGTA	ethyleenglycol-bis-(2-amino ethyl)-tetra acetic acid
ELISA	enzyme-linked immunoassay
Endo H	endo-β-*N*-acetylglucosaminidase H
HNF	hepatic nuclear factor
ER	endoplasmic reticulum
GH	growth hormone
HDL	high density lipoprotein
HL	hepatic lipase
IDL	intermediate density lipoprotein
IgG	immunoglobulin
kDa	kiloDalton
LPL	lipoprotein lipase
LDL	low density lipoprotein
MEM	Eagles minimum essential medium
nt	nucleotide
OCT-1	octamer binding protein 1
PAGE	poly-acryl-amide-gel electrophoresis
PBS	phosphate-buffered saline (pH 7.4)
PEDAT	phosphatidylethanolamine:dolichol acyltransferase
PL	phospholipid
PLase	phospholipase activity AI
PMSF	phenylmethanesulphonyl fluoride
RT-PCR	reverse-transcribtase polymerase chain reaction
RER	rough endoplasmic reticulum
SEM	standard error of the mean
SD	standard deviation
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TG	triglyceride
TGase	triglyceridase activity
TH	thyroid hormone
TPCK	L-1-tosylamido-2-phenylethylchloromethyl-ketone
VLDL	very low density lipoprotein

CHAPTER 1

General Introduction

1.1 INTRODUCTION TO HEPATIC LIPASE

Hepatic lipase is synthesized and constitutively secreted by liver parenchymal cells (1-3), and is subsequently bound extracellularly in the space of Disse of the liver (4-6). The protein is also found in adrenals, ovaries and testes (7-10). However, in adrenals and ovaries an altered, shorter mRNA is expressed and no mature HL protein is synthesized (3, 9, 11, 12). Thus, liver parenchymal cells may uniquely synthesize and secrete a fully active HL. HL affects the metabolism of several lipoproteins and is thought to protect against the development of atherosclerosis (Chapter 1.1.1). Therefore it is of importance to understand the mechanisms which regulate HL expression. Several cell types serve as a model system for secretion of HL (13-17). The studies described herein were performed with the human-derived HepG2 cell-line and freshly isolated rat hepatocytes.

1.1.1 ROLE OF HEPATIC LIPASE IN LIPOPROTEIN METABOLISM

Hepatic lipase plays an important role in lipoprotein metabolism. As depicted in figure 1, HL is involved in the metabolism of the high density lipoproteins (HDL), intermediate density lipoproteins (IDL) and chylomicron-remnants (see for review (18-20)). HL hydrolyses phospholipid and triacylglycerol present in high and intermediate density lipoproteins. This was recently confirmed by the changes in lipoproteins concentrations, which occurred after over-expressing of human HL in rabbits (21), mice (22), and in HL-deficient mice (23). The phopholipase A_1-activity of HL stimulates the delivery of cholesterol(esters) from HDL to rat liver cells (24-27). Hence, HL may facilitate the selective uptake of cholesterol by transforming HDL_2 to HDL_3 (28, 29) and /or pre-β_1 HDL (30, 31). Further, HL is involved in the removal of both chylomicron and VLDL-remnant particles by the liver (32-37). HL may delay the development of premature atherosclerosis, as chylomicron remnants are implicated in the progression of coronary artery disease (38). Moreover, elevated HDL levels are reciprocally related to the prevalence of coronary artery disease (39, 40). Hence, by contributing to reverse cholesterol transport, and by the lowering of atherogenic remnant particles in the circulation, HL is thought to protect against the development of premature atherosclerosis. Indeed, post-heparin HL activity was lower in patients with than without stenotic coronary arteries (41). HL activity inversely correlated with progression of coronary atherosclerosis in patients on a lipid-lowering diet (42). In line with this, transgenic mice over expressing HL were shown to accumulate less cholesterol in the aortic wall than non-expressing littermates (22). Further, HL deficiency was shown to correlate with hyperlipidaemia (36, 43-47). The elevated level of plasma triglycerides, associated with HL deficiency (48, 49), is an independent risk factor for coronary heart disease (50). In several human patients, HL deficiency is associated with premature atherosclerosis (45, 51).

1.1.2 ACTIVITY OF HEPATIC LIPASE

HL is involved in the conversion of IDL to LDL (21, 52) and of HDL_2 to HDL_3 and pre-β HDL (28-31). In addition, HL is involved in the removal of both chylomicron and VLDL remnant particles by the liver (32-37). HL may exert its action on lipoprotein metabolism via its enzymatic activity or by acting as a ligand towards lipoproteins. Some studies have shown the involvement of HL activity in chylomicron remnant clearance (33, 53). On the other hand, several studies indicated that

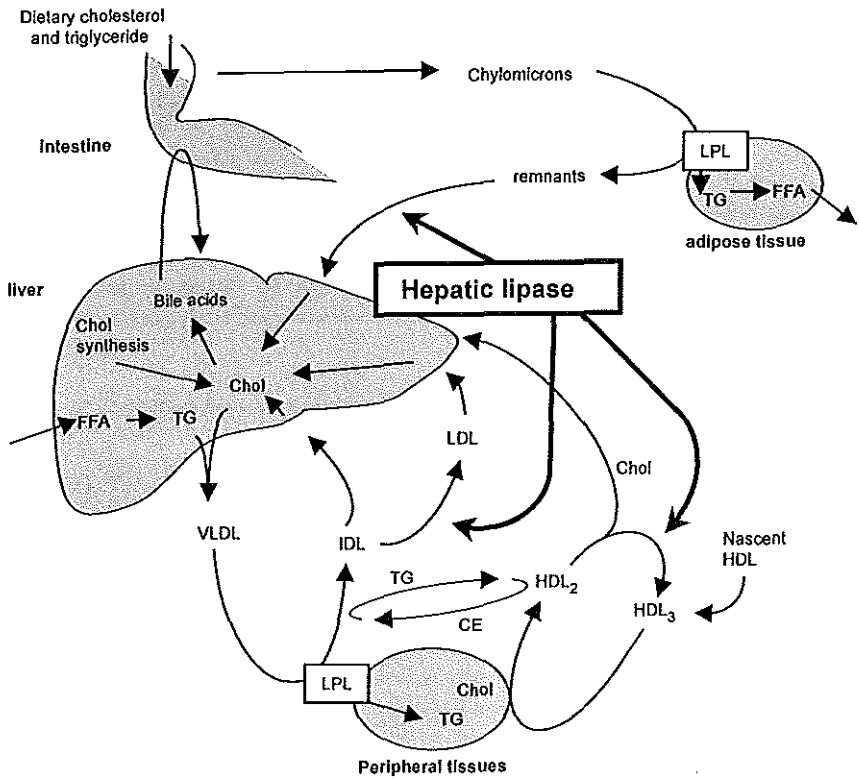

Figure 1 **Role of hepatic lipase in the lipoprotein metabolism.**
The action of hepatic lipase, which is located in the space of Disse in the liver affects endogenous (VLDL) and exogenous (chylomicrons) lipoprotein metabolism. The effect of hepatic lipase on the turnover of HDL and its containing cholesterol is another important physiological action. Of the other proteins involved in the lipoprotein metabolism only lipoprotein lipase (LPL) is indicated. VLDL, Very Low Density Lipoprotein; LDL, Low Density Lipoprotein; IDL, Intermediate Density Lipoprotein; HDL, High Density Lipoprotein; TG, triglycerides; Chol, cholesterol; CE, cholesterolesters; FFA, free fatty acids. (Adapted from Sheperd(J. Shepherd (1994) Drugs 47, suppl.2, 1-10)

the effect of HL on remnant clearance is independent of its activity, and that HL may serve as a binding site for lipoproteins (34, 35, 54-57).

Active HL hydrolyses triacylglycerol and phospholipids. The triglyceridase (TGase) activity of HL is insensitive to high salt concentrations, which enables discrimination between LPL and HL TGase activity (1). The enzyme activity of HL has a pH optimum of 8.0-8.5. Activity of HL may be measured by a triacylglycerol hydrolase assay at pH 8.5 in 0.6 M NaCl using a gum acacia-stabilized, glycerol([^{3}H] or [^{14}C])trioleate emulsion as substrate (1). For HL activity in a liver cell-homogenate this assay must be performed with care, as not all activity could be inhibited with antibodies to HL, and liver cell homogenates may contain an inhibitor of the TGase activity (58). In vivo, the TGase activity of HL may mainly hydrolyse TG present in the HDL_2 subfraction (18, 59, 60). The phospholipase A_1 (PLase) activity of HL is mainly directed towards phosphatidylethanolamine and phosphatidylcholine present in HDL-particles (28, 61). Furthermore,

the PLase activity of HL may facilitate the selective uptake of cholesterol(esters) from HDL in the liver (26, 27).

The TGase and PLase activity of HL are thought to be independent of any co-factor. This is unlike other members of the lipase family, which depend on cofactors for activity. Pancreatic lipase is active only in the presence of colipase (62-64), and LPL is activated by apolipoprotein CII (65, 66). Although no cofactor is identified for HL activity, several apolipoproteins (Apo) were shown to affect the enzyme activity of HL in vitro. Apolipoproteins CI, CII, CIII, AI and AII inhibit hydrolysis of triglycerides and phospholipids by HL (67, 68). The effects of Apo AI and AII on HL activity are ambiguous. In vitro, Apo AII could also stimulate HL activity (69). Hime et al (70) presented data, which showed that Apo AI stimulated the V_{max} for TG and PL hydrolysis by HL, and that Apo AII stimulated the affinity of HL towards TG and PL. Another apolipoprotein, which has been shown to affect HL is Apo E. This apolipoprotein stimulates mainly the HL-mediated hydrolysis of phophatidylcholine present in Apo E-rich HDL phospholipids (68, 71). Stimulation of HL activity by Apo E depends on low surface pressure and may involve adhesion of HL to the water-emulsion interface, and subsequent activation of HL (71).

Recently, HL has been shown to possess phosphatidylethanolamine:dolichol acyltransferase (PEDAT) activity (72). In contrast to the TGase and PLase activity of HL, the PEDAT-activity is dependent on a plasma co-factor. This co-factor has been shown to be apolipoprotein AIV (73). Apo AIV had marked effects on the properties of HL enzyme activity. Not only did it change the pH optimum for PEDAT-activity towards a more physiological one (pH 8 to 7.5), the substrate specificity of HL shifted from both TGase and PLase towards merely PLase activity. The presence of all the above described apolipoproteins may affect the actual HL activity in vivo. Whether the presence of Apo AIV on lipoprotein substrates will shift HL activity towards phospholipids as the major physiological substrate of HL remains an important question.

1.1.3 STRUCTURE-FUNCTION RELATIONSHIP OF HL PROTEIN

HL is a member of the lipase super family, which includes pancreatic(-like) lipases, lipoprotein lipase and some drosophila yolk proteins (74, 75). The genes of human HL and LPL have similar intron/exon organization for the different domains of the protein. The nine exons of HL code for a protein of 477 amino acids (76). Sequence analysis (77) showed that Exon 1 encodes a signal peptide targeting newly synthesized protein to the ER (22 amino acids). Exon 4 codes for a region with binding properties to lipoproteins. Exon 5 is an evolutionary highly conserved region, which may be of importance for the catalytic function (74). Furthermore, exon 5 codes for Asp^{176}, which together with Ser^{147} and His^{263} form the catalytic triad. The catalytic triad is highly conserved, and is similar to the triad of serine proteases (78, 79). Exons 6 and 9 encode sequences rich in basic amino acids, which are thought to be important for heparin binding (77). Further, exon 6 codes for the surface loop covering the catalytic triad. The amino-acid residues of this covering lid are less conserved in the lipase family (75).The entire amino acid sequence of human and rat HL contains several putative N-glycosylation sites (four and two, respectively). The similar genetic organization and the strong amino acid sequence homology within the lipase superfamily indicate a common

folding pattern.

The three-dimensional structure of pancreatic lipase, as determined by X-ray crystallography (80) has been used as a model for the three-dimensional structure of LPL and HL (81, 82). This model (Fig. 2) shows that HL consists of a C- and a N-terminal domain, which are connected by a hinge region. The N-terminal domain contains the catalytic triad, which is covered by a surface loop ('the lid').

Numerous chimeras have been constructed with the different domains of HL and LPL to define the relationship between structural features and functionality. Exchange of the C-terminal domains suggests that this domain influences the substrate and heparin binding (83-85). The N-terminal domain mainly influences the kinetic parameters of the enzymes. The lid, which covers the catalytic triad in the N-domain may specify the substrate specificity (86, 87). The N-domain of LPL and C-domain of pancreatic lipase bind a cofactor (apoCII and colipase, respectively) (85, 88), which activates the enzymes (62-66, 84). So far, no cofactors have been established for HL, although several apolipoproteins were shown to stimulate HL activity (see Chapter 1.1.2).

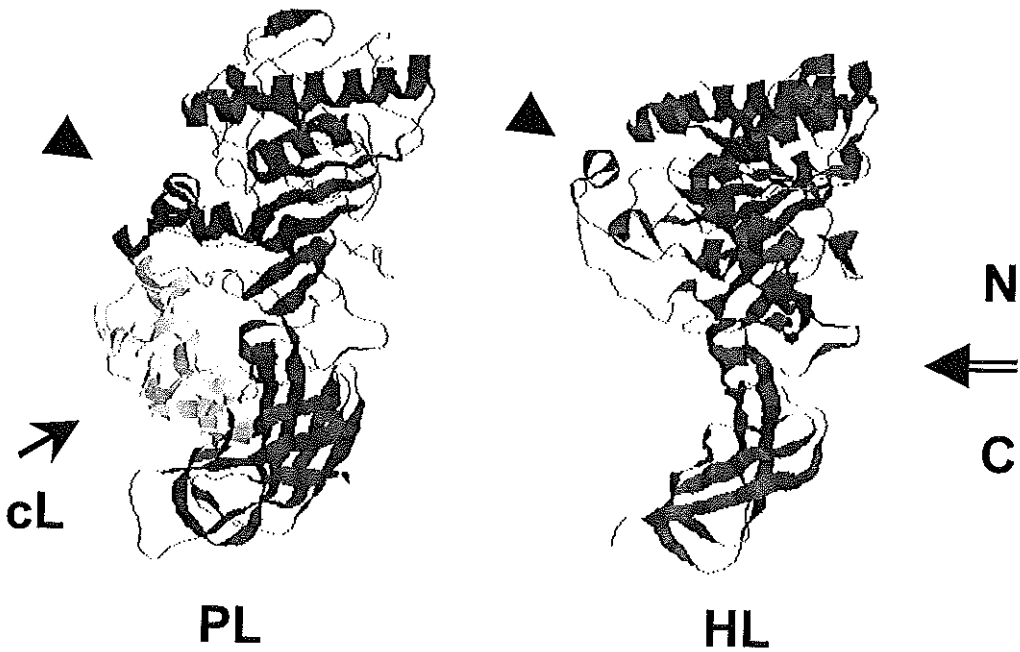

Figure 2 **Structure of human hepatic lipase.**

The structure of human pancreatic lipase (PL) with bound colipase (cL) (van Tilbeurgh, H., Sarda, L., Verger, R., Cambillau, C. (1992) Nature, 359, 159-162 ; Winkler, F.K., A, D.A. and Hunziker, W. (1990) Nature, 343, 771-774) was retrieved from the 3D protein database on the Internet (Brookhaven protein databank, 21LPA.PDB). The structure of hepatic lipase (HL) was deduced from the crystalline structure of PL (Derewenda, Z.S. and Cambillau, C. (1991) J. Biol. Chem., 266, 23112-23119), and was also retrieved from the Internet (Swiss model repository, LIPH_Human). The double arrow indicates the hinge region between the N-terminus (N) and C-terminus (C). The lid-structure (arrow head) of PL is in 'open' conformation due to the presence of colipase and micelles, whereas the lid of HL covers the catalytic crypt.

The amino acid sequence of human and rat HL contains four and two putative glycosylation sites, respectively. The relative dimensions of the four N-glycan chains to HL is shown in the human model for HL (Fig. 3). The physiological relevance of N-glycosylation for the function of HL is discussed in more detail in chapter 1.1.4.

1.1.4 N-GLYCOSYLATION OF HEPATIC LIPASE

Introduction to protein glycosylation

N-Glycosylation of proteins occurs co-translationally in the ER via a complex sequence of events. Dolichol-containing glycolipids donate the initial oligosaccharide chain (Glc_3-Man_9-$GlcNac_2$) to the asparagine residue in the consensus sequence (Asn-X-Ser/Thr) (Fig. 4). The N-linked glycan chain is sequentially modulated by a variety of enzymes in the ER and Golgi compartment (89).

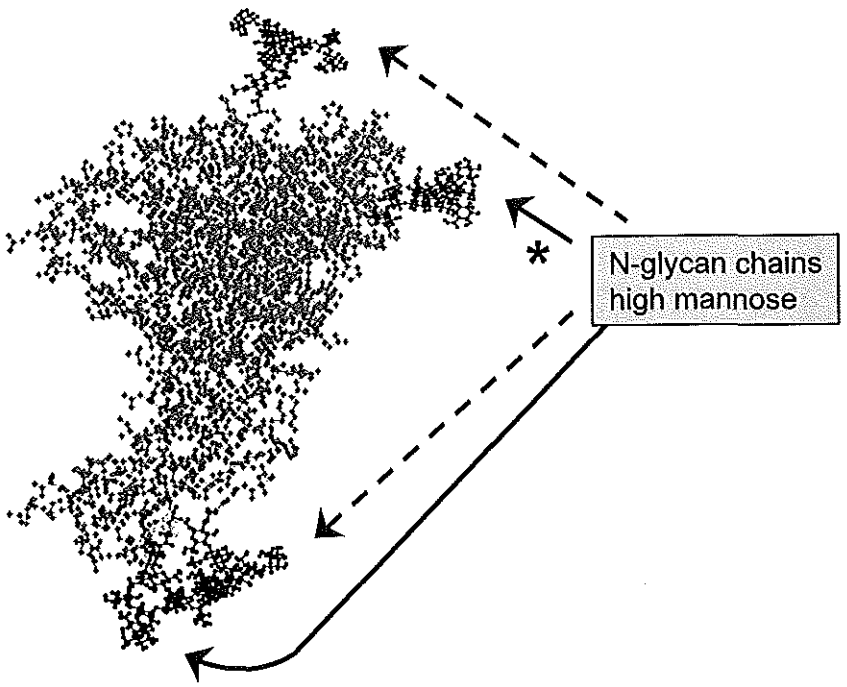

Figure 3 **Impression of oligosaccharide chains on human hepatic lipase.**

To visualize the relative dimensions of glycosylation-chains on hepatic lipase (HL), an impression of glycosylated HL was constructed. The structure of human HL was deduced from the crystalline structure of pancreatic lipase (Derewenda, Z.S. and Cambillau, C. (1991) J. Biol. Chem., 266, 23112-23119) and was retrieved from the Internet. The end of the C-terminus and N-terminus, including Asn 20, is excluded from this model, because the difficult prediction of loose peptide structures. The high mannose oligosaccharide was obtained from the structural data of the glycosylated adhesion domain of human CD2 (Brookhaven protein databank, 1GYA). The in vivo glycosylated Asn-residues were localized in the HL structure, and the high mannose oligosaccharides were placed arbitrarily near the Asn residues. The asterisk marks the glycosylation site Asn 57, which is essential for secretion of active HL. The solid arrows point to glycosylation sites preserved in HL and LPL of different species, and the broken arrows point to the additional glycosylation sites of human HL.

The oligosaccharides are divided into high-mannose, hybrid and complex-type chains (Fig. 5). The enzyme endo-*β-N*-acetylglucosaminidase H (Endo H) has proven to be useful in monitoring the state of glycosylation. Endo H cleaves high-mannose oligosaccharides from a protein, but is inactive towards complex oligosaccharide chains. Specific inhibitors of the different steps during oligosaccharide remodelling (Fig. 4) have permitted research on the role of glycosylation in intracellular protein traffic and on the function of the various glycan chains (90, 91).
The majority of secreted proteins in mammals are N-glycosylated (92). The addition and modulation of the oligosaccharide chains is cell-type and protein specific (93, 94). Glycan chains can modulate the intrinsic biological/biochemical properties of proteins (92, 94, 95). For example, the negative charge of the sialic acid residues increases the solubility, and may affect the adhesive properties of the glycoprotein. Decreased galactosylation of IgG is related to human diseases, like rheumatoid arthritis (93). In addition, desialysation of glycoproteins may lead to internalisation via the asialoglycoprotein receptor, and subsequent degradation of the protein (96, 97).

Intracellularly, N-glycosylation can affect maturation and secretion of proteins. This was clearly shown for the different glycosylated forms of α_1-acid glycoprotein, which are secreted with different rates (98, 99). Glycosylation may assist the initial folding of proteins, and subsequently may stabilize their conformation. Although generally glycosylation and folding reactions are co-operative, they can also compete in vivo (100).

The oligosaccharide chain is an important factor in binding of glycoproteins to intracellular chaperones. In the absence of N-glycosylation the influenza virus hemagglutinin associates with the chaperone BiP, which retains the protein in the ER (101, 102). When glycosylated, the hemagglutinin associates with calnexin, which may release the fully folded protein after removal of the last glucose residue of the oligosaccharide chain (see (2) in figure 4) (103). Calnexin and calreticulin bind the mono-glucosylated glycan chains, and retain glycoproteins in the ER until they are correctly folded (103-105). A wide variety of glycoproteins associate with calnexin and/or calreticulin (104), including the proteins α_1-antitrypsin and α_1-antichymotrypsin. For these proteins it was shown that proper glucose trimming is important for secretion by HepG2 cells (106). In HepG2 cells several secretory glycoproteins have different half times of interaction with calnexin (107). Other factors than glycan chain binding, like ATP- or calcium-binding may affect the association of glycoproteins with calnexin (108).

N-Glycosylation may direct glycoproteins to different localizations. Phosphorylation of a mannose residue in the oligosaccharide of a protein in the Golgi causes lysosomal targeting (109). Furthermore, N-glycosylation may act as an apical sorting signal, as demonstrated for growth hormone. When non-glycosylated the growth hormone was secreted from both sides of Madin-Darby canine kidney cell layers, but when glycosylated it was secreted mainly from the apical site (110).

Glycosylation of HL

HL carries two (rat) to four (human) oligosaccharide chains (9, 111-113). Mature rat HL has an apparent molecular weight of 58 kDa, and bears two complex-type oligosaccharide chains (9, 16, 17, 111). Intracellularly, an additional HL-form with an apparent molecular weight of 53 kDa resides, which is Endo H sensitive and carries high-mannose type chains (9, 16, 17, 111). The

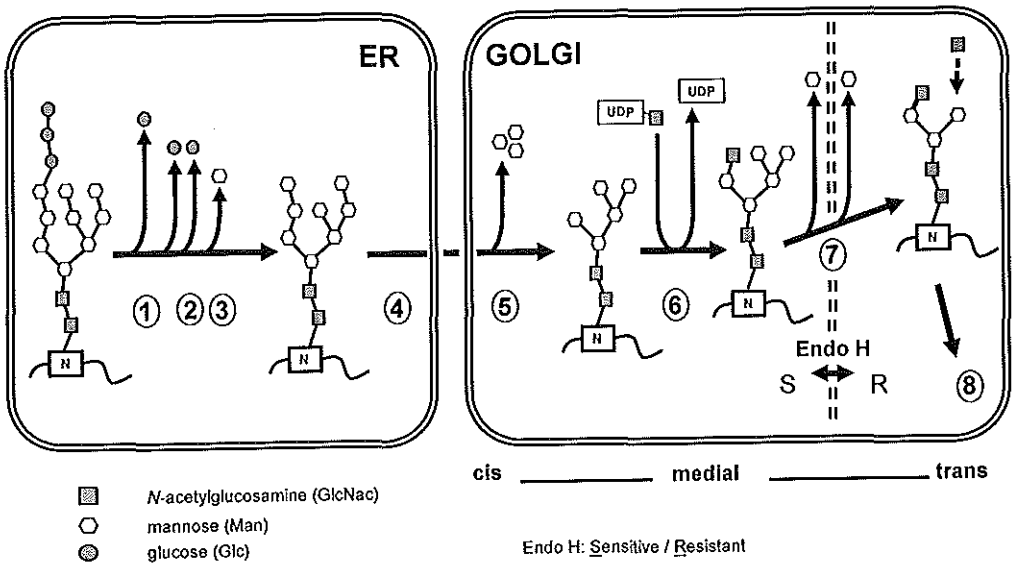

Figure 4 **Oligosaccharide processing in the ER and Golgi.**

Processing of the glycan chains is a complex sequence of events. Initially the oligosaccharide is donated to the protein by a transferase. When cells are incubated with tunicamycin glycosylation is blocked at this stage. The first step of oligosaccharide processing is trimming of the glucose residues by glucosidase I (1) and glucosidase II (2). N-methyldeoxynojirimycin (MdN) and castanospermine are frequently used to inhibit these glucosidases. After the glucose residues are removed, several of the mannose residues are cleaved off. The first mannose is removed by the mannosidase located in the ER (3), which is inhibited by 1-deoxymannojirimycin (dMM). Before the next mannose is cleaved off the protein is transported to the cis-Golgi stacks (4). Transport of the protein depends on correct folding, and may be inhibited by association of the protein with calnexin. The assembly of the transport vesicle may also regulate the transport of glycoproteins. In the Golgi, three of the protruding mannoses are cleaved off from the oligosaccharide chain by Golgi mannosidase I, which is also sensitive to dMM. Then, a GlcNac residue may be added to the oligosaccharide by N-acetylglucosamine transferase I (6). Thereafter the oligosaccharide is sensitive to cleavage of two additional mannoses by mannosidase II (7). Swainsonine is known to inhibit Golgi mannosidase II. These processes are common for all glycoproteins. Frequently, the oligosaccharide is further processed (8) to a complex type chain (Fig. 5). Vesicular transport of proteins occurs from ER to Golgi (4), between cis, medial and trans Golgi-stacks and towards the cell membrane. This transport can be blocked by CCCP, monensin and colchicin, respectively. When cells are incubated with brefeldin A the anterograde vesicle transport between ER and Golgi is selectively inhibited, and the ER and Golgi compartments fuse. (Adapted from Molecular biology of the Cell (Third edition, Chapter 13; Alberts, B., Bray, Lewis J., Raff, M., Robberts, K., Watson, J.D.), Elbein (Elbein, A.D. (1991) Faseb J., 5, 3055-3063) and Fuhrmann et al (Fuhrmann, U., Bause, E. and Ploegh, H. (1985) Biochim. Biophys. Acta, 825, 95-110))

nonglycosylated HL protein has an apparent molecular weight of 47 kDa (17, 111, 114). The two glycosylation sites found in rat (Asn 56/57 and Asn 375/378) are preserved in HL and LPL of different species (75). The dimension of high-mannose glycan chains relative to the structure of HL is shown in the human model for HL (Fig. 3).

Glycosylation affects protein folding and may therefore alter the enzyme activity of HL. Only a few glycoproteins are known to depend on glycosylation for their biological activity (95). Over-expression of the α-subunit of human chorionic gonadotropin results in over-glycosylation, and prevents proper dimerization with the β-subunit (115). Thus, over-glycosylation prevented formation of active heterodimers. Moreover, LPL depends on glycosylation for formation of an active dimer (116-118). Enzymatically active human HL, secreted by stably transfected Chinese

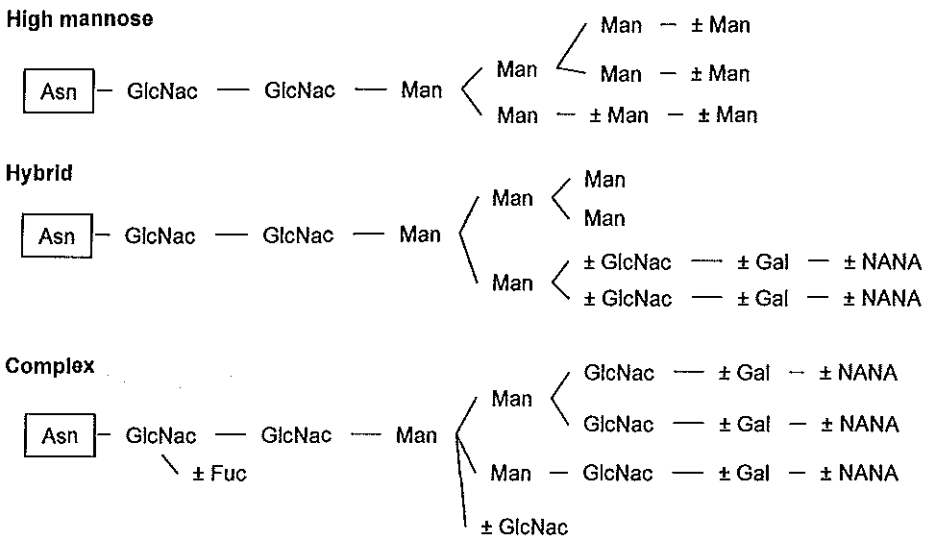

Figure 5 **Structure of different glycan chains.**

Depicted are the main classes of N-linked oligosaccharides found attached to mammalian glycoproteins. High-mannose oligosaccharides gain no new sugars in addition to the original oligosaccharide, while hybrid and complex oligosaccharides often obtain more N-acetylglucosamine, and galactose, sialic acid and sometimes fucose residues. Asn: Asparagine; GlcNac: N-acetylglucosamine; Gal:galactose; NANA: N-acetylneuraminic acid (sialic acid); Fuc: fucose. (Adapted from Kornfeld (Kornfeld, R. and Kornfeld, S. (1985) Annu. Rev. Biochem., 54, 631-664))

hamster ovary cells is in the dimeric form (119). In addition, gel-filtration experiments yielded, in some cases, a dimeric form of human and rat HL (120-122). For rat HL, however, glycosylation-dependent dimerization may be of no importance as its active unit is a monomer (123). However, dimerization of HL might be of importance for the HL-mediated removal of remnant particles from the circulation. In this process, HL acts as a ligand and catalytic enzyme activity is not required (Chapter 1.1.2).

In addition to folding, HL function may be affected by differences in complexity of glycosylation. Complex-type glycan chains can contain several sialic acid residues (Fig. 5), which add negative charge to the protein. Binding of HL to the extracellular matrix is an non-covalent interaction, which is disrupted by heparin, polyanions and 0.3 M salt (124) and, therefore, may be affected by negatively charged sialic acids.

N-Glycosylation of HL transfected in Xenopus laevis oocytes affected the secretion, but not the activation of HL (114). However, other studies have shown that N-glycosylation is required for secretion of a fully active lipase (14, 112, 113, 125). Occupation of only the Asn 56/57 with an oligosaccharide is sufficient for secretion of active HL or LPL (112, 126, 127). Furthermore, proper processing of the oligosaccharide in the ER is required for both HL and LPL to become catalytically active (58, 125, 128-130). As described in the introduction to protein glycosylation, maturation of

proteins may depend on glycosylation-mediated association with chaperones. Hence, glycosylation affects the intracellular trafficking of HL, which might be mediated by association with chaperones such as calnexin.

The importance of the intracellular pathway is demonstrated in the mice syndrome *cld/cld* (131, 132). *Cld/cld* is characterised by a combined deficiency of HL and LPL expression, whereas no mutations in either genes of the *cld/cld* mice occur. Further, no mutations in the cDNA of HL could be identified (133). Several reports showed that both lipase genes are transcribed into mRNA and that there is synthesis of HL protein in liver, and of LPL protein in extra hepatic tissues. However, LPL and HL remained intracellularly, mainly in the rough ER (130, 134, 135). In addition, the inactive LPL in adipocytes of *cld/cld* mice acquires activity when the ER and Golgi fuse upon incubation of the cells with brefeldin A (136). This suggests that there is impaired transport or maturation of the proteins.

1.2 REGULATION OF HEPATIC LIPASE EXPRESSION

Expression of HL is under hormonal and dietary control. Several hormones are known to decrease the HL activity. HL is lower in woman than in man, probably due to estrogen. In postmenopausal women, estrogen-supplementation decreases the expression of HL activity (29). In rats, HL activity is reduced moderately by estradiol (137-139). Further, glucocorticoids and catecholamines have been shown to lower the expression of HL activity in rats (140-142). ACTH lowers the expression of HL activity both in rat and human liver (124, 143, 144). Among the positive modulators of HL expression are androgenic steroids, which have been shown to increase the HL activity in humans (145-148). Thyroid hormone increases HL expression in the human derived HepG2 cells (149), whereas in hypothyroid rats HL is down regulated (150). Insulin, EGF and growth hormone have been shown to induce activity of HL in rats (138, 150-152).

HL is constitutively secreted by liver parenchymal cells. Regulation of HL expression may occur at several levels, which are depicted in figure 6. First, the transcription of the HL gene into mRNA may be rate limiting for synthesis of HL protein. Second, the stability of HL mRNA, and the translation velocity may determine the amount of HL protein synthesized. Next, the intracellular processing of HL, including glycosylation, folding and activation of HL protein, may affect the secretion of active HL. Further, regulated extracellular binding of HL may be a mechanism to control HL activity in the liver. Last, the uptake and subsequent recycling or degradation of HL may determine the expression of HL activity.

Transcriptional regulation of hepatic lipase expression

Part of the hormonal and dietary regulation of HL expression in human and rat liver is at the level of transcription. The C3P and C/EBP, HNF-4 and HNF-1 responsive elements identified in the human and/or rat HL promoter region may account for the liver specific transcription of HL (153-156). Several negative regulatory elements are present in the upstream regulatory region and first exon of the human HL gene (153, 155, 156). HL expression may be modulated by the cholesterol homeostasis of hepatocytes: The rat HL gene contains a putative sterol responsive element in its

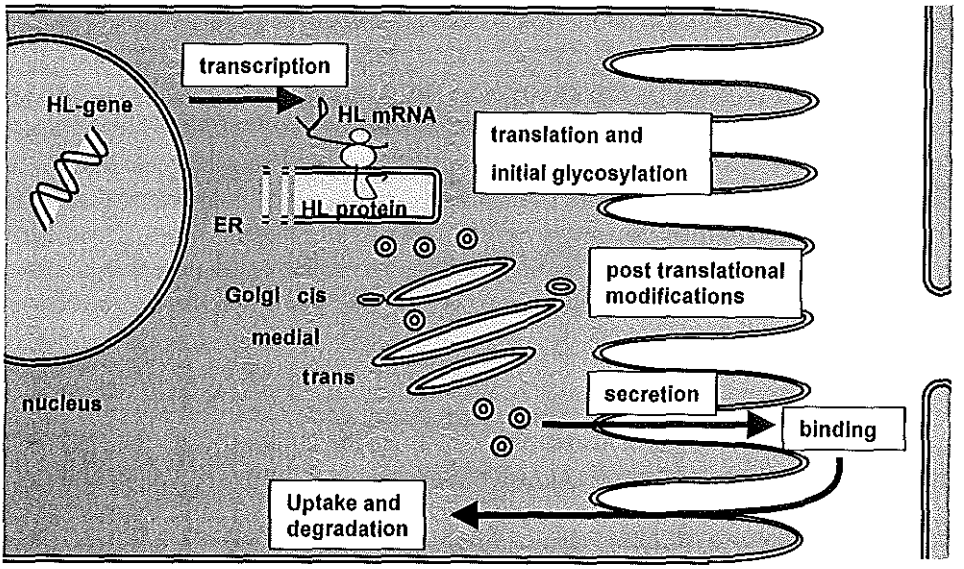

Figure 6 **Potentially regulated steps in hepatic lipase expression.**

Schematic representation of processes, which may determine the expression of HL activity in the liver (more details in text of chapter 1.2). Depicted are the synthesis and secretion of active HL in parenchymal liver cells, the adjacent space of Disse and the sinusoidal endothelial cell layer.

promoter region (154). In addition, cholesterol/cholate fed rats have decreased HL mRNA levels (157, 158). Mevinolin-treatment of HepG2 cells, and thus inhibiting the cholesterol biosynthesis of these cells, increases HL mRNA and activity (159). Further, inhibition of HMG-CoA reductase in cultured rat hepatocytes increased HL activity significantly (160). Modulation of HL expression by cholesterol homeostasis might be a kind of feed back mechanism, as HL plays a role in the reversed transport of cholesterol to the liver.

The 5' flanking region of rat HL gene contains putative elements for AF-1 (apolipoprotein factor 1), cAMP and AP-2, OCT-1, C/EBP, sterol, estrogen, glucocorticoid and thyroid hormone receptors (154). In line with this, glucocorticoids, estrogens, vasopressin, and thyroid hormone are shown to decrease the transcription of the HL gene in rats (138, 140, 150, 161). However, not all changes in HL activity are parallelled by expression of HL mRNA levels. Hence, post-transcriptional regulation may also determine the HL expression in the liver.

Post-transcriptional regulation of hepatic lipase expression

Post-transcriptional regulation is suggested by the short term effects of insulin and catecholamines on HL activity (138, 141, 142). In addition, substitution treatment of orchidectomized rats with sex steroids leads to a decrease in HL activity, while the HL mRNA is increased (139). Growth hormone stimulates the synthesis of HL mRNA in hypothyroid rats, but the total HL activity in the liver remains decreased (150). In fenofibrate-treated rats the extracellular HL activity in the liver seems to follow the decrease in HL mRNA levels. Intracellularly, however, HL activity increased, suggesting fenofibrate may have post-translational effects in addition to the inhibition of

transcription (162). Mevalonic acid decreases the secretion of HL by $HepG_2$ cells, but has no effect on HL mRNA levels. In this case, isoprenylation of a regulatory protein has been hypothesized to mediate the reduction of HL secretion (159). Further, TNF-α production by Kupffer-cells in mice decreases the HL activity significantly with no alterations of mRNA levels (163). These conditions, in which changes in HL activity are not parallelled by levels of mRNA demonstrate that HL expression in vivo is also regulated on a post-transcriptional level.

Figure 6 shows the multiple sites for post-transcriptional regulation of HL expression. For some regulators the nature of post-transcriptional effects is defined. Fenofibrate may affect the intracellular processing and/or secretion of HL (162). The heparin-induced secretion of HL activity by liver hepatocytes is mediated by a decreased internalization of HL, and subsequent lower degradation (16). The corticotrophin-induced decrease of HL expression is due to a reduction of HL binding sites in the liver (124, 144). The mechanisms by which other post-transcriptional modulators affect HL expression remain to be investigated.

1.3 SCOPE OF THIS THESIS

HL affects lipoprotein and cholesterol metabolism (Chapter 1.1.1). HL may be an important factor directing cholesterol transport to the liver. When HL activity in liver is low and up regulated in the adrenals and reproductive organs, HL may direct transport to these cholesterol-utilizing organs (7). Expression of HL activity is regulated at a transcriptional and post-transcriptional level (Chapter 1.2). Post-transcriptional regulation can cause rapid changes of HL activity, which may facilitate redirection of cholesterol-fluxes. Further, post-transcriptional regulation of HL activity may be important in the post-prandial state, when remnants and dietary cholesterol in the plasma are rapidly elevated. Our objective was to study the post-transcriptional regulation more closely. We studied HL expression in rat hepatocytes and in human-derived HepG2 cells under conditions where regulation at the post-transcriptional level had been implicated. We hypothesized that in these conditions N-glycosylation and oligosaccharide processing (Chapter 1.1.4) play a role in regulating HL expression.

Post-transcriptional regulation

Our first approach was to study the effect of catecholamines on HL activity. Rat hepatocytes treated with adrenaline secrete less HL than cells in which the protein de novo synthesis is blocked with cycloheximide. This observation led to the hypothesis that at least part of the inhibitory effect was at the post-translational level. In chapter 2 of this thesis experiments are described in which we studied in detail the mechanism by which adrenaline decreases the HL activity.

Second, we focussed on the discrepancy between mRNA levels and HL activity in hypothyroid rats supplemented with growth hormone. Growth hormone-supplementation of hypothyroid rats results in normalization of HL mRNA, but the HL activity in the liver is not restored, which implicates post-transcriptional regulation. The results of this study are described in chapter 3.

Third, we studied the effects of fenofibrate treatment of rats on the secretion and maturation

of HL. The non-heparin releasable HL pool in the liver after fenofibrate treatment was elevated, whereas mRNA levels and HL activity in the total liver was decreased. These findings suggested that fenofibrate had post-transcriptional effects on HL secretion. We examined this hypothesis in chapter 4.

N-linked glycosylation of HL

Secretion of a catalytically active HL requires N-linked glycosylation. Besides glycosylation, proper processing of the glycan chains, in particular trimming of the terminal glucose residues in the ER appears to be crucial for gaining activity. We examined the role of N-linked glycosylation in the secretion and activation of human HL in HepG2 cells (Chapter 5.1) and of rat HL in suspensions of freshly isolated hepatocytes (Chapter 5.2).

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CHAPTER 2

(Post)translational regulation of hepatic lipase expression by adrenaline

CHAPTER 2.1

Acute effects of adrenaline on hepatic lipase secretion by rat hepatocytes.

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2.1 ACUTE EFFECTS OF ADRENALINE ON HEPATIC LIPASE SECRETION BY RAT HEPATOCYTES.

ABSTRACT

Catecholamines are responsible for the daily changes in hepatic lipase (HL) expression associated with feeding and fasting. We have studied the mechanism by which adrenaline decreases HL secretion in suspensions of freshly isolated rat hepatocytes. Adrenaline acutely inhibited HL activity through activation of the α_1-adrenergic pathway. The cells had significantly less HL activity in the presence of adrenaline versus cycloheximide, where protein de novo synthesis is completely blocked. The specific enzyme activity of secreted HL was not affected. Intracellular HL activity was decreased by adrenaline-treatment. Pulse-labelling with [^{35}S]methionine showed that the de novo synthesis of the 53 kDa Endo H-sensitive HL protein was unaffected by adrenaline. During subsequent chase of the control cells, the 53 kDa form was converted to a 58 kDa Endo H-resistant HL protein, which was rapidly secreted into the medium. In the presence of adrenaline, formation of the 58 kDa protein was markedly reduced whereas the 53 kDa protein disappeared at a rate similar to the rate of controls. This suggests that part of the HL protein was degraded. In contrast to adrenaline, inhibition of HL secretion by colchicine was accompanied by an intracellular accumulation of HL activity and of the 58 kDa protein. We conclude that adrenaline inhibits HL secretion posttranslationally by retarding the maturation of the 53 kDa HL precursor into an active 58 kDa protein, possibly by stimulating degradation of newly synthesized HL protein.

INTRODUCTION

Hepatic lipase (HL) is synthesized and constitutively secreted by liver parenchymal cells and subsequently bound extracellularly in the liver (1-3). Here, it plays an important role in lipoprotein metabolism. HL hydrolyses phospholipid and triacylglycerol present in high- and intermediate-density lipoproteins and chylomicron remnants, and facilitates the selective uptake of cholesterol from high density lipoproteins and the removal of remnant particles by the liver (see Jackson et al for review (4)). Thus, by contributing to reverse cholesterol transport and to the decrease of the atherogenic remnant particles in the circulation, HL is thought to protect against the development of premature atherosclerosis. Indeed, post-heparin HL activity was lower in patients with versus those without stenotic coronary arteries (5). In addition, HL activity was inversely correlated with progression of coronary atherosclerosis in patients on a lipid-lowering diet (6). The factors that determine the level of HL activity in liver are poorly understood.

The amount of HL activity present in the liver of rat and man is under hormonal and dietary control (7-10). An important role is played by the "stress" hormones, glucocorticoids and catecholamines, which all reduce HL expression (11, 12). Catecholamines and long-term treatment with corticosteroids are associated with profound changes in plasma lipoprotein turnover and the development of atherosclerosis (11-15). Decreased HL may contribute to the changes in lipoprotein

metabolism. The catecholamines were recently shown to be responsible for the daily changes in the HL expression in rat (16). Depending on the feeding condition, HL activity in the liver changes over a twofold to threefold range, and is lowest during fasting periods.

Whereas most hormones including glucocorticoids have been shown to alter HL expression at the level of transcription (12), the mechanism by which catecholamines decrease HL expression is less clear. In contrast to the other hormones, the catecholamines have an acute effect on HL secretion, which can be demonstrated in suspensions of freshly isolated hepatocytes (11, 16). Cells treated with adrenaline secrete less HL activity than cells in which protein de novo synthesis is completely blocked with cycloheximide (16). This observation led to the hypothesis that at least part of the inhibitory effect occurs at the post-translational level. In the present study, the mechanism by which adrenaline lowers secretion of HL activity was addressed directly. We show here that HL de novo synthesis is unaffected by adrenaline. Instead, intracellular processing of newly synthesized HL into a mature protein is inhibited and degradation is increased.

MATERIALS AND METHODS

Materials

Cycloheximide and CHAPS were purchased from Boehringer Mannheim (Mannheim, Germany). Benzamidine and amino acids were from Merck, (Darmstadt, Germany). Adrenaline was obtained from Centrafarm (Etten-Leur, The Netherlands) and prazosin from Pfizer (Brussel, Belgium). Trasylol was from Bayer (Mijdrecht, The Netherlands) and heparin from Leo Pharmaceutical Products (Weesp, The Netherlands). Ham's F10 and methionine-free MEM were from Gibco (Paisle, U.K.). Endo H was from Genzyme (Cambridge, MA, U.S.A). Glycerol tri[9,10(n)-^{3}H]oleate was purchased from Amersham (Amersham, U.K.) and Tran-^{35}S-label, 1100 Ci/mmol, was from ICN (Costa Mesa, CA, U.S.A.). All other chemicals were from Sigma (St. Louis, MO, U.S.A.).

Hepatocyte isolation and incubation.

Male Wistar rats (250 to 300 g body weight) were fed ad libitum with a standard chow diet (Hope Farm, Wilnis, The Netherlands). Hepatocytes were isolated by in situ perfusion with collagenase type I and nonparenchymal cells were removed by differential centrifugation according to the method of Seglen (17). The cells were washed with Ham's F10 medium containing 25 U/ml of heparin to remove residual extracellularly bound HL. The cells were resuspended at 3 to 5x10^6 cells/ml Ham's F10 medium containing 25 U/ml of heparin and 20% heat-inactivated dialysed bovine serum (1). The cell suspensions were incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 in a shaking water bath. Cell viability ranged from 85 to 95%, as determined by Trypan blue exclusion and remained essentially unaltered throughout the incubation.

At the indicated times, 0.5 ml-samples were collected from the incubation media and put on ice. Cells and media were separated by centrifugation (5 s, 10,000*g*, 4°C) and the cell-free media were used for analysis of secreted HL. For analysis of intracellular HL, the cells were washed twice with Ham's F10 medium and then resuspended in the original volume of PBS containing 4 mM CHAPS, 25 U/ml of heparin, and the protease inhibitors leupeptin (1 µg/ml), antipain (1 µg/ml), chymostatin (1 µg/ml), pepstatin (1 µg/ml), benzamidine (1 mM), Trasylol (10 IU/ml) and EDTA (1 mM) (18, 19). The cells were lysed by sonification (10 s, ar amplitude 14 µ with the MSE Soniprep 150), either immediately or after overnight storage at -80°C. Thereafter, the homogenates were centrifuged (10 min, 10,000*g*, 4°C) and the supernatants were used for further analysis.

HL activity and protein

HL activity was determined by a triacylglycerol hydrolase assay at pH 8.5 in 0.6 M NaCl using a gum acacia-stabilized glycerol [^{3}H]trioleate emulsion as substrate (1). Assays were performed for 30 min at 30°C. Enzyme activities are

expressed as mU (nmoles of free fatty acids released per min). Lipase activity in the cell-free media was completely inhibited by goat anti-HL IgGs (20). Of the lipase activity present in the cell homogenates, approximately 0.17 ± 0.03 mU/10^6 cells was resistant to immuno-inhibition with anti-HL; this value was not affected by any of the incubation conditions used (data not shown). For determination of intracellular HL activity, this value was subtracted from the total lipase activity in the cell homogenates.

The amount of HL protein in cell-free media was measured by a solid-phase ELISA, in which HL is sandwiched between goat polyclonal and a mixture of monoclonal anti-HL IgG's, as described previously (20). Absorbance was read against a standard curve prepared by serial dilutions of rat HL. The latter was prepared from post-heparin rat liver perfusates by affinity chromatography on Sepharose-heparin. HL activity was eluted with a linear 0.2 to 1.0 M NaCl gradient in 1% BSA; peak fractions were pooled and kept at -80°C until use.

Protein de novo synthesis

Freshly isolated hepatocytes were pre incubated for 30 min in methionine-free MEM containing 25 U/ml of heparin and 20% heat-inactivated dialysed bovine serum in the absence of adrenaline. Then, 50 µCi/ml of Tran-^{35}S-label was added with or without adrenaline. After 10 min, the incubation was stopped on ice and cold methionine was added at a final concentration of 1 mM. The cells were collected by centrifugation (2 min, 50*g*, room temp.). After washing once in Ham's F10 medium, the cells were lysed in lysis buffer (1% Triton X-100, 0.1% sodium deoxycholate, 25 U/ml heparin, 1 mM methionine and 1 mM cysteine, and the cocktail of protease inhibitors described above) (18). After 30 min on ice, the lysates were centrifuged for 10 min at 10,000*g* and 4°C, and the supernatants were used for immunoprecipitation of [^{35}S]HL (see below). After separation of the immunoprecipitated proteins by SDS-PAGE, the HL bands were quantified by overnight exposure to a phosphor screen in the GS363 Molecular Imager system from Bio-Rad (Richmond, OA, U.S.A.).

To determine overall protein de novo synthesis, 25 µl of the lysates were spotted onto Whatman 3MM filter paper (Whatman, Maidstone, UK). After boiling in 5% TCA, the filters were washed successively with ethanol:ether (1:1) and ether, and radioactivity in the TCA-precipitable material was determined by a 1-hour exposure in the GS363 Molecular Imager system.

Pulse-chase experiments

Cells were pulse-labelled in the absence of adrenaline with 80 µCi/ml of Tran-^{35}S-label for 5 to 10 min, as described earlier. After washing and resuspending the cells, the incubation was continued in Ham F10 medium containing 25 U/ml of heparin, 20% bovine serum and 1 mM cold methionine in the absence or presence of adrenaline. After the indicated chase times, the incubations were stopped on ice and lysisbuffer was added. In some experiments, cells and media were first separated by centrifugation and then the cells were lysed in lysis buffer as described earlier.

Immunoprecipitations

HL protein was immunoprecipitated by goat polyclonal anti-HL IgGs immobilized onto Sepharose (20). Twenty mg of the goat antibody preparation was coupled per 1 gram of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturers instructions. Similarly, non-immune IgGs from a control goat were also immobilized onto Sepharose. Samples (0.5 to 1 ml) of cell-free media and cell lysates were first incubated for 2 h at 4°C with 50 µl of a 50%-slurry of immobilized non immune IgGs. After removal of the beads by centrifugation (20 s, 10,000*g*, 4°C), the samples were incubated overnight at 4°C with 50 µl of a 50%-slurry of the immobilized anti-HL IgGs. The beads were collected by centrifugation and then washed twice with 1 ml of successively PBS, 1 M NaCl in PBS, 0.2% Tween-20 in PBS, and finally PBS (all at 4°C). The bound proteins were released by boiling in Laemmli sample buffer without ß-mercaptoethanol. After removal of the beads the proteins were reduced with ß-mercaptoethanol and then resolved by SDS-PAGE on 10% gels. Radioactive bands were visualized by fluorography using Amplify (Amersham), and their molecular masses were estimated using broad-range markers from Bio-Rad electrophoresed in parallel. To quantify the radioactivity in the protein bands, the dried gels were analysed by a 24-hour exposure in the GS363 Molecular Imager system.

Transferrin was immuno-precipitated from the cell-free media as outlined above, using 10 µl of a 1:10 diluted anti-serum against rat transferrin (a kind gift from Prof. Dr. H.G. van Eijk, Rotterdam, The Netherlands) followed by 20 µl of a 50% slurry of protein A-Sepharose (Pharmacia, Uppsala, Sweden).

Deglycosylation with Endo H

Following pulse-chase labelling and immunoprecipitation, [^{35}S]HL was removed from the Sepharose-beads and denatured by heating for 5 min 95°C in 50 mM NaPi buffer (pH 6.0) containing 0.5% SDS. Aliquots (10 µl) were incubated with or without 80 mU/ml Endo H for 16h at 37°C in NaPi buffer with 0.2% SDS. Samples were heated for 5 min 95°C in Laemmli sample buffer and subjected to SDS-PAGE and the phosphor imaging with the Molecular Imaging system. Sensitivity to Endo H was evidenced by increased electrophoretic mobility (18).

Statistics

Statistical significances were determined by two-way ANOVA, followed by Student-Newman-Keuls test (21).

RESULTS

Secretion of HL activity and protein

HL activity in suspensions of freshly isolated rat hepatocytes was reduced by adrenaline in a concentration-dependent manner (Fig. 1). In the presence of 1 µM adrenaline secretion was inhibited by 30%; maximal inhibition of 50% was obtained with 100 µM adrenaline. This effect

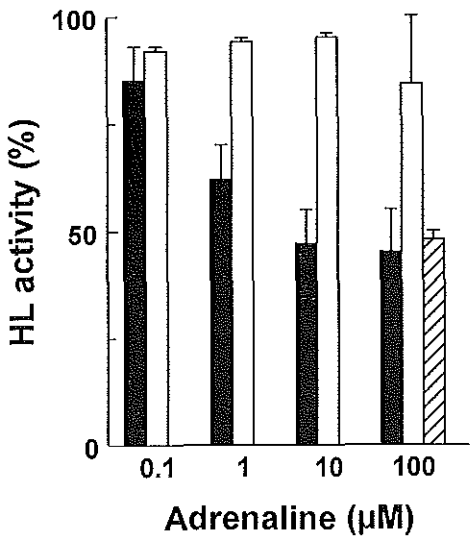


Figure 1 **Effects of different adrenaline concentrations on secretion of HL activity.**
Freshly isolated hepatocytes were incubated in control medium, or in the presence of different amounts of adrenaline, either alone (filled bars) or with 1 µM prazosin (open bars) or 1 µM propranolol (hatched bar) added at time zero. After one hour samples were collected on ice and the cell-free media were assayed for HL activity. Data are expressed as percentage of control (1.31 ± 0.53 mU/10^6 cells) and are mean ± S.D. for 3 independent experiments.

was completely abolished by co-incubation with 1 μM of the α_1-blocker prazosin but not with 1 μM of the β-blocker propranolol. A 50% inhibition of HL secretion was also obtained with 10 μM phenylephrine, a selective α_1-agonist. Therefore, the effect of adrenaline was mediated by α_1-adrenergic receptors.

Throughout the first 90 min of incubation with adrenaline, HL activity was secreted at a constant rate of approximately 0.63 ± 0.18 mU/h/10^6 cells (mean ± S.D.; n = 5), compared to 1.16 ± 0.26 mU/h/10^6 cells in control suspensions (Fig. 2). Already 30 min after addition of adrenaline, HL activity in the medium was significantly lower than in parallel controls, indicating that adrenaline acted almost immediately. This time course of secretion differed markedly from that observed with cycloheximide (10 μg/ml), which completely and instantaneously blocked protein de novo synthesis (19). With cycloheximide the secretion of HL activity initially continued unaffected, but plateaued after 60 min (Fig. 2). This lag period apparently reflects the intracellular transport time of newly synthesized HL protein. At the 30 and 60 min time points, extracellular HL activity was significantly lower with adrenaline than with cycloheximide ($p<0.05$). Under all conditions tested, secretion of HL protein as measured by ELISA parallelled the extracellular appearance of HL activity. Hence, the specific enzyme activity of secreted HL remained unaffected by incubation with adrenaline (Fig. 2, insert).

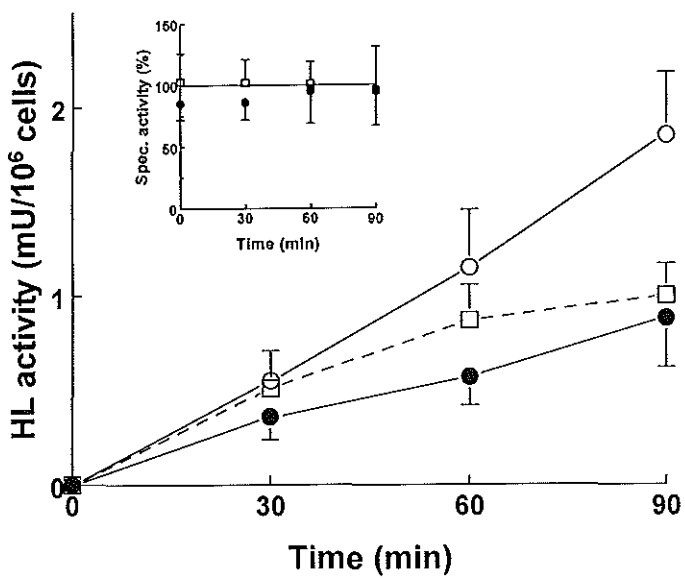


Figure 2 **Acute inhibition of HL secretion.**
Hepatocytes were incubated in control medium (○) and in medium containing 10 μM adrenaline (●) or 10 μg/ml cycloheximide (□) added at the start of the incubation. At the times indicated samples were collected and cell-free media were assayed for HL activity and HL protein. The data on HL activity represent mean ± S.D. of 5 independent experiments. At all time points the difference between adrenaline and control data was statistically significant ($p<0.05$). At 30 and 60 min time points, the adrenaline data were also significantly different from the cycloheximide data ($p<0.05$).
Insert:
Effect on the specific HL activity, calculated from the HL activity and amount of HL protein in the cell-free media. Data are expressed as % of control and are representative for 3 similar experiments.

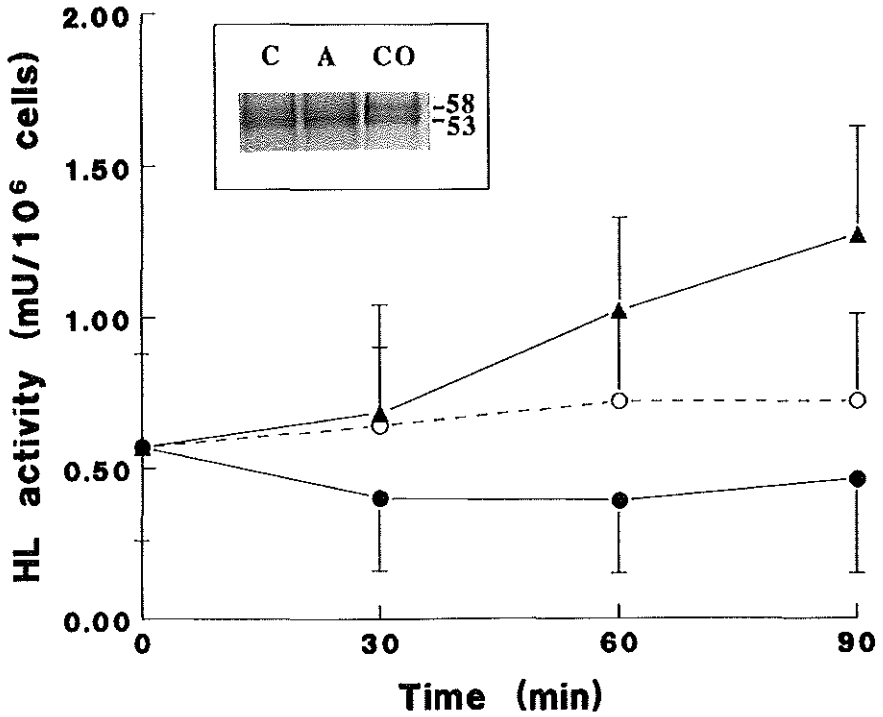


Figure 3 **Effect of adrenaline on intracellular HL.**

Cells were incubated in control medium (○), or in medium containing 10 μM adrenaline (●) or 100 μM colchicine(▲). At the indicated times samples were taken for determination of intracellular lipase activity. Data are the mean ± S.D. (n=3). Statistically significant differences were found between control and adrenaline-treated cells at 60 and 90 min ($p<0.05$). **Insert:** immunoprecipitated HL from cells labelled for 10 min with [^{35}S]methionine and chased for 60 min in the absence (C) or presence of 10 μM adrenaline (A) or 100 μM colchicine (CO). At the end of the incubation, HL was immunoprecipitated from the cell lysates and resolved by SDS-PAGE and fluorography; the molecular weight of the bands is indicated in kDa.

Intracellular HL activity and protein

Freshly isolated hepatocytes contained 0.57 ± 0.17 mU of HL activity / 10^6 cells of HL activity (mean ± SD; n = 4), which was constant throughout the 90 min incubation (Fig. 3). In the presence of adrenaline, HL activity decreased during the first 30 min to 60% of control values. Upon prolonged incubation, intracellular HL activity remained constant at this low level. Hence, the adrenaline-induced reduction in HL secretion was accompanied by a loss rather than increase in intracellular HL activity. In contrast, the HL activity in cycloheximide-treated cells continued to fall to negligible levels after 90 min (Table I). An intracellular accumulation of HL activity could be induced by treating cells with 0.1 M colchicine, which inhibits protein secretion posttranslationally (22, 23). Herein, intracellular HL activity almost doubled in about 90 min of incubation (Fig. 3). Secretion of HL activity (and protein) was inhibited to a similar extent as by 10 μM adrenaline (Table I).

Since the ELISA for HL protein proved unreliable on cell lysates, we used ^{35}S-labelling followed by immunoprecipitation with anti-HL IgGs as a relative measure for intracellular HL protein. Cells that had been pulsed for 10 min with [^{35}S]methionine, were chased for 60 min in the absence or presence of adrenaline (Fig. 3; insert). In control cells, intracellular [^{35}S]HL mainly migrated at the 53 kDa position, whereas a faint band was visible at 58 kDa (Fig. 3, insert). The 58 kDa band comigrated with HL present in extracellular media and therefore represents mature HL. In contrast to the 58 kDa band, the 53 kDa band was Endo H-sensitive (see later) and thus corresponds to the high-mannose-type HL precursor (18). Intracellular [^{35}S]HL was not affected by adrenaline, despite the inhibition of HL secretion. In the presence of adrenaline, intracellular [^{35}S]HL mainly migrated at the 53 kDa position. In colchicine-treated cells, inhibition of HL secretion and the increase in intracellular HL activity coincided with the accumulation of [^{35}S]HL at the 58 kDa position. These observations suggest that intracellular HL activity is associated with the mature 58 kDa HL protein rather than with the 53 kDa precursor protein.

Table I **Distinct effects on intracellular and secreted HL activity.**

	Medium		Cells	
Treatment	mU/10^6 cells	%	mU/10^6 cells	%
Control	1.87 ± 0.63	100	0.55 ± 0.26	100
Cycloheximide	1.16 ± 0.41	62	0.10 ± 0.14	18
Adrenaline	0.94 ± 0.45	50	0.29 ± 0.28	52
Colchicine	0.56 ± 0.13	30	1.10 ± 0.33	200

Cells were incubated in control medium, or in medium containing either 10 µg/ml cycloheximide, 10 µM adrenaline, or 100 µM colchicine. After 90 min, samples were collected on ice, and the HL activity in the cell-free media and cell homogenates was measured. Data are expressed as the mean ± S.D. (n=3), or as percentage of control. HL activity in the treatment groups were all significantly different from the controls ($p<0.05$).

Effect on HL translation

Parenchymal cells were incubated with [^{35}S]methionine for 10 min with or without adrenaline. Thereafter, the incorporation of radioactivity into HL protein and into total TCA-precipitable material was determined. In control cells, approximately 0.02% of the ^{35}S-radioactivity in total TCA precipitable material was present in HL protein (the 53 plus 58 kDa bands) (Table II). With 10 µM adrenaline incorporation of ^{35}S-label into HL protein was slightly, but not significantly lower than in control cells. Similar values were found in cells incubated with the combination of adrenaline and prazosin. Overall protein synthesis was not affected by adrenaline. This demonstrated that the short term effects of adrenaline on inhibition of HL expression are not mediated at the level of translation.

Effect on intracellular HL processing

In [^{35}S]methionine pulse-chase experiments, cells were pulsed in the absence of adrenaline and then chased with or without adrenaline. After a 5 min pulse, a protein band with an apparent molecular mass of 53 kDa was immunoprecipitated with anti-HL IgGs from the total cell suspensions (Fig.4A).

Table 2 **Effect of adrenaline on protein and HL de novo synthesis.**

	control	adrenaline	adrenaline + prazosin
HL (cpm/ml)	33 ± 13	31 ± 12	30 ± 10
total protein (cpm/µl)	144 ± 46	145 ± 43	131 ± 43

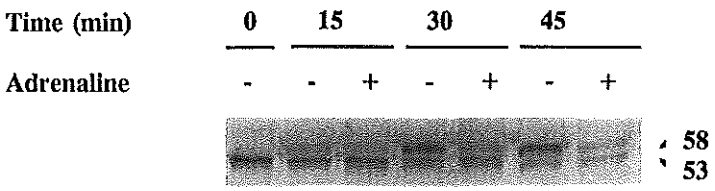
Hepatocytes were pulsed with [^{35}S]methionine for 10 min with or without 10 µM adrenaline, either alone or in combination with 1 µM prazosin. Thereafter the radioactivity in total TCA-precipitable material and immunoprecipitated HL (the 53 plus 58 kDa bands on SDS-PAGE) was determined. Data represent the amount of ^{35}S-labelled protein, which was calculated in terms of cpm/ml) cell suspension by taking into account the different times of exposure to the phosphor screens. Data are expressed as mean ± SD of 3 independent experiments, each performed in duplicate. No statistically significant differences were found between the treated values and controls ($p>0.05$)

During the chase, this band gradually shifted towards the 58 kDa position. In similar pulse-chase experiments, when HL was immunoprecipitated separately from cell lysates and cell-free media, we observed that the 53 kDa band was the major intracellular protein.

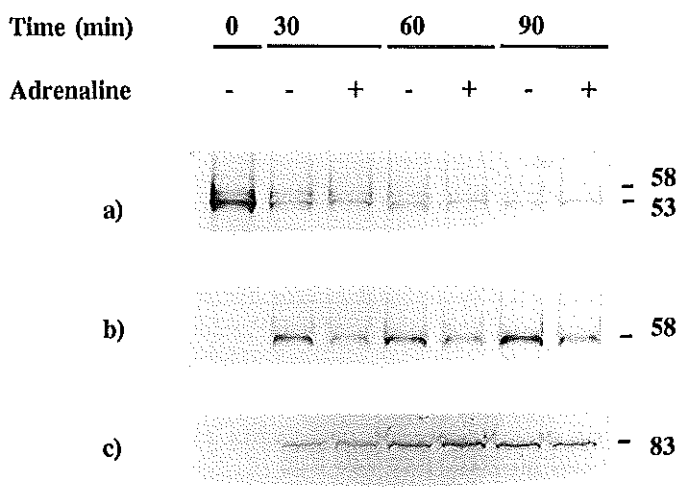
The ^{35}S-labelled 53 kDa protein was never observed extracellularly. The 58 kDa protein was rapidly secreted into the medium (Fig. 4B; a and b). Upon incubation of the 53 kDa band with Endo H, the apparent molecular weight decreased to about 50 kDa, demonstrating that the 53 kDa protein represented Endo H-sensitive HL protein bearing high-mannose type oligosaccharide chains. In contrast, the apparent molecular weight of the 58 kDa band was not affected by Endo H-treatment, and thus represents the mature, complex type HL protein. When adrenaline was included in the chase medium, less of the 58 kDa band appeared (Fig. 4A). ^{35}S-labelled HL remained predominantly in the 53 kDa form. Secretion of [^{35}S]HL was markedly reduced in the presence of adrenaline compared to control (Fig. 4B; panel b). Concomitantly, the secretion rate of [^{35}S]transferrin, a glycoprotein unrelated to HL, was not affected by adrenaline, or even slightly increased (Fig. 4B; panel c). This finding argues against a general effect of adrenaline on glycoprotein secretion.

Figure 5 shows the quantitative analysis of the pulse-chase experiments. Although the appearance of the 58 kDa band was markedly reduced by adrenaline compared to control cells, the disappearance of radioactivity from the 53 kDa band was hardly affected. As a result the total radioactivity in HL protein gradually decreased, so that at the end of the chase, total ^{35}S-radioactivity in HL protein was approximately 25% lower in the adrenaline-treated suspensions than in the controls. The radioactivity lost from the adrenaline-treated suspensions was not recovered in any immunoreactive protein, and may reflect complete degradation. This observation suggests that adrenaline induces the degradation of newly synthesized HL protein.

A



B



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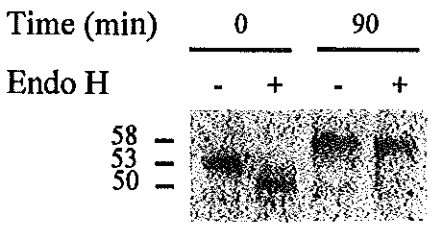

Figure 4 **Chase experiments in presence or absence of adrenaline.** Hepatocytes were pulsed with [^{35}S]methionine for 5 min in the absence of adrenaline. After washing the cells in fresh medium the cell suspension was divided in two. One part was incubated in control medium and the other part was incubated in medium containing 10 µM adrenaline. At the times indicated samples were collected and HL was immunoprecipitated from the whole suspensions (A). (B)Suspensions were first separated into cells and media, then HL was immunoprecipitated from the cell lysates (*a*) and cell-free media (*b*); then transferrin was immunoprecipitated from the same cell-free media (c). Samples were resolved by SDS-PAGE and fluorography. Data represent 4 similar experiments.
(C) Effect of Endo H on the mobility of the 53 kDa and 58 kDa bands in SDS-PAGE. The apparent molecular weight of the bands is indicated in kDa.

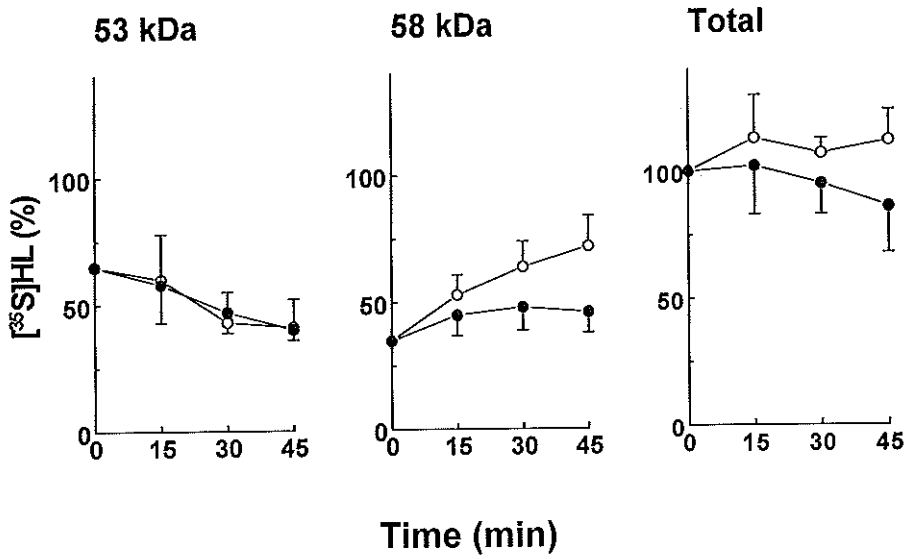

Figure 5 **Effect of Adrenaline on the maturation of HL into a 58 kDa protein.**

Experiments were performed as outlined in the legend to figure 4A. The ^{35}S-labelled bands of immunoprecipitated HL on SDS-PAGE were analysed quantitatively. The data show the disappearance of the 53 kDa protein, the appearance of the 58 kDa protein and the sum of both signals for the chase in the absence (○) or presence of 10 μM adrenaline (●). Data are expressed as a percentage of the total radioactivity in the HL bands at the start of the chase (14 ± 5 cpm/ml cell suspension) and are the mean ± SD of 3 similar experiments.

DISCUSSION

Our study confirms previous reports that adrenaline acutely inhibits secretion of HL activity by rat hepatocytes (11, 16, 24). Up to 50% inhibition was observed within 60 min of exposure to 1 to 100 μM of adrenaline, which was mediated through activation of the α_1-adrenergic pathway. We show here that the acute inhibition of HL expression occurs mainly posttranslationally, and that HL de novo synthesis is not affected by adrenaline. This contrasts with the effect of adrenaline on lipoprotein lipase expression in 3T3 adipocytes, which was recently reported to be mediated at least in part by a reduced translation of the LPL mRNA (25, 26).

Adrenaline has been previously hypothesized to affect HL secretion at the posttranslational level (16, 24). In line with this, we found that secretion of HL activity in the presence of adrenaline was significantly lower than with cycloheximide, where protein de novo synthesis is completely blocked. Which posttranslational event in the maturation of HL protein is sensitive to inhibition by adrenaline is unknown. Neither the specific catalytic activity of secreted HL protein, as determined by ELISA, nor its mobility on SDS-PAGE were affected by adrenaline. Pulse-chase experiments in which adrenaline was added after the pulse showed that the extracellular appearance of [^{35}S]HL was

retarded compared to controls levels. After a 30 min-chase, secretion of [^{35}S]HL was 25 to 30% lower than in the controls, similar to the observed effect on secretion of HL activity. In the presence of adrenaline, maturation of the 53 kDa Endo H-sensitive protein into the mature 58 kDa Endo H-resistant HL protein occurred at a lower rate than in control cells; after 45 min intracellular [^{35}S]HL was mainly in the immature form. At this time, recovery of total ^{35}S-label in HL protein was only 75% of that in parallel controls, thus suggesting that adrenaline increases intracellular degradation of HL protein. Retardation of HL maturation may render the 53 kDa HL more susceptible to degradation.

The decreased secretion of HL into the extracellular medium was not accompanied by an intracellular increase in HL activity. On the contrary, intracellular HL activity was markedly lower than in control cells. We recently showed that in rat hepatocytes, HL is initially synthesized as an inactive protein in the rough endoplasmatic reticulum (RER). Subsequent oligosaccharide processing by RER glucosidases is necessary for the protein to leave the RER and to become catalytically active (19, 20). Here, we show that intracellular HL activity is mainly associated with the presence of the 58 kDa protein form (Fig. 3). In adrenaline-treated cells, HL protein was predominantly in the 53 kDa precursor form, which was found to be associated with a low specific enzyme activity. Taken together, our data indicate that adrenaline acts at an early stage during the maturation of HL by inhibiting the processing of the inactive precursor into the mature active protein.

How the extracellular presence of adrenaline is signalled to the posttranslational modification of HL remains unclear, but the mechanism presumably involves binding to α_1-adrenoceptors and the subsequent increase in intracellular calcium. Indeed, secretion of HL activity is also inhibited by other Ca-mobilizing agonists such as vasopressin and angiotensin II as well as by the Ca-ionophore A23187 (11, 27). These calcium signals may affect vesicular transport from RER to the Golgi complex, thus inhibiting maturation and secretion of HL. However, this mode of action would be expected to affect overall constitutive secretions. In light of our results with transferrin, this possibility appears unlikely. Alternatively, a factor required for the maturation of a limited number of glycoproteins including HL may be involved. Such a factor has been implicated in mice suffering from combined lipase deficiency (28, 29). In these mice, HL and lipoprotein lipase are normally synthesized in liver and non hepatic tissues, respectively, but the protein accumulates in an inactive, high-mannose type form within the RER without being secreted (30). No other glycoproteins appear to be affected in this syndrome. A factor that specifically interacts with HL in the RER and is impaired by the *cld* mutation may be the target of adrenaline. Further studies are required to delineate the mechanism by which catecholamines interfere with posttranslational processing of HL.

Acknowledgments

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CHAPTER 2.2

Maturation and secretion of rat hepatic lipase is inhibited by α_{1B}-adrenergic stimulation through changes in Ca^{2+}-homeostasis. Thapsigargin and EGTA both mimic the effect of adrenaline.

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Biochemical Journal (1998) 330 (2), 701-706

2.2 MATURATION AND SECRETION OF RAT HEPATIC LIPASE IS INHIBITED BY α_{1B}-ADRENERGIC STIMULATION THROUGH CHANGES IN CA^{2+}-HOMEOSTASIS. THAPSIGARGIN AND EGTA BOTH MIMIC THE EFFECT OF ADRENALINE.

ABSTRACT

In rats, the daily changes in hepatic lipase (HL) activity in the liver follow the diurnal rhythm of the catecholamines. To study the underlying mechanism, the effect of adrenaline on maturation and secretion of HL was determined in freshly isolated rat hepatocytes. Adrenaline (10 μM) acutely inhibited the secretion of HL. This effect was abolished by 0.1 μM prazosin, but not by 1 μM propranolol, indicating the involvement of the α_1-adrenergic pathway. Prazosin was at least 1000-fold more potent than WB4101, a selective α_{1A}-antagonist. Adrenaline had no effect on HL secretion in hepatocytes pretreated with chloroethylclonidine, an irreversible α_{1B}-selective antagonist. Inhibition of HL was not induced by 10 μM methoxamine, a α_{1A}-selective agonist. Thus, adrenaline inhibited HL secretion through activation of the α_1-adrenoceptors subtype B, which have been shown to signal through Ca^{2+} as well as cAMP. A similar reduction in HL secretion was induced by the Ca^{2+}-mobilizing hormones angiotensin II (100 nM) and vasopressin (12 nM), the Ca^{2+}-ionophore A23187 (2 μM), and by thapsigargin (1 μM), which inhibits the ER Ca^{2+}-ATPase pump. HL secretion was unaffected by elevating cAMP with 10 μM forskolin or 1 μM 8-Br-cAMP. These results suggest that the α_{1B}-adrenergic effects on HL expression are mainly mediated through elevation of intracellular Ca^{2+}. Chelation of extracellular calcium and subsequent lowering of intracellular calcium with EGTA also inhibited HL secretion. In pulse-chase experiments, adrenaline was shown to inhibit the maturation of HL from the 53 kDa, Endo H sensitive precursor to the Endo H resistant, catalytically active protein of 58 kDa. In addition, adrenaline induced intracellular degradation of newly synthesized HL. Similar post-translational effects, both qualitatively and quantitatively, were observed with A23187, thapsigargin and EGTA. We conclude that the inhibition of HL maturation and increase in intracellular degradation induced by catecholamines, A23187, thapsigargin and EGTA is evoked by changes in Ca^{2+}-homeostasis, possibly through lowering ER-Ca^{2+}.

INTRODUCTION

Hepatic lipase (HL) plays an important role in lipid metabolism. HL hydrolyses phospholipid and triacylglycerol present in high and intermediate density lipoproteins, and facilitates the selective uptake of cholesterol from high density lipoproteins and the removal of remnant particles by the liver (see for review (1)). HL may affect lipoprotein metabolism via its enzymatic activity (2), or by its ligand function towards lipoproteins (3-6). By contributing to reverse cholesterol transport and by lowering atherogenic remnant particles in the circulation, HL is thought to protect against the development of premature atherosclerosis. A low HL has been shown to be a risk factor for premature atherosclerosis (7, 8). In addition, HL activity inversely correlated with progression of

coronary atherosclerosis in patients on a lipid-lowering diet (9). In line with this, HL transgenic mice were shown to accumulate less cholesterol in the aortic wall than non-expressing littermates (10).

Catecholamines are responsible for the diurnal changes in HL activity observed in rat liver, with HL being low when plasma catecholamines are elevated (11). In freshly isolated hepatocytes adrenaline decreases HL secretion via activation of α_1-adrenoceptors (11-13), which are generally thought to exert their action via intracellular calcium. Recently, several subclasses of this receptor have been identified and shown to signal differentially (14-17). The α_{1A}-adrenergic receptor signals by stimulating Ca^{2+}-influx from the extracellular medium and mediates a tonic response (18, 19). The α_1-subclasses B, C and D induce a rapid IP_3 formation and subsequent release of Ca^{2+} from intracellular stores (20, 21). In addition to calcium mobilization, the latter subclasses were also shown to elevate cAMP (21, 22). Hepatocytes from different species express different subclasses of α_1-adrenergic receptors. Rats, mice and hamsters mainly express α_{1B}-adrenoceptors (23). In isolated rat hepatocytes, the α_{1B}-adrenoceptors were reported to directly stimulate cAMP accumulation, rather than indirectly in response to elevated calcium (22). The cAMP accumulation, induced by the α_1-receptors, depends on the maturity of the rats from which the hepatocytes are isolated (24). This may indicate age-related changes in α_1-adrenoceptor subclass expression. The isolation procedure may also cause changes in expression of α_{1B}-adrenoceptors by rat liver cells (25). Therefore, we decided to re-investigate the signalling pathway involved in the adrenaline-induced reduction of HL secretion in isolated hepatocytes.

In earlier studies, we showed that adrenaline acutely decreases the maturation of HL and increases degradation of newly synthesized HL protein (13). To distinguish between the various α_1-adrenoceptors, we incubated hepatocytes with compounds known to have different affinities for the α_1-subtypes (26). We show here that the α_1-adrenoceptor subclass B mediates the inhibitory effects of adrenaline on HL expression. Although α_{1B}-stimulation induces elevated Ca^{2+} and cAMP levels, the post-translational effects of adrenaline on HL expression appear to be only mediated by Ca^{2+}. In order to study the mechanism of action, the role of intracellular calcium in the inhibition of HL secretion by freshly isolated hepatocytes was studied.

MATERIALS AND METHODS

Materials

Adrenaline was obtained from Centrafarm B.V. (Etten-Leur, The Netherlands) and prazosin from Pfizer (Brussel, Belgium). Propranolol, thapsigargin, angiotensin II, vasopressin, 8-bromo-cAMP and EGTA were purchased from Sigma (St. Louis, MO, U.S.A.). Methoxamine, WB4101 and chloroethylclonidine were from Research Biochemicals International (Natick, MA, U.S.A). A23187 was from Boehringer Mannheim (Mannheim, Germany). Benzamidine and amino acids were purchased from Merck (Darmstadt, Germany). Trasylol was from Bayer (Mijdrecht, The Netherlands) and heparin from Leo Pharmaceutical Products (Weesp, The Netherlands). Protein A-Sepharose and CNBr-activated Sepharose 4B were obtained from Pharmacia (Uppsala, Sweden). Ham's F10 and methionine-free MEM were from Gibco BRL (Paisle, U.K.). Glycerol tri[9,10(n)-^{3}H]oleate (5-20 Ci/mmol) was purchased from Amersham (Amersham, U.K.), Tran-^{35}S-label (1100 Ci/mmol) was from ICN (Costa Mesa, CA, U.S.A.). Broad range markers for SDS-PAGE came from Bio-Rad (Richmond, CA, U.S.A.). All other chemicals were from Sigma.

Hepatocyte isolation and incubation

Hepatocytes were isolated from male Wistar rats (200g-300g) by in situ perfusion with collagenase type I, whereafter

non-parenchymal cells were removed by differential centrifugation according to Seglen (27). The cells were washed with Ham's F10 medium containing 25 U/ml of heparin to remove residual extracellularly bound HL. Then the cells were resuspended at 3-5*10^6 cells/ml in Ham's F10 medium containing 25 U/ml of heparin and 20% heat-inactivated, dialysed bovine serum (28). The cell suspensions were incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 in a shaking water bath. Cell viability ranged from 85 to 95%, as determined by Trypan blue exclusion and remained essentially unaltered during all incubations. At the indicated times, 0.5 ml-samples were collected from the incubations and put on ice. Cells and media were separated by centrifugation (5 s, 10 000g, 4°C) and the cell-free media were used for analysis of secreted HL.

HL activity and protein

HL activity was determined by a triacylglycerol hydrolase assay at pH 8.5 in 0.6 M NaCl using a gum acacia-stabilized glycerol[^{3}H]trioleate emulsion as substrate (28). Assays were performed for 45 min at 30°C. Enzyme activities are expressed as mU (nmoles of free fatty acids released per min). The lipase activity in the cell-free media was completely inhibited by goat anti-HL IgGs (29).

The amount of HL protein in cell-free media was measured by a solid-phase ELISA as described previously (29), with minor modifications. HL was sandwiched between goat polyclonal and rabbit polyclonal anti-HL IgGs. Absorbances were read against a standard curve prepared by serial dilutions of partly purified rat HL. The latter was prepared from post-heparin rat liver perfusates by affinity chromatography on Sepharose-heparin. HL activity was eluted with a linear 0.2-1.0 M NaCl gradient in 1% BSA; peak fractions were pooled and kept at -80°C until use.

Pulse-chase experiments with [^{35}S]methionine

Pulse-chase experiments were performed as described before (13). In short, freshly isolated hepatocytes were pre-incubated for 30 min in methionine-free MEM containing 25 U/ml of heparin and 20% heat-inactivated, dialysed bovine serum. After a 5 min pulse with 80 µCi/ml of Tran-^{35}S-label, cold methionine was added at a final concentration of 1 mM. After washing twice by centrifugation (2 min, 50g, room temperature) and resuspending in Ham's F10, the cells were incubated further in Ham F10 medium containing 25 U/ml of heparin, 20% serum and 1 mM cold methionine. After the indicated chase times the incubations were stopped on ice. The whole cell suspension was lysed with 1% Triton X-100, 0.1% sodium deoxycholate, 25 U/ml heparin, 1 mM methionine and the protease inhibitors leupeptin (1 µg/ml), antipain (1 µg/ml), chymostatin (1 µg/ml), pepstatin (1 µg/ml), benzamidine (1 mM), Trasylol (10 IU/ml) and EDTA (1 mM). After a 30-min incubation on ice, the lysates were centrifuged for 10 min at 10 000g at 4°C, and the supernatants were collected. These post-nuclear supernatants were used for immunoprecipitation.

Immunoprecipitations

HL protein was immunoprecipitated by goat polyclonal anti-HL IgGs immobilized onto Sepharose (29). Twenty mg of the goat antibody preparation was coupled per 1 gram of CNBr-activated Sepharose 4B according to the manufacturers instructions. The post-nuclear supernatants (1 ml) were incubated overnight at 4°C with 50 µl of a 50%-slurry of the immobilized anti-HL IgGs. The beads were collected by centrifugation, and then washed twice with 1 ml of successively PBS, 1 M NaCl in PBS, 0.2% Tween-20 in PBS, and finally PBS (all at 4°C). The bound proteins were released by a 5-min incubation at 95°C in Laemmli sample buffer without ß-mercaptoethanol. After removal of the beads the proteins were reduced with ß-mercaptoethanol and then resolved by SDS-PAGE on 10%-gels. Protein bands were visualized by staining with Coomassie Brilliant Blue, and their molecular masses were estimated using broad range markers run in parallel. To visualize and quantify the radioactivity in the bands, the dried gels were analysed by phosphor imaging using CS-screens and the GS363 Molecular Imager (Bio-Rad, Richmond, CA, U.S.A.).

Transferrin was immunoprecipitated from the cell free media as outlined above, using 10 µl of a 1:10 diluted antiserum against rat transferrin (a kind gift from Dr H.G. van Eijk, Rotterdam, The Netherlands) followed by 20 µl of a 50% slurry of protein A-Sepharose (13).

Statistics

Statistical significances were determined by one-way analysis of variance (one-way-ANOVA), followed by the Student-Newman-Keuls test (30).

RESULTS

Characterisation of the α_1-adrenoceptor subtype involved in HL secretion

In the presence of heparin, freshly isolated hepatocytes secreted 1.34 ± 0.19 mU/10^6 cells of HL activity (mean ± S.D.; n=3) into the medium in one hour. When adrenaline (10 μM) was present from the start of the incubation, the secretion of HL activity and HL mass decreased to approximately 60% of control (Table I). Propranolol (1 μM), which was included in these experiments to exclude possible interference by the β-adrenergic pathway, did not alter the secretion of HL activity and mass in control, nor in adrenaline-treated hepatocytes (13). On the other hand, the response to adrenaline was abolished by co-incubation with 1 μM prazosin, confirming the involvement of the α_1-adrenergic receptor pathway.

Table I **Secretion of HL activity in the presence of different α_1-adrenergic (ant)agonists**

Treatment	HL activity (%)	HL mass (%)	HL specific activity (%)
Control	100	100	100
Adrenaline (10 μM)	57 ± 6*	48 ± 6*	119 ± 15
Adrenaline (10 μM) + prazosin (1 μM)	98 ± 6	91 ± 24	108 ± 31
Adrenaline (10 μM) + WB4101 (10 μM)	67 ± 7*	51 ± 13*	131 ± 30
Adrenaline (10 μM) + chloroethylclonidine (100 μM)	97 ± 11	103 ± 10	94 ± 14
Methoxamine (10 μM)	91 ± 12	96 ± 18	95 ± 11

Freshly isolated hepatocytes were incubated with 1 μM propranolol in control medium and in medium containing the indicated compounds. Part of the hepatocytes were preincubated with chloroethylclonidine for 20 min and then adrenaline was added. After 60 min, samples of the cell-free media were assayed for HL activity and mass. Data are expressed as percentage of control (1.34 ± 0.19 mU/10^6cells and 20.4 ± 4.9 mU/μg respectively) and represent mean ± S.D. (n=3). Statistically significant differences from control are indicated by the asterisks ($p<0.05$).

To distinguish between the various α_1-adrenoceptor subtypes, we incubated freshly isolated hepatocytes with adrenaline and propranolol in combination with antagonists that have different affinities for the α_1-adrenoceptor subtypes (26). In the presence of 1 μM WB4101, an α_{1A}-antagonist, the effect of adrenaline on secretion of HL activity and HL mass was hardly affected (Table I). Adrenaline was less effective when higher concentrations (up to 100 μM) of WB4101 were added (Fig. 1). Adrenaline was inactive in the presence of only 0.1 μM prazosin, demonstrating that prazosin was at least 1000-fold more potent than WB4101. Adrenaline had no effect on HL secretion when hepatocytes were pre-incubated with 100 μM chloroethylclonidine, an irreversible α_{1B}-selective antagonist (Table I). Moreover, the effect of adrenaline on secretion of HL activity and HL mass was not mimicked by 10 μM methoxamine, an α_{1A}-selective agonist. Under all conditions tested, the specific enzyme activity of secreted HL was not significantly changed (Table I). These findings indicate that adrenaline inhibited HL secretion by hepatocytes through activation of the α_1-adrenoceptors subtype B.

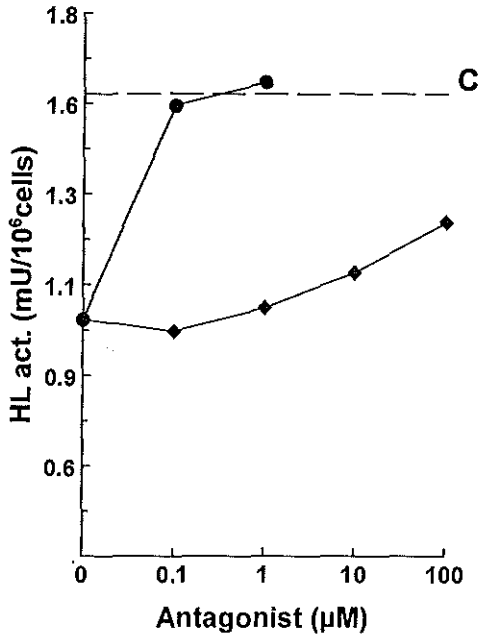


Figure 1 **Secretion of HL activity in the presence of different concentrations of α_1-adrenergic antagonists.**

Freshly isolated hepatocytes were incubated with 1 μM propranolol in control medium (C) and in medium containing 10 μM adrenaline either alone or in combination with different concentrations of WB4101 (◆) or prazosin (●). After 60 min of incubation the cell-free media were assayed for HL activity.

The effect of adrenaline is mediated by changes in intracellular calcium

In hepatocytes of adult rats α_{1B}-adrenoceptors are shown to signal simultaneously through Ca^{2+}-mobilization and cAMP accumulation (22). We therefore studied through which signal adrenaline induces inhibition of HL secretion. When hepatocytes were incubated with the Ca^{2+}-ionophore A23187 (2 μM), the HL activity and HL mass secreted into the cell-free medium was reduced to levels comparable to that with adrenaline (Table II). Thapsigargin (1μM), which elevates intracellular Ca^{2+} by mobilizing Ca^{2+}-stores from the ER (31), also induced a 40% fall in HL activity and HL mass in the medium. In addition, the Ca^{2+}-mobilizing hormones angiotensin II (100 nM) and vasopressin (12 nM) reduced secretion of HL similar to adrenaline. On the other hand, secretion of HL activity and HL mass was not affected by incubation with 10 μM forskolin or 1 μM 8-Br-cAMP. Under all conditions tested, the specific enzyme activity of the secreted HL was not significantly affected (Table II). These results imply that the α_{1B}-adrenergic effect of adrenaline on HL secretion is mainly mediated by the Ca^{2+} signal.

Opposite changes in intracellular Ca^{2+} levels inhibit the secretion of HL

As shown above, elevation of intracellular Ca^{2+} by A23187, thapsigargin, angiotensin II or vasopressin results in a similar inhibition of HL secretion as with adrenaline. Chelation of extracellular Ca^{2+} with EGTA has also been shown to inhibit secretion of HL activity (32). To establish at which concentration of extracellular free Ca^{2+} HL secretion is affected, freshly isolated hepatocytes were incubated for one hour in medium containing 0.3 mM Ca^{2+} and different concentrations of EGTA. Secretion of HL activity (Figure 2) and HL protein (not shown)

Table II

Secretion of HL activity is inhibited by increasing intracellular Ca^{2+} but not cAMP levels

	HL activity (%)	HL mass (%)	HL specific activity (%)
Control	100	100	100
Adrenaline (10 μM) + propranolol (1 μM)	57 ± 6*	48 ± 6*	119 ± 15
A23187 (2 μM)	67 ± 17*	56 ± 5*	120 ± 19
Thapsigargin (1 μM)	57 ± 10*	56 ± 2*	102 ± 27
Angiotensin II (100 nM)	72 ± 15*	63 ± 4*	114 ± 28
Vasopressin (12 nM)	70 ± 10*	59 ± 3*	119 ± 20
Forskolin (10 μM)	96 ± 6	77 ± 19	125 ± 32
8-Br-cAMP (1 μM)	92 ± 18	74 ± 6	124 ± 22

Hepatocytes were incubated for 60 min in the presence of the compounds indicated, whereafter the cell-free media were assayed for HL activity and mass. Data are expressed as percentage of control (1.47 ± 0.62 mU/10^6 cells and 20.4 ± 4.9 mU/μg respectively) and represent mean ± S.D. for 3-5 independent experiments. Asterisks indicate statistically significant differences from control ($p<0.05$).

was unaffected by EGTA up to 0.4 mM. Between 0.4 and 0.5 mM EGTA, secretion of HL abruptly fell by 40%. A further increase in EGTA concentration (up to 1 mM) had no additional inhibitory effect. Calculation of free Ca^{2+}-concentrations (33, 34) in the media show that the threshold value was between 300 and 160 nM free extracellular Ca^{2+}. Half maximal reduction in HL secretion was observed at approximally 200 nM free Ca^{2+}, which is close to intracellular Ca^{2+} concentrations reported for rat hepatocytes (22, 35-37). Apparently, the inhibitory effect of EGTA on HL secretion occurs when the free extracellular Ca^{2+}-concentration falls to levels close to or below intracellular Ca^{2+}.

Effects of adrenaline, thapsigargin and EGTA on HL maturation

The inhibitory effect of the Ca^{2+}-mobilizing hormones, as well as of A23187, thapsigargin and EGTA are evident within the first hour of incubation. During this period, mainly pre-synthesized HL is secreted (13, 29). Thus, the acute effects on HL secretion are mainly post-translational. In pulse-chase experiments with [^{35}S]methionine, the mechanism by which adrenaline, thapsigargin, A23187 and EGTA lower HL secretion by rat hepatocytes was compared. To study post-translational processing of HL, hepatocytes were pulsed with [^{35}S]methionine for 5 min in control medium. During the chase, equal parts of the cell suspension were incubated without any addition, or with either adrenaline, A23187, thapsigargin or EGTA.

After a 5-min pulse, HL was immunoprecipitated as a 53 kDa, Endo H sensitive protein (13, 38). During the chase in control suspensions, the 53 kDa protein gradually decreased (Figure 3A,B). In parallel the mature, Endo H resistant HL-protein of 58 kDa was formed. Total [^{35}S]HL, which was determined by the sum of the radioactivity in the 53 and 58 kDa protein, was not altered during the 45-min of chase, indicating that there was no degradation of HL under these conditions. When adrenaline (10 μM) was present during the chase, the intracellular 53 kDa protein disappeared at a rate similar to control (Figure 3A,B; Adr). However, the appearance of the mature 58 kDa protein

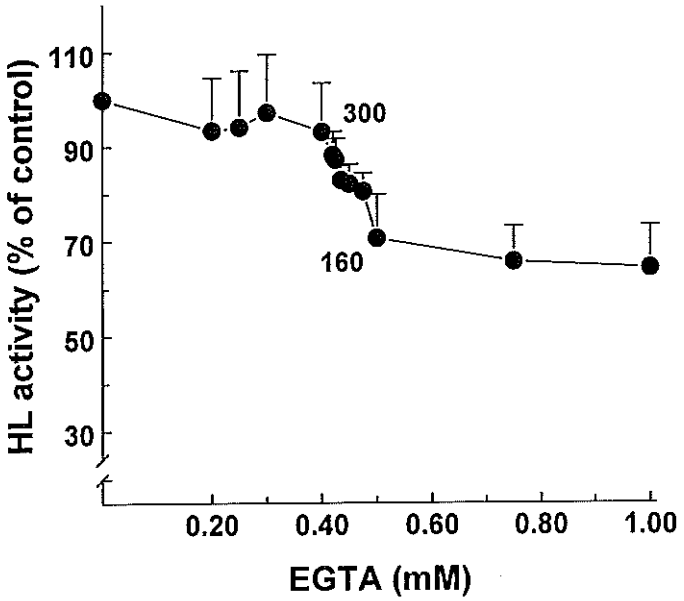


Figure 2 **Effect of chelating extracellular Ca^{2+} on secretion of HL activity.**

Freshly isolated hepatocytes were incubated in Ham's F10 medium containing 0.3 mM Ca^{2+} and different concentrations of EGTA. After 60 min the cell-free media were assayed for HL activity. Data are expressed as percentage of control (1.54 ± 0.80 mU/10^6 cells) and represent mean ± S.D. for 2-6 independent experiments. The numbers indicate the free extracellular calcium concentrations (nM) calculated for these conditions (33, 34).

A

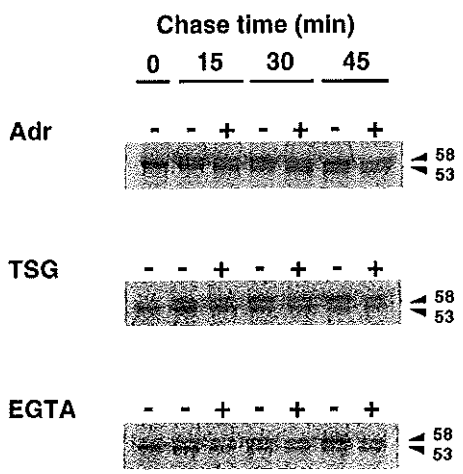
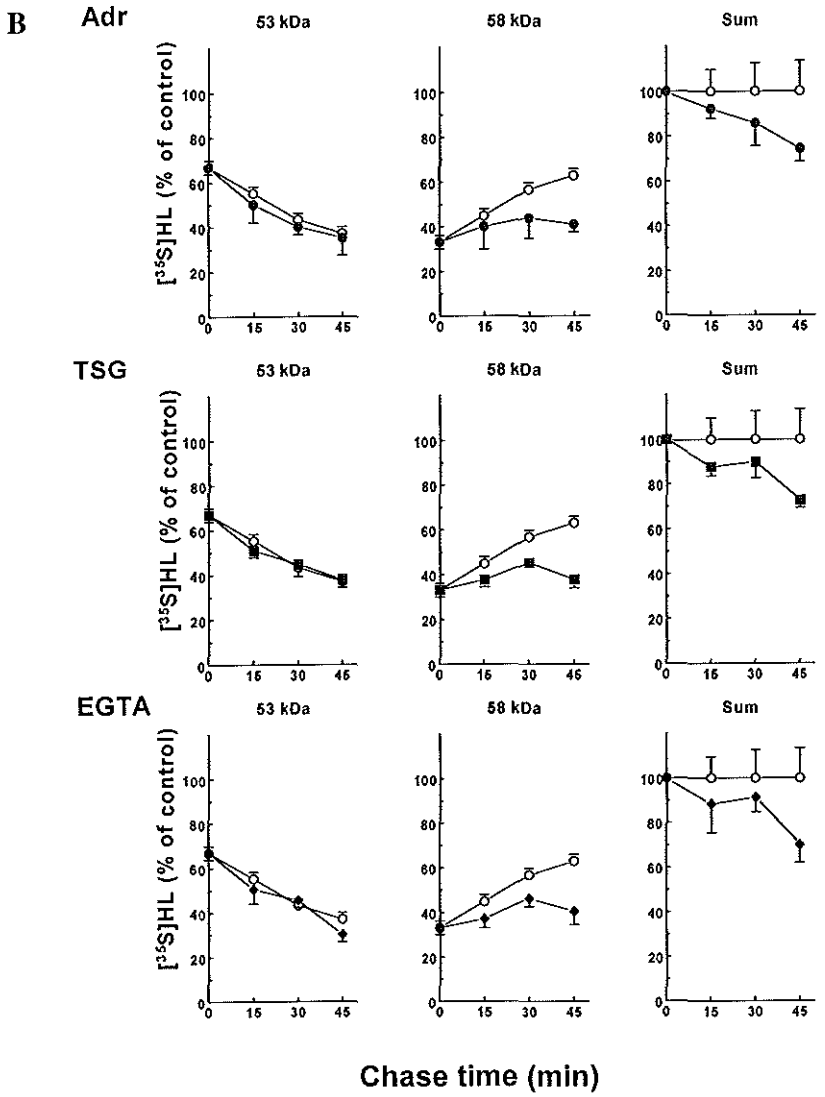
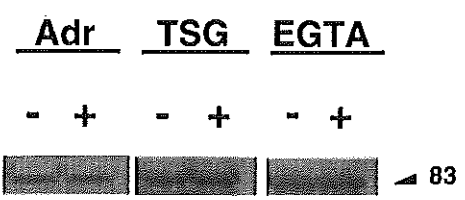


Figure 3 **Maturation of HL in the presence of adrenaline, thapsigargin or EGTA.**

Hepatocytes were pulsed with [^{35}S]methionine for 5 min in the absence of any agent. After washing the cells in fresh medium, part of the cell suspension was incubated in control medium, and an other part was incubated in medium containing either 10 µM adrenaline (Adr), 1 µM thapsigargin (TSG) or 500 µM EGTA. At the times indicated samples were collected and HL was immunoprecipitated from the whole cell suspensions. The [^{35}S]HL was analysed by SDS-PAGE followed by phosphor imaging.
(A) A representative image for a chase in the absence (-) and presence (+) of Ca^{2+}-modulating agents. The apparent molecular weight of the bands is indicated in kDa.



C



(B) The quantified data are expressed as percentage of total radioactivity in the HL bands at the start of the chase (mean ± SD, n=3). They show, from left to right, the disappearance of the 53 kDa protein, appearance of the 58 kDa protein, and total radioactivity in the 53 plus 58 kDa bands in the absence (O) or presence of either adrenaline (Adr, ●), thapsigargin (TSG, ■) or EGTA (◆). (C) Fluorograph of [^{35}S]transferrin, which was immunoprecipitated from the extracellular medium after 45 min of chase in the absence (-), or presence (+) of either adrenaline (Adr), thapsigargin (TSG) or EGTA.

was retarded compared to control cells. After 45 min of incubation, total [^{35}S]HL was 30% less than in control incubations. The loss in total [^{35}S]HL during incubation with adrenaline was not recovered in any immunoreactive protein, and may reflect complete degradation. Overall, the results indicate that adrenaline inhibited the maturation of HL protein and increased the degradation of HL.

When hepatocytes were chased in the presence of 1 μM thapsigargin, the ^{35}S-labelled 53 kDa protein disappeared at a similar rate as in control or adrenaline-treated cells (Figure 3A,B; TSG). The formation of the 58 kDa protein was retarded compared to control cells to an extent similar to adrenaline-treated cells. At the end of the chase with thapsigargin, total [^{35}S]HL was decreased to about 70% of control. With 2 μM A23187 (not shown) or with 0.5 mM EGTA (Figure 3A, B) the effects on the disappearance of the 53 kDa protein, the appearance of the 58 kDa protein, and loss of total [^{35}S]HL were superimposable to those observed with thapsigargin and adrenaline. In contrast to secretion of [^{35}S]HL, the secretion of [^{35}S]transferrin was unaffected up to 45 min of incubation with either adrenaline, thapsigargin or EGTA (Figure 3C).

DISCUSSION

In freshly isolated hepatocytes secretion of HL was shown to be inhibited by adrenaline through activation of the α_1-adrenoceptor pathway (11-13). Rat liver was reported to be enriched in the α_{1B}-subtype (23, 39). In our isolated rat hepatocytes WB4101, an α_{1A}-antagonist, was at least 1000-fold less potent than prazosin in preventing the effect of adrenaline on HL secretion. When pre-incubated with chloroethylclonidine, an α_{1B}-antagonist, the adrenaline-induced inhibition of HL secretion was completely abolished. The effect of adrenaline was mimicked by phenylephrine (13), but not by the selective α_{1A}-agonist methoxamine. Thus, adrenaline inhibited the secretion of HL via activation of the α_{1B}-adrenergic receptors. This adrenoceptor subtype was recently described to signal simultaneously through elevation of intracellular Ca^{2+} and cAMP (22). Several observations suggest that the effect of adrenaline is mediated via Ca^{2+} rather than cAMP. First, similar inhibition of HL secretion was observed with the Ca^{2+}-mobilizing hormones vasopressin and angiotensin II. In addition, the Ca^{2+}-ionophore A23187 and thapsigargin had similar effects on the secretion of HL. Second, the effect of adrenaline could not be mimicked by elevating cAMP with forskolin or 8-Br-cAMP. cAMP may be important for potentiating the IP_3-induced Ca^{2+}-release from the ER (40, 41). However, co-incubation of hepatocytes with forskolin and adrenaline showed that the forkolin-mediated elevation of cAMP did not modulate the inhibitory effect of adrenaline on HL secretion (not shown).

The acute effects of the Ca^{2+}-mobilizing hormones, A23187, thapsigargin and EGTA on HL secretion are mainly post-translational. They are evident within the first hour, during which mainly pre-synthesized HL is secreted (13, 29). Pulse-chase experiments with [^{35}S]methionine showed that adrenaline, A23187, thapsigargin, as well as EGTA retard the maturation of the 53 kDa, high mannose precursor to the mature 58 kDa HL protein, and stimulate the intracellular degradation of HL. Thus, the mechanisms by which HL secretion was inhibited by these agents were highly similar.

Maturation and secretion of HL was inhibited when the intracellular Ca^{2+}-concentration was increased by incubation with Ca^{2+}-mobilizing hormones, A23187 and thapsigargin, suggesting that

the intracellular Ca^{2+}-concentration itself may influence the processing of HL. However, HL maturation was inhibited similarly when the free extracellular Ca^{2+} was reduced with EGTA to concentrations close to or below intracellular Ca^{2+}. Under these conditions, intracellular Ca^{2+} may be reduced, and EGTA may inhibit HL processing by lowering intracellular Ca^{2+}. These results imply that, besides elevation of intracellular Ca^{2+}, also the lowering of intracellular Ca^{2+} mimics the effect of adrenaline on maturation and secretion of HL. EGTA may induce a different process than the Ca^{2+}-mobilizing agents, which leads to a similar inhibition of HL maturation and increase in HL degradation. Alternatively, this apparent contradiction may be resolved by considering the effects on Ca^{2+}-levels in the ER. The α_{1B}-adrenoceptor stimulates the release of Ca^{2+} from IP_3-sensitive stores, such as the ER (20-22). The Ca^{2+}-ionophore A23187 will carry Ca^{2+} across the ER membrane along the gradient of Ca^{2+}, thereby lowering ER-Ca^{2+} (42, 43). Thapsigargin increases intracellular Ca^{2+} by inhibition of the Ca^{2+}-ATPase in the ER membrane; Ca^{2+} is no longer retrieved from the cytosol and the Ca^{2+}-level in the ER decreases (31, 43). Incubation of hepatocytes with EGTA depletes non-mitochondrial Ca^{2+}-pools, including the ER (43). Therefore, the decreased Ca^{2+}-level in the ER, induced by incubation of hepatocytes with adrenaline, A23187, thapsigargin and EGTA, may evoke the effects on HL processing.

In which order the retardation of HL maturation and degradation of HL protein occurs is unclear. Lowering ER-Ca^{2+} may increase the degradation process by increasing the activity of Ca^{2+}-sensitive proteases in the ER. Maturation of α_1-antitrypsin and the asialoglycoprotein-receptor was also reported to be decreased by lowering Ca^{2+} in the ER (44, 45). In contrast, secretion and maturation of the glycoprotein transferrin was not affected by adrenaline, thapsigargin or EGTA. Depletion of ER-Ca^{2+} was also shown to selectively increase the degradation of transfected T-cell antigen receptor-β and CD3-δ, but not of CD3-γ and CD3-ϵ-β dimers in CHO cells (46). Alternatively, the effects on HL processing may be mediated by processes other than the Ca^{2+}-dependent stimulation of proteases in the ER. A low Ca^{2+}-level in the ER may change the affinity of HL for possible chaperones, whereafter HL may become more susceptible to ER degradation. Several chaperones resident in the ER are reported to be calcium-dependent (47). The existence of a chaperone with selectivity towards HL and other lipases was implicated from mice suffering from combined lipase deficiency (48, 49). Thus, a specific Ca^{2+}-sensitive chaperone may be involved in HL maturation. We conclude that the inhibition of HL maturation and increase in intracellular degradation by catecholamines, A23187, thapsigargin and EGTA are mediated by changes in Ca^{2+}-homeostasis, possibly through lowering ER-Ca^{2+}.

Acknowledgements

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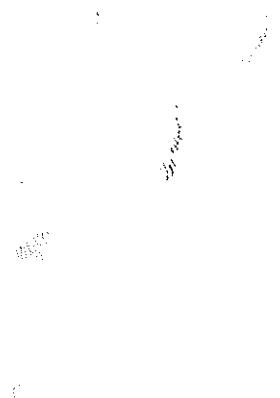


CHAPTER 2.3

Adrenaline-induced degradation of hepatic lipase in rat hepatocytes is sensitive to the protease inhibitor ALLN.

Bernadette P. Neve, Adrie J.M. Verhoeven, Ina Kalkman and Hans Jansen.

2.3 ADRENALINE-INDUCED DEGRADATION OF HEPATIC LIPASE IN RAT HEPATOCYTES IS SENSITIVE TO THE PROTEASE INHIBITOR ALLN.

ABSTRACT

Adrenaline acutely decreases the expression of HL in freshly isolated rat hepatocytes. Pulse-chase experiments showed that in the presence of adrenaline the rate of disappearance of the immature, Endo H-sensitive 53 kDa HL protein was similar to control. However, the appearance of the mature, EndoH-resistant HL protein of 58 kDa was impaired compared to control. The amount of total [^{35}S]HL (the radioactivity in the 53 kDa plus 58 kDa protein) was decreased, indicating degradation of newly synthesized HL protein. Thus, adrenaline reduces maturation of HL and increases degradation. The adrenaline-mediated degradation of HL was not sensitive to chloroquine or leupeptin, and therefore appears to be extra-lysosomal.

Other protease inhibitors we studied were ALLN, TPCK, E-64, PMSF and chymostatin. Only ALLN partly reverted the adrenaline-mediated degradation of HL protein. In the presence of 40 µg/ml ALLN, the adrenaline-induced disappearence of the 53 kDa immature protein was retarded. The appearance of the mature 58 kDa remained inhibited, as was the secretion of HL protein and activity.

CCCP, which inhibits HL maturation by decreasing ER-to-Golgi transport had no effect on HL degradation. In the presence of CCCP, [^{35}S]HL accumulated as a 53 kDa HL protein, which was slowly converted to the 58 kDa form after 30 min of chase. The total [^{35}S]HL remained constant during the 45-min chase. Thus, inhibition of HL maturation in itself does not trigger HL degradation.We conclude that the adrenaline-induced degradation of HL is mediated by ALLN-sensitive proteases.

INTRODUCTION

Hepatic lipase (HL) is synthesized and constitutively secreted by liver parenchymal cells (1, 2), whereafter it binds extracellularly in the space of Disse in the liver (3, 4). HL is involved in the metabolism of high and intermediate density lipoproteins and chylomicrons (5, 6), and facilitates the selective uptake of cholesterol from high density lipoproteins and the removal of remnant particles by the liver (7, 8). HL may affect lipoprotein metabolism via its enzymatic activity (9), or act as a ligand towards lipoproteins (10, 11). Deficiency of HL is associated with elevated levels of plasma triglycerides and large HDL particles, and increased the risk for coronary heart disease (12-15). Further, a low HL has been shown to be a risk factor for premature atherosclerosis (16, 17). HL is thought to protect against the development of atherosclerosis, by contributing to reverse cholesterol transport and by reducing atherogenic remnant particles in the circulation. In line with this, overexpression of HL in transgenic mice was shown to decrease the accumulation of cholesterol in the aortic wall (18).

Catecholamines are associated with profound changes in plasma lipoprotein turnover and

the development of atherosclerosis (19, 20). Decreased HL activity may contribute to the changes in lipoprotein metabolism. In freshly isolated hepatocytes adrenaline decreases the HL activity via the α_{1B}-adrenoceptor (21-24). Catecholamines are responsible for the diurnal changes in HL activity observed in rat liver, with HL being low when plasma catecholamines are elevated (22). In earlier studies we have shown that adrenaline has profound post-translational effects on the expression of HL in rat hepatocytes. Adrenaline acutely decreases the maturation of HL and increases degradation of newly synthesized HL protein (23). To gain more insight in the effect of adrenaline we studied the degradation process more closely. We show that the degradation of HL protein takes place early in the secretion pathway, and that the adrenaline-mediated degradation of HL was sensitive to the protease inhibitor ALLN.

MATERIALS AND METHODS

Materials.

Adrenaline was obtained from Centrafarm (Etten-Leur, The Netherlands) and heparin from Leo Pharmaceutical Products (Weesp, The Netherlands). Chloroquine, leupeptin, ALLN, TPCK and chymostatin were purchased from Sigma (St. Louis MO, U.S.A.). E64, cycloheximide and CHAPS were from Boehringer Mannheim (Mannheim, Germany). CCCP was from Calbiochem (La Jolla CA, U.S.A). Ham's F10 and methionine-free MEM were obtained from Gibco BRL (Paisle, U.K.). Glycerol tri[1-^{14}C]oleate (50-80 mCi/mmol) was purchased from Amersham (Amersham, U.K.), whereas Tran-^{35}S-label (1100 Ci/mmol) was from ICN (Costa Mesa CA, U.S.A.). PMSF, benzamidine and amino acids were from Merck (Darmstadt, Germany). Trasylol was from Bayer (Mijdrecht, The Netherlands) Protein A-Sepharose and CNBr-activated Sepharose 4B were obtained from Pharmacia (Uppsala, Sweden). Broad range markers for SDS-PAGE came from Bio-Rad (Richmond CA, U.S.A.). All other chemicals were from Sigma.

Hepatocyte isolation and incubation.

Hepatocytes were isolated from male Wistar rats (± 250g) by in situ perfusion with collagenase type I and non-parenchymal cells were removed by differential centrifugation according to Seglen (25). Cells were washed with Ham's F10 medium containing 25 U/ml of heparin to remove residual extracellularly bound HL. Thereafter, the hepatocytes were resuspended at 3-5*10^6 cells/ml in Ham's F10 containing 25 U/ml of heparin and 20% heat-inactivated, dialysed bovine serum (1). The cell suspensions were incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 in a shaking water bath. When indicated ALLN, TPCK, E-64, PMSF and chymostatin were added from 1000-fold stocks in DMSO; other agents were added from stock solutions in PBS. Cell viability ranged from 85 to 95%, as determined by Trypan blue exclusion and remained essentially unaltered during all incubations.

At the indicated times, 0.5 ml-samples were collected from the incubations and put on ice. Cells and media were separated by centrifugation (5 sec, 10 000g, 4°C) and the cell-free media were used for analysis of secreted HL. For analysis of intracellular HL, the cells were washed twice with Ham's F10 and then resuspended in the original volume of PBS containing 4 mM CHAPS, 25 U/ml of heparin, and the protease inhibitors leupeptin (1 µg/ml), antipain (1 µg/ml), chymostatin (1 µg/ml), pepstatin (1 µg/ml), benzamidine (1 mM), Trasylol (10 IU/ml) and EDTA (1 mM) (2, 26). The cells were lysed by sonification (10 sec, amplitude 14 µ, MSE Soniprep 150) either immediately, or after overnight storage at -80°C. These homogenates were centrifuged (10 min, 10 000g, 4°C) and the supernatants were used for analysis of intracellular HL.

HL activity and protein

HL activity was determined by a triacylglycerol hydrolase assay at pH 8.5 in 0.6 M NaCl using a gum acacia-stabilized

glycerol tri[^{14}C]oleate emulsion as substrate (1). Assays were performed for 45 min at 30°C. Enzyme activities are expressed as mU (nmoles of free fatty acids released per min). The lipase activity in the cell-free media was completely inhibited by goat anti-rat HL IgGs, whereas approximately 80% of the intracellular lipase activity was sensitive to inhibition (23).

The amount of HL protein in cell-free media was measured by a solid-phase ELISA as described previously (26), with minor modifications. HL was sandwiched between goat polyclonal and rabbit polyclonal anti-HL IgGs. Absorbances were read against a standard curve prepared by serial dilutions of partly purified rat HL. The latter was prepared from post-heparin rat liver perfusates by affinity chromatography on Sepharose-heparin. HL activity was eluted with a linear 0.2-1.0 M NaCl gradient in 1% BSA; peak fractions were pooled and kept at -80°C until use.

Pulse-chase experiments with [^{35}S]methionine.

Pulse-chase experiments were performed as described before (23). In short, freshly isolated hepatocytes were pre-incubated for 45 min in methionine-free MEM containing 25 U/ml of heparin and 20% heat-inactivated, dialysed bovine serum. After a 5-min pulse with 80 µCi/ml of Tran-^{35}S-label, cold methionine was added at a final concentration of 1 mM. After washing twice by centrifugation (2 min, 50g, room temperature), the cells were resuspended in Ham's F10. The cell suspension was divided into equal parts, and the incubation was continued in Ham's F10 containing 25 U/ml of heparin, 20% serum, 1.3 mM methionine and the agents under study. After the desired chase time, the incubations were stopped on ice and lysis buffer was added. The whole cell suspension was lysed with 1% Triton X-100, 1% sodium deoxycholate, 25 U/ml heparin, 1 mM methionine and the protease inhibitors described above. After a 30-min incubation on ice, the lysates were centrifuged for 10 min at 10 000g and 4°C, and the post-nuclear supernatants were used for immunoprecipitation of HL.

Immunoprecipitation of HL

HL protein was immunoprecipitated by goat polyclonal anti-HL IgGs immobilized onto Sepharose (27). Twenty mg of the goat antibody preparation was coupled per 1 gram of CNBr-activated Sepharose 4B according to the manufacturers instructions. The post-nuclear supernatants (1 ml) were incubated overnight at 4°C with 50 µl of a 50%-slurry of the immobilized anti-HL IgGs. The beads were collected by centrifugation, and then washed twice with 1 ml of successively PBS, 1 M NaCl in PBS, 0.2% Tween-20 in PBS, and finally PBS (all at 4°C). The bound proteins were released by a 5-min incubation at 95°C in Laemmli sample buffer without ß-mercaptoethanol. After removal of the beads the proteins were reduced with ß-mercaptoethanol and then resolved by SDS-PAGE on 10%-gels. Protein bands were visualized by staining with Coomassie Brilliant Blue, and their molecular masses were estimated using broad range markers run in parallel. To visualize and quantify the radioactivity in the bands, the dried gels were analysed by phosphor imaging using CS-screens and the GS363 Molecular Imager (Bio-Rad, Richmond, CA, U.S.A.).

Statistics

All data were expressed as mean ± SD. Statistical significances ($p<0.05$) were determined by one-way-ANOVA, followed by the Student-Newman-Keuls test (28).

RESULTS

Effects of adrenaline on HL expression

Hepatocytes were pulsed with [^{35}S]methionine for 5 min in the absence of adrenaline. Newly synthesized [^{35}S]HL migrated as a 53 kDa protein (Fig. 1A) that was Endo H sensitive (2, 23). After the pulse, cells were chased for the indicated times in the absence or presence of 10µM adrenaline. In control cells the 53 kDa band matured into the Endo H resistant HL of 58 kDa.When adrenaline was present during the chase, the ^{35}S-radioactivity of the 53 kDa protein decreased at a similar rate

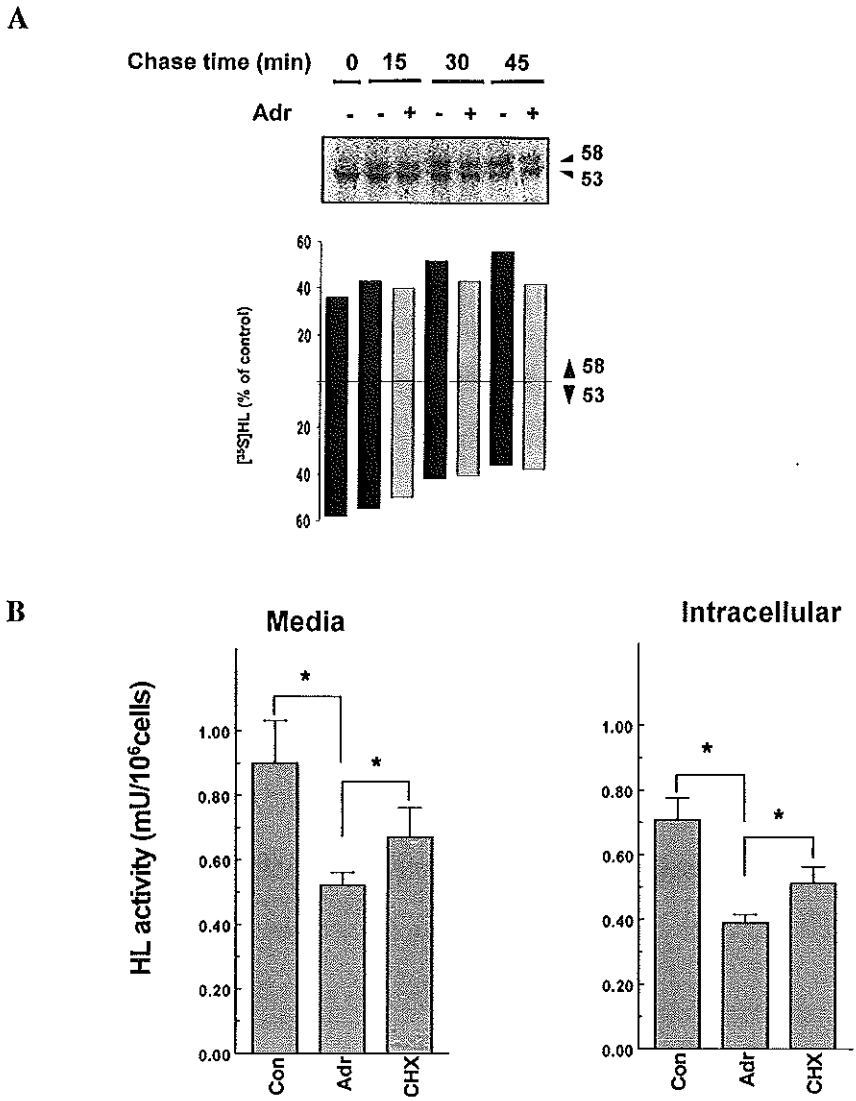


Figure 1 **Effect of adrenaline on HL maturation**

(A) Freshly isolated hepatocytes were pulsed with [^{35}S]methionine for 5 min in the absence of adrenaline. After washing, the cell suspensions were chased in medium with (+) or without (-) adrenaline (Adr). At the indicated time, samples were collected and HL was immunoprecipitated from the whole cell suspension. The [^{35}S]HL was analysed by SDS-PAGE and phosphor imaging. The ^{35}S-radioactivity in the 53 kDa band (down) and 58kDa band (up) is depicted underneath the phosphor image. The total [^{35}S]HL is represented by the total bar for the control (dark gray bar) and adrenaline (light gray bar) incubation. Radioactivity is expressed as percentage of total [^{35}S]HL in the control incubation after 5 min pulse. The apparent molecular weight of the HL bands is indicated in kDa.

(B) Hepatocytes were incubated in control medium (Con), in medium containing 10 µM adrenaline (Adr), or in medium containing 10 µg/ml cycloheximide (CHX). After 60 min, the incubation was stopped on ice, and the cell-free media and cell-homogenates (intracellular) were assayed for HL activity. Data are mean ± S.D of 3 independent experiments. Statistically significant differences from control are indicated by an asterisk ($p<0.05$).

as in control incubations. However, less of the 58 kDa protein was formed. The total radioactivity of both HL proteins remained constant during the chase in control medium, but was decreased to 75 ± 6% (n=4) when chased in the presence of adrenaline. These results confirm that adrenaline retards the maturation of HL protein and suggest that adrenaline induces the intracellular degradation of newly synthesized HL protein (23).

The effect of adrenaline on the secretion of HL activity was measured after the freshly isolated hepatocytes were one hour incubated in heparin-containing medium. Control cells secreted 0.90 ± 0.13 mU/10^6 cells of HL activity into the medium (Fig.1B). Intracellularly, the HL activity remained constant at 0.71 ± 0.07 mU/10^6 cells. When adrenaline (10 μM) was present from the start of the incubation, the secretion of HL activity decreased to 58 ± 9% of control. These changes were paralelled by changes in HL protein, as determined by ELISA (not shown). Simultaneously, intracellular HL activity decreased to 55 ± 6% of control (Fig 1B). When incubated in the presence of 10 μg/ml cycloheximide, which completely blocks de novo protein synthesis the secretion of HL activity was reduced to 75 ± 5% of control, whereas intracellular HL activity was decreased to 78 ± 4% of control. The extra- and intracellular HL activity in the presence of adrenaline was significantly lower in the presence of adrenaline than in parallel incubations with cycloheximide. Taken together, these data indicate that the acute effects of adrenaline on HL are predominantly at the post-translational level (23).

Role of lysosomal degradation in the adrenaline-mediated effects on HL expression

To study the potential involvement of the lysosomes in the adrenaline-induced effects on HL expression, hepatocytes were pulsed with [^{35}S]methionine for 5 min in control medium. Thereafter the cells were chased in the absence or presence of 10 μM adrenaline with or without 0.5 μM chloroquine or 10 μg/ml leupeptin. After 45 min of chase, chloroquine and leupeptin alone had no effect on the ^{35}S-radioactivity in the 53 kDa and 58 kDa HL proteins (Fig. 2A). In the presence of adrenaline, the fall in ^{35}S-label in the 53 kDa protein and the reduced formation of 58 kDa [^{35}S]HL were also unaffected. The total [^{35}S]HL protein (53 plus 58 kDa form) was reduced by 25 ± 9%, 23 ± 4% and 32 ± 4% in the presence of adrenaline alone, or in combination with chloroquine or leupeptin, respectively (n=2-3). Hence, the adrenaline-induced disappearance of ^{35}S-labelled 53 kDa, the retarded maturation of the 58 kDa and the decrease of total [^{35}S]HL were unaffected by co-incubation with chloroquine or leupeptin (Fig 2A).

In control cells the HL activity secreted into the medium and the intracellular HL activity was not affected by the presence of either chloroquine or leupeptin (Fig. 2B). When adrenaline was present, the HL activity secreted into the medium was decreased to 57 ± 11% of control in the absence and presence of chloroquine or leupeptin. Secretion of HL protein, as determined by ELISA, decreased in parallel to the enzyme activity, suggesting that the specific enzyme activity of the secreted HL was unaltered (not shown). Intracellularly, the adrenaline-induced fall in HL activity was also unaffected by chloroquine or leupeptin, and remained 57 ± 9% of control (Fig.2B). Thus, disrupting the lysosomal degradation with chloroquine or leupeptin did not alter the effects of adrenaline on HL protein, suggesting that the degradation is extra-lysosomal.

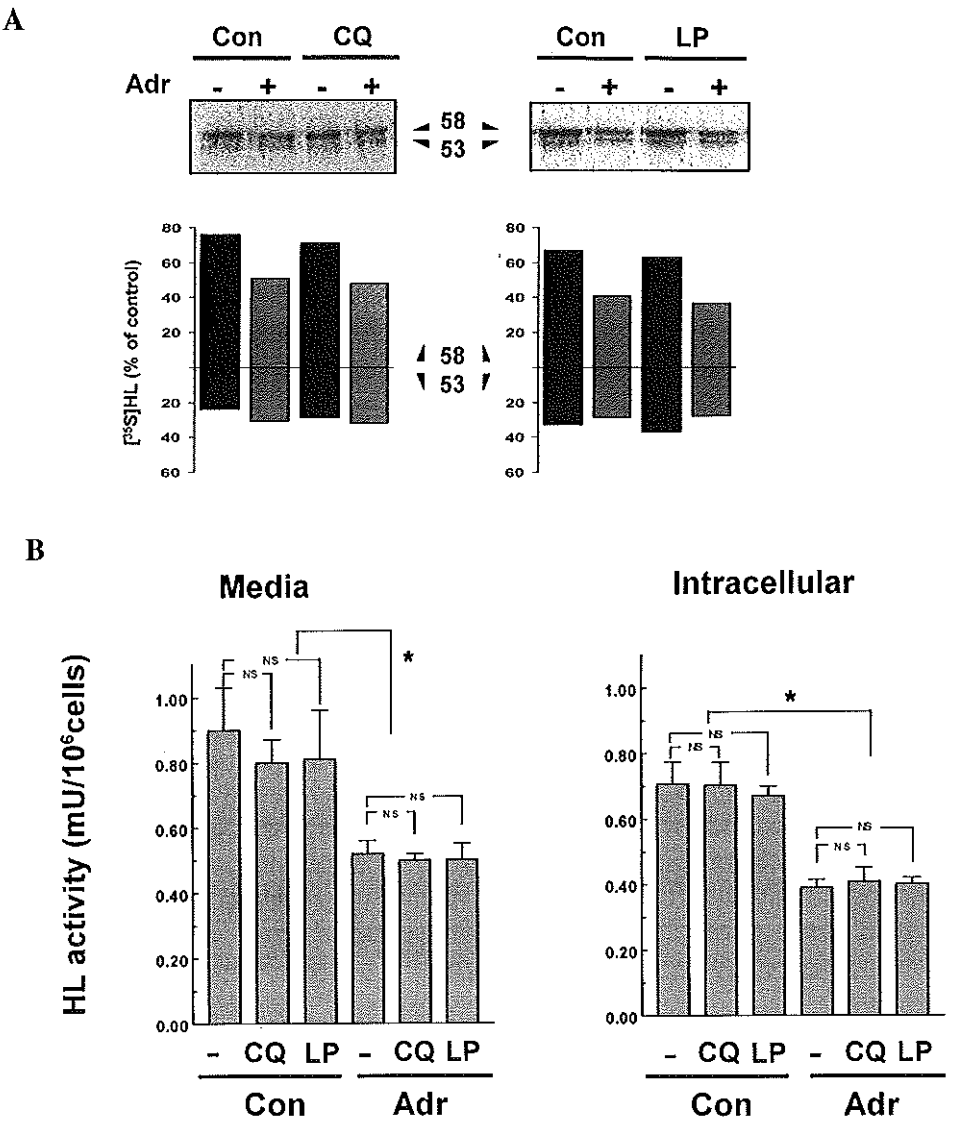


Figure 2 **Effect of lysosomal degradation inhibitors on HL in the presence of adrenaline**

(A) Hepatocytes were pulsed with [^{35}S]methionine for 5 min in the absence of adrenaline. Thereafter the cells were incubated in control medium (Con) or in medium containing 0.5 μM chloroquine (CQ) or 10 μg/ml leupeptin (LP), in the absence (-) or presence (+) of 10 μM adrenaline (Adr). Samples were collected after 45 min. The [^{35}S]HL was immunoprecipitated from the whole cell suspension and analysed by SDS-PAGE and phosphor imaging. The upper part of the figure shows a phorphor image of a representive experiment. The apparent molecular weight of the HL bands are indicated in kDa. The quantified data are depicted in bars underneath the corresponding phosphor image. Shown are the ^{35}S-radioactivity in the 53 kDa band (down) and in the 58kDa band (up) separately, the total [^{35}S]HL (53 plus 58kDa protein) is indicated by the length of the total bars.

(B) Hepatocytes were incubated in control medium (Con) or in medium containing 0.5 μM chloroquine (CQ) or 10 μg/ml leupeptin (LP), in the absence (-) or presence of 10 μM adrenaline. After 60 min, the incubation was stopped on ice and the cell-homogenates were assayed for HL activity. Data represent mean ± S.D of 3 similar experiments. Significant differences are indicated by an asterisk ($P<0.05$; NS=not significant).

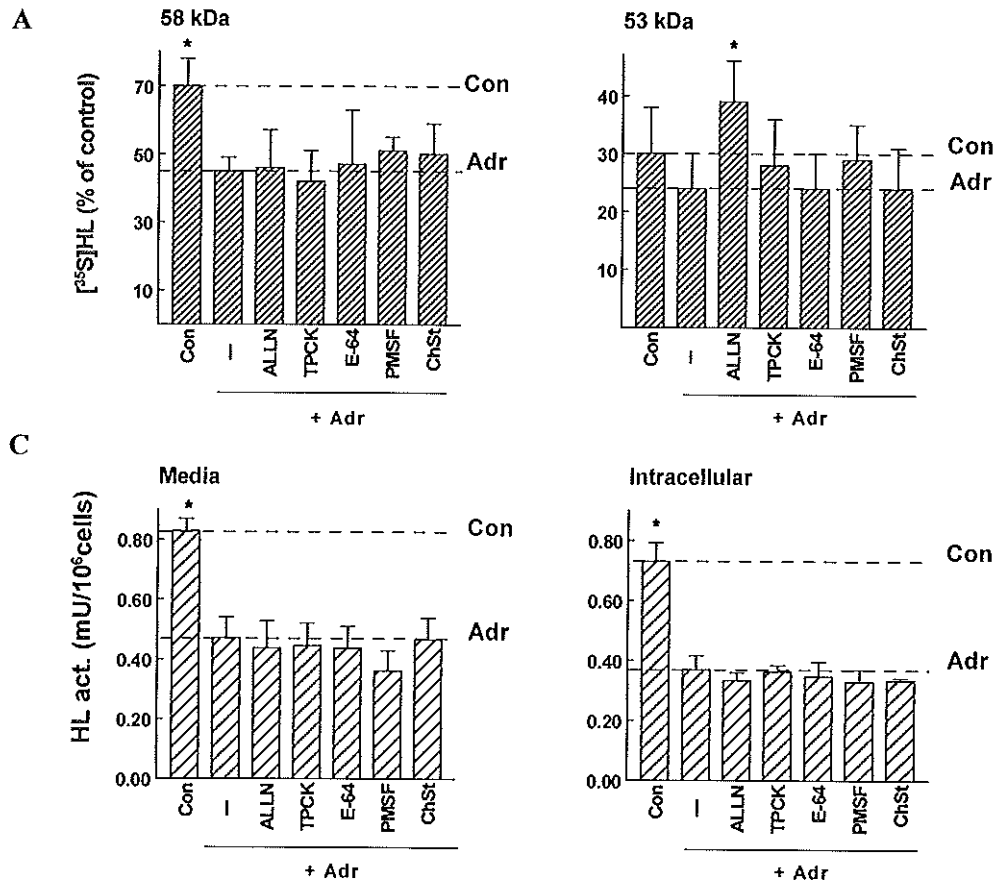
Role of intracellular proteases

To study the role of intracellular proteases in HL degradation [^{35}S]methionine-pulsed cells were chased in the presence of either the serine-plus cysteine protease inhibitors TPCK or PMSF, the cysteine inhibitor E-64, the calpain I protease inhibitor ALLN, or the chymotrypsin inhibitor chymostatin. The cells were chased for 45 min in the absence or presence of adrenaline in combination with the protease inhibitors all at a final concentration of 40 µg/ml. After 45 min of chase, the ^{35}S-radioactivity of the immature protein of 53 kDa was 30 ± 8% of the total [^{35}S]HL (53 kDa plus 58 kDa), and that of the mature, Endo H resistant 58 kDa protein was 70 ± 8% (Fig.3A). When the cells were chased with adrenaline, the ^{35}S-radioactivity of the 53 kDa plus 58 kDa HL was decreased to 31 ± 3% of control. Most of this decrease was accounted for by the retarded formation of the mature 58 kDa HL protein. The 53 kDa HL protein was hardly affected compared to control (24 ± 6 vs 30 ± 8 for adrenaline-treated and control cells, respectively). The amount of radioactivity recovered in the 58 kDa HL protein in adrenaline-treated cells was not affected by any of the protease inhibitors used. However, more ^{35}S-labelled 53 kDa protein was immunoprecipitated when the cells were incubated in the presence of ALLN in addition to adrenaline (39 ± 7% vs 24 ± 6 for with and without ALLN, respectively; Fig.3A). Further, the 53 kDa [^{35}S]HL protein slightly increased with co-incubation of adrenaline with TPCK or PMSF, but the differences were not statistically significant. In the presense of ALLN and adrenaline, the total ^{35}S-label in the 53 and 58 kDa HL protein was 85 ± 17% of control (n=4). This was significanly lower than the recovery in control cells, but significantly higher than in cells treated with adrenaline alone. Apparently, ALLN partly prevented the adrenaline-induced degradation of newly synthesized HL protein.

The HL activity present in the medium of adrenaline-treated cells reduced to 57 ± 10% of control, which was similar to co-incubation with either ALLN, TPCK, E-64, PMFS or chymostatin (Fig 3B). The HL mass in the medium, as determined by ELISA paralleled the enzyme activity, suggesting that the specific enzyme activity of secreted HL was unaffected (not shown). The intracellular HL activity was 49 ± 8% of control in the presence of adrenaline, whether or not co-incubated with the protease inhibitors. Thus, relative accumulation of the 53 kDa, [^{35}S]HL protein in the presence of ALLN and adrenaline was not accompanied by an increase in intracellular HL activity.

Effects of CCCP on the maturation of HL

Degradation of HL may result from activation of proteases, or from the inhibition in maturation and subsequent longer exposure to proteases. To clarify the sequence of events, we prevented the maturation of HL by inhibiting ER to Golgi transport with CCCP. Hepatocytes were preincubated in control medium, or in the presence of 10 µM CCCP. Then, the cells were pulsed with [^{35}S]methionine for 5 min and chased in either control or CCCP-containing medium (Fig.4, n=1). After the 5-min pulse with or without CCCP, newly synthesized ^{35}S-labelled HL migrated as a 53 kDa protein. During the subsequent chase in the presence of CCCP, significantly less 58 kDa was formed compared to control. Only after 45 min, a 58 kDa band could be immunoprecipitated, indicating that the maturation of HL was indeed retarded. After 45 min of chase, [^{35}S]HL was equally divided over the 53 and 58 kDa form, whereas in control suspensions [^{35}S]HL mainly



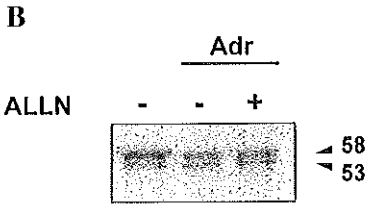


Figure 3 Effect of protease inhibitors on HL in the presence of adrenaline

(A) Hepatocytes were pulsed with [^{35}S]methionine for 5 min in the absence of adrenaline. After washing the cells, part of the cell suspension was incubated in control medium, while the other part was incubated in medium containing 10 µM adrenaline. In addition, 40 µg/ml of either ALLN, TPCK, E-64, PMSF or chymostatin (ChSt) was added. After 45 min the samples were put on ice and HL was immunoprecipitated from the whole cell suspensions. The [^{35}S]HL was analysed by SDS-PAGE and phosphor imaging. Data are expressed as percentage of the total radioactivity of the HL bands in the incubation without adrenaline (n=3-6). The amount of ^{35}S-labelled 58 kDa and 53 kDa HL-protein are depicted in bars whereas the broken line depicts the level of [^{35}S]HL in incubations with (Adr), or without adrenaline (Con).

(B) A typical phosphor image of incubations in the presence of ALLN. The apparent molecular weight of the HL bands are indicated in kDa.

(C) Hepatocytes were incubated in control medium (Con) or medium containing 10 µM adrenaline (Adr). In addition, 40 µg/ml of the above described protease inhibitors were added. After 60 min, the cell-free media and cell homogenates (intracellular) were assayed for HL activity. The broken line depicts the level of HL activities in incubations with (Adr) and without (Con) adrenaline. Data represent mean ± S.D. of three independent experiments. In A and C, statistically significant differences from the incubation with adrenaline alone are indicated by an asterisk ($p<0.05$).

consisted of mature, 58 kDa HL protein. The total amount of [^{35}S]HL (53 kDa plus 58 kDa) remained constant during the chase in the presence of CCCP (100 ± 6%), as in control suspensions. Hence, incubation with CCCP resulted in retardation of maturation from a 53 kDa into a 58 kDa HL protein. However, degradation of newly synthesized HL protein was not induced by CCCP.

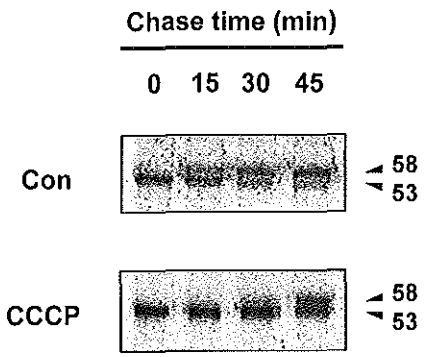


Figure 4 **Effect of CCCP on the maturation of HL protein.**

Hepatocytes were preincubated for 45 min with or without 10 μM CCCP in methionine-free medium. Thereafter 80 μCi/ml of [^{35}S]methionine was added. After 5 min, the cells were washed and further incubated in control medium (Con) or in medium containing 10 μM CCCP. At the times indicated, samples were withdrawn and put on ice. HL was immunoprecipitated from the whole cell suspensions and analysed by SDS-PAGE and phosphor imaging. The apparent molecular weight of the bands is indicated in kDa.

DISCUSSION

Pulse-chase experiments with [^{35}S]methionine showed that adrenaline retards the maturation of the 53 kDa, high mannose precursor into the 58 kDa HL protein. Further, adrenaline induced the loss of total immunoreactive [^{35}S]HL during the chase, suggesting an enhanced intracellular degradation of newly synthesized HL. In control incubations no degradation of HL was detected, which may be due to the presence of heparin in the extracellular media (2). The effects of adrenaline on secretion of HL activity and intracellular HL activity of freshly isolated hepatocytes are evident within the first hour of incubation, during which mainly pre-synthesized HL is secreted (23, 27). When protein de novo synthesis was blocked with cycloheximide, HL activity in the media and cells was also decreased, but to a lesser extent than with adrenaline. This underlines the post-translational nature of the acute adrenaline effects, as reported before (22, 23).

In pulse-chase experiments neither chloroquine nor leupeptin, both inhibitors of lysosomal degradation had any effect on the adrenaline-mediated decrease of total HL protein. Thus, degradation of newly synthesized HL may take place extra-lysosomal. The disappearance of the 53 kDa [^{35}S]HL was partly prevented by ALLN. Formation and secretion of catalytically active HL in the presence of adrenaline was not affected by ALLN. The immature ^{35}S-labelled 53 kDa HL protein accumulated in the presence of ALLN, suggesting that the degradation occurs before the 53 kDa HL protein has matured into the 58 kDa, active HL protein. Therefore, degradation probably takes place early in the secretion pathway.

The effect of ALLN on the adrenaline-induced degradation may indicate the involvement

of the cytosolic proteasome, which have been shown to be inhibited by ALLN (29). This would, however, require translocation of HL from the ER or Golgi compartment to the cytosol. Several secretory proteins are reported to be translocated from the ER to the cytosol, and than degraded by the proteasome (30). If HL would be translocated into the cytosol, inhibition of the proteasomal degradation with ALLN is unlikely to restore normal processing in the ER or Golgi lumen, which might explain the lack of restoration of HL maturation.

Alternatively, the adrenaline-induced degradation may involve cysteine-endoproteases in the ER, which have also been shown to be sensitive to ALLN (31-33). The ER-60 protease has recently been implicated in the degradation of apolipoprotein B100 (34).

In which order the retardation of HL maturation and degradation of HL protein occurs in the adrenaline-treated cells is unclear. Degradation of HL may result from the stimulation of proteases degrading the immature protein before maturation occurred. Alternatively, HL maturation may be inhibited, leaving the immature protein longer exposed to proteases. Inhibiting protein transport from ER to Golgi by CCCP (35) resulted in accumulation of immature 53 kDa HL protein without stimulating HL degradation. The accumulation of 53 kDa HL in the ER may not be sufficient to initiate degradation, implying that adrenaline specifically induces the degradation process. On the other hand, CCCP may induce a fall in cytoplasmic ATP, which may inhibit degradation by the protease.

In earlier studies, we found that the effects of adrenaline are mediated at the level of calcium in the ER (24). Degradation of HL may be increased by stimulation of calcium sensitive proteases. ALLN is known to inhibit cysteine proteases, which are calcium sensitive (36). Furthermore, low ER calcium has been shown to stimulate degradation of several other proteins (37). Alternatively, a low ER calcium may change the affinity of HL to possible chaperones, whereafter HL may become more susceptible to degradation. Several chaperones resident in the ER are reported to be calcium-dependent (38). Inhibiting the degradation may not restore the normal interaction between HL and the chaperone, and thus not restore maturation. The existence of a chaperone with selectivity towards HL and other lipases was implicated from mice suffering from combined lipase deficiency (39, 40). Thus, a specific Ca^{2+}-sensitive chaperone may be involved in HL maturation.

We conclude that the adrenaline-induced degradation of HL is sensitive to ALLN. In contrast with the effects of adrenaline on HL, the retarded maturation of HL protein by CCCP was not accompanied by degradation of newly synthesized HL protein. Where the adrenaline-mediated degradation occurs, either in the ER or cytosol, and whether chaperones are involved remains to be established.

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CHAPTER 3

(Post)transcriptional regulation of HL by growth hormone in hypothyroid rats

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3 GROWTH HORMONE RESTORES HEPATIC LIPASE MRNA LEVELS BUT THE TRANSLATION IS IMPAIRED IN HEPATOCYTES OF HYPOTHYROID RATS.

ABSTRACT

During hypothyroidism hepatic lipase (HL) activity is decreased. The low HL may be due to thyroid hormone insufficiency or to the concomitant fall in growth hormone (GH) activity. We studied HL expression in hepatocytes freshly isolated from hypothyroid rats with and without additional GH-substitution. In all animals HL mRNA was detected by RT-PCR in the hepatocytes, but not in the non-parenchymal cells. In hypothyroid cells HL mRNA levels were reduced by 40%, and the in vitro secretion of HL-activity and HL-protein was decreased by about 50%. In cells from GH-substituted hypothyroid rats, HL mRNA level was normalised, but the secretion of HL remained low. The specific enzyme activity of secreted HL was similar under all conditions. The discrepancy between HL mRNA and HL secretion in GH-supplemented rats may be due to (post)translational effects. Therefore we studied the HL synthesis and maturation in hepatocytes from hypothyroid and GH-substituted rats. Pulse-labelling experiments with [^{35}S]methionine showed that the incorporation of [^{35}S]methionine into HL protein was lower both in hypothyroid cells and in GH-supplemented cells than in control cells. During the subsequent chase, the intracellular processing and transport of newly synthesized HL protein in the hepatocytes from hypothyroid rats, whether or not supplemented with GH, was similar to control cells. We conclude that in livers of hypothyroid, GH-substituted rats translation of HL mRNA is inhibited despite restoration of HL mRNA levels.

INTRODUCTION

Hepatic lipase (HL) is involved in the metabolism of several lipoprotein fractions, high density lipoproteins, intermediate density lipoproteins and chylomicron-remnants (1-3). HL may function via its enzymatic activity (see for review (3,4)) or by its ligand function towards lipoproteins (5-8). A low HL has been implicated in the development of premature atherosclerosis (9-11). Therefore, factors that influence HL expression are of importance. During hypothyroidism HL activity is strongly decreased, which at least partly explains concomitant changes in lipoproteins and the atherogenicity of this condition (12-14).

By what mechanism thyroid hormone exerts its influence on HL activity is not clear. In rat liver, HL mRNA levels are lowered during experimentally induced hypothyroidism. However, in hypothyroidism not only thyroid hormones are low but the synthesis, secretion and activity of growth hormone (GH) is also impaired (see for review (15)). It is not clear to what extent HL activity is lowered by the decrease of thyroid hormone levels itself or by the accompanying deficiency of GH. Earlier we have shown that GH-substitution of hypothyroid rats leads to a complete restoration of the HL mRNA level in whole livers, suggesting that it is mainly the GH-deficiency that leads to the lowering of HL mRNA (16).

In spite of the restoration of the HL mRNA levels, HL activity in the liver remained

depressed during GH substitution (16). In order to explain the apparent inconsistency of HL expression at the mRNA and enzyme-activity level, we examined the effect of GH in hypothyroid rats more closely. We studied the in vitro secretion of HL activity by liver parenchymal cells isolated from hypothyroid animals, with and without GH-substitution and performed pulse-chase experiments with [^{35}S]methionine to study the intracellular transport of HL protein.

MATERIALS AND METHODS

Materials.

Vegetarian chow was obtained from Puyk Diervoeders (The Netherlands). Human growth hormone (Norditropin®) was a generous gift from Novo-Nordisk A/S (Gentofte, Denmark). 2-Mercapto-1-methyl-imidazole (methimidazole) was from Janssen Chimica (Beerse, Belgium). Trasylol was from Bayer (Mijdrecht, The Netherlands) and heparin from Leo Pharmaceutical Products (Weesp, The Netherlands). Benzamidine and amino acids were from Merck (Darmstadt, Germany). Ham's F10 and methionine-free MEM were from Gibco BRL (Paisle, U.K.). Glycerol tri[9,10(n)-^{3}H]oleate, [α-^{32}P]dCTP (3000 Ci/mmol) and Amplify® were obtained from Amersham (Amersham, U.K.). RNase A and T1, T7 RNA polymerase, restriction enzymes and DNA size marker VI were from Boehringer Mannheim (Mannheim, Germany); pBluescript KS^{-} was from Stratagene (La Jolla, CA, U.S.A.). Tran-^{35}S-label (1100 Ci/mmol) was from ICN (Costa Mesa, CA, U.S.A.) and both Percoll and CNBr-activated Sepharose 4B were from Pharmacia (Uppsala, Sweden). All other chemicals were from Sigma (St. Louis, MO, U.S.A.).

Animal treatment.

Male Wistar rats (220 ± 10 gram), were housed at 21°C on a 12 hour light cycle. To avoid the possible intake of any thyroid hormone, the animals were fed vegetarian chow, a mixture of grains, herbs and seeds. Both chow and drinking water were available ad libitum. Hypothyroidism was induced by the addition of 0.05% (w/v) 2-mercapto-1-methyl-imidazole to the drinking water, resulting in serum T4 levels below 1 nM in all animals after 14 days of treatment (17). To one group of hypothyroid rats human growth hormone (GH) was administered subcutaneously for 10 days (twice a day 0.1 IU per 100 g bodyweight, dissolved in 0.1 ml 0.9% NaCl) (16,18,19). Before use, animals were fasted overnight.

Cell isolation and incubation.

Rat liver cells were isolated by recirculating perfusion of the liver in situ with 0.1% collagenase in Krebs-Ringer buffer and parenchymal cells were obtained by differential centrifugation (20). In some experiments the hepatocytes were further purified by centrifugation through 60% Percoll, whereas non-parenchymal cells were collected from the 25-50% Percoll interfase (21). Cell viability, determined by Trypan blue exclusion, ranged from 85 to 95%. For in vitro secretion experiments the cells were resuspended to 3-5*10^{6} cells/ml in Ham F10 containing 25 U/ml of heparin and 20% heat-inactivated, dialysed bovine serum (22). The cell suspensions were incubated at 37°C under an atmosphere of 5% CO_2/95% O_2 in a shaking water bath. At the indicated times, 0.5 ml-samples were collected on ice. Cells and media were separated by centrifugation (5 sec, 10 000*g*, 4°C) and the cell-free media were used for analysis of secreted HL.

HL mRNA measurements.

Expression of HL mRNA was determined by RT-PCR and RNAse protection assays. Total RNA was prepared from isolated parenchymal and non-parenchymal cell fractions by the method of Chomczynski and Sacchi (23). In RT-PCR, 1 µg of total RNA was reverse-transcribed using oligo(dT)$_{16}$, followed by amplification in 25 cycles (95 °C - 55 °C - 72 °C; 1 min each) using the HL-specific primers HL-1 (5'-GTG GGC ATC AAA CAG CCC-3', nt 697-714; numbering according to Komaromy and Schotz (24)) and HL-2 (5'-CAG ACA TTG GCC CAC ACT G-3', nt 1292-1274), as described previously (25).

For the RNAse protection assays (26), a *Pst I/Pst I* fragment of rat HL cDNA (bp 237-605), was cloned into pBluescript KS^{-}. After linearization with *Fsp I*, a ^{32}P-labelled antisense riboprobe was prepared by in vitro transcription using T7 RNA polymerase. This probe (10^{5} dpm) was hybridized in solution with 50 µg of total RNA from the

parenchymal cells. Subsequently, single-strand RNA was digested with RNase A and T1, and the protected fragments were analyzed by electrophoresis through a 6 % denaturing polyacrylamide gel. The radioactivity in the 185nt protected RNA-fragment was quantified by counting in the Instant Imaging system from Packard (Meridan, CT, U.S.A.).

Hepatic lipase determination.

To determine the total heparin-releasable HL activity in the liver, rat livers were perfused in vitro through the portal vein and the perfusate was collected through the inferior vena cava. After a 10 min perfusion with Krebs-Ringer buffer (pH 7.4), the liver was perfused for 20 min with the same buffer containing 20 U/ml of heparin. Thereafter, the perfusates were collected on ice and the liver was homogenized in PBS containing 5 U/ml heparin; both fractions were used for further analysis.

HL activity was determined by a triacylglycerol hydrolase assay at pH 8.5 in 0.6 M NaCl using a gum acacia-stabilized glycerol [^{3}H]triolein emulsion as substrate (22). Enzyme activities were expressed as mUnits (nmol of free fatty acids released per min).

The amount of HL protein was determined by a solid-phase ELISA described previously (28). In short, microtiter plate wells were coated with 20 µg of goat anti-HL IgG. After blocking with gelatin, the wells were incubated with successively sample, a mixture of five monoclonal mouse antibodies against rat HL and alkaline-phosphatase-conjugated goat anti-mouse IgGs. Alkaline phosphatase activity was assayed using p-nitrophenol phosphate. Absorbances were read against a standard curve of rat HL purified from post-heparin liver perfusates,exactly as described by Jensen and Bensadoun (29) with omission of the last gel-filtration step.

[^{35}S]methionine incorporation studies.

Freshly isolated hepatocytes were pre-incubated for 30 min in methionine-free MEM containing 25 U/ml of heparin and 20% heat-inactivated, dialysed bovine serum. Then, 80 µCi/ml of Tran-^{35}S-label was added. After a 10-min pulse, cold methionine was added to a final concentration of 1 mM. In pulse-labelling experiments the cells were reisolated and lysed as described below. In pulse-chase experiments, the labelled cells were washed once by centrifugation (2 min, 50*g*, room temp.) and then incubated further in Ham's F10 containing 25 U/ml of heparin, 20% serum and 1 mM cold methionine. After the indicated chase times the incubations were stopped on ice. Cells and media were separated by centrifugation (2 min, 50*g*, room temp.). The cells were washed once with Ham's F10 and then lysed in the original volume of PBS containing 1% Triton X-100, 0.1% sodium deoxycholate, 25 U/ml heparin, 1 mM methionine, 1 mM cysteine, and a cocktail of protease inhibitors (1 µg/ml leupeptin, 1 µg/ml antipain, 1 µg/ml chymostatin, 1 µg/ml pepstatin, 1 mM benzamidine, 10 IU/ml Trasylol and 1 mM EDTA) (28,30). After a 30 min incubation on ice, the lysates were centrifuged for 10 min at 10 000*g* and 4°C, and the supernatants were used for immunoprecipitation.

HL protein was immunoprecipitated from the cell-free media and cell-lysates by goat polyclonal anti-HL IgGs immobilized onto Sepharose (27). Twenty mg of the goat antibody preparation was coupled per 1 gram of CNBr-activated Sepharose 4B according to the manufacturers instructions. Cell-free media and cell lysates were incubated overnight at 4°C with 50 µl of a 50% slurry of the immobilized anti-HL IgGs. The beads were collected by centrifugation (20 sec, 10 000*g*, 4°C), and then washed twice with 1 ml of successively PBS, 1M NaCl in PBS, 0.2% Tween-20 in PBS, and finally PBS (all at 4°C). The bound proteins were released by boiling in Laemmli sample buffer without ß-mercaptoethanol. After removal of the beads the proteins were reduced with ß-mercaptoethanol and then resolved by SDS-PAGE on 10% gels. Radioactive bands were visualized by fluorography using Amplify, and their molecular masses were estimated using broad-range markers from BioRad (Richmond, U.S.A.) run in parallel. To quantify the bands, fluorographs were scanned with the HP ScanJet II CX densitometer, and the blackness of the bands was expressed in arbitrary units.

Statistics.

Differences between groups were tested for statistical significancy by one-way ANOVA followed by the Dunn's test.

RESULTS

Hepatic lipase mRNA in liver parenchymal cells.

A parenchymal and a non-parenchymal cell fraction was isolated from control, hypothyroid and GH-supplemented hypothyroid rats. From each cell fraction, RNA was isolated and subjected to RT-PCR. The expected 596-bp cDNA product of the HL mRNA encoding exon 5-8 was readily detected in each of the parenchymal cell preparations (Fig. 1A). Under the same conditions, no HL cDNA product was formed with RNA from the non-parenchymal cells, suggesting that expression of HL mRNA is restricted to the parenchymal cell fraction. Product formation with parenchymal cells from hypothyroid rats appeared to be slightly lower than in cells from GH-supplemented rats, or euthyroid controls.

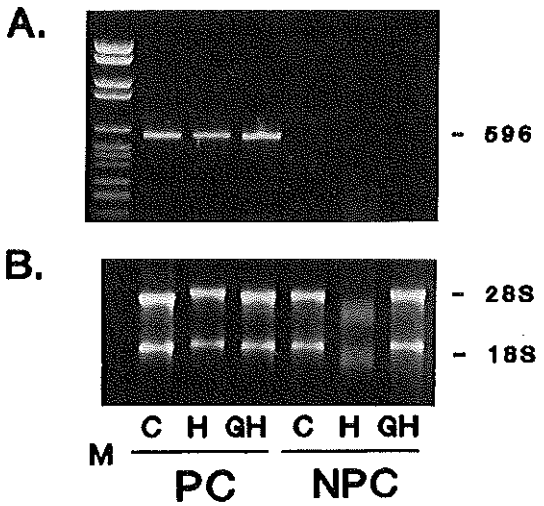

Figure 1 **Detection of HL mRNA in parenchymal and non-parenchymal cells.** RNA isolated from parenchymal (PC) and non-parenchymal (NPC) cell fractions of control rats (C), hypothyroid (H) and GH-supplemented (HG) rats was subjected to RT-PCR. The used combination of primers yielded a cDNA product of 596-bp representing the HL mRNA (A). The lane with DNA size marker VI is indicated with M. One μg of total RNA from the indicated cell-fractions was separated by electrophoresis through a formaldehyde-containing agarose gel; the position of the ribosomal RNA bands are indicated (B).

Quantification of HL mRNA in the parenchymal cell fractions was performed by an RNAse protection assay targeted at exon 3-5 (Fig. 2A). Per microgram of total RNA, hypothyroid cells contained 40% less HL mRNA than controls (Fig. 2B,C). Upon GH-supplementation of hypothyroid rats, HL mRNA levels strongly increased to values above the euthyroid controls. This observation extends our previously reported findings based on dot-blot assays on whole liver RNA's (16) and confirms that the reduction in HL mRNA levels in hypothyroid rats can be completely reversed by GH-supplementation.

Hepatic lipase activity in rat liver

Total HL activity in livers of hypothyroid rats was only 40% of that in control, euthyroid animals (Table I). Upon GH-supplementation, HL increased only to about 50% of control levels. In all three animal groups, the HL activity in the liver was mainly present in a heparin-releasable pool. The

differences in the heparin-releasable HL activity between the groups were quantitively similar to that in total liver activity. The residual, non-releasable HL activity was also decreased, although not significantly, in the hypothyroid rats and remained so after GH-supplementation. Hence, GH substitution failed to normalize both the heparin-releasable and non-heparin releasable HL activity in hypothyroid rats despite the complete restoration of HL mRNA levels.

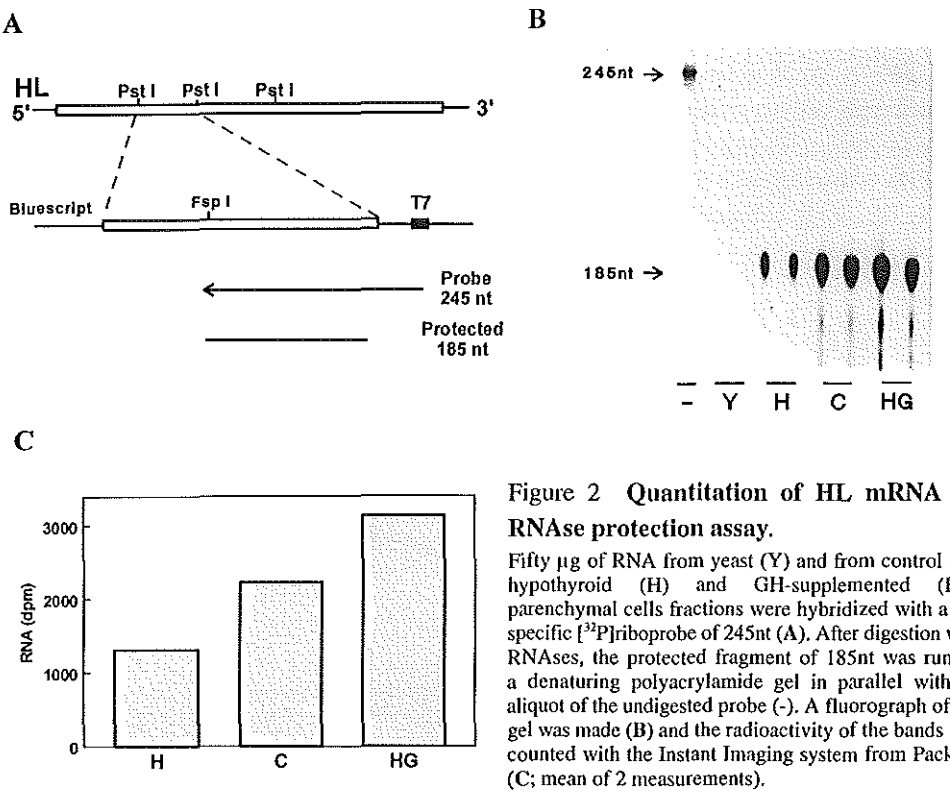

Figure 2 **Quantitation of HL mRNA by RNAse protection assay.**

Fifty µg of RNA from yeast (Y) and from control (C), hypothyroid (H) and GH-supplemented (HG) parenchymal cells fractions were hybridized with a HL specific [^{32}P]riboprobe of 245nt (A). After digestion with RNAses, the protected fragment of 185nt was run on a denaturing polyacrylamide gel in parallel with an aliquot of the undigested probe (-). A fluorograph of the gel was made (B) and the radioactivity of the bands was counted with the Instant Imaging system from Packard (C; mean of 2 measurements).

Table I. **Heparin-releasable and non-releasable HL activities in rat livers.**

Condition (#)	Total	Releasable	Residual
Control (5)	6000 ± 1230	5450 ± 1100	540 ± 160
Hypothyroid (4)	2490 ± 430*	2170 ± 400*	320 ± 80
GH-supplemented (7)	3050 ± 790*	2610 ± 720*	440 ± 160

Rat livers were perfused with 20 IU/ml heparin, whereafter the liver was homogenized. HL activity was determined both in the perfusate (heparin-releasable) and in the homogenate (residual). The total activity was calculated from the HL releasable plus residual activity. Data are expressed in mU/liver and represent mean ± S.D. of the number of experiments listed between brackets. Statistically significant differences from the control group are marked with an asterix ($p<0.05$).

In vitro secretion of HL.

In vitro secretion experiments were performed in the presence of heparin to prevent cell-surface association and degradation of secreted HL (30). Freshly isolated hepatocytes from hypothyroid rats secreted, in the presence of heparin HL activity into the medium at a constant rate of 0.93 ± 0.30 mU/10^6 cells/h (Fig.3), which was significantly lower than with control cells (1.53 ± 0.58 mU/10^6 cells/h, $p<0.05$; n=11). Treatment of hypothyroid rats with GH for 10 days had no effect on the HL activity secreted by the isolated parenchymal cells. Secretion of HL activity occurred at a rate of 0.92 ± 0.32 mU/10^6cells/h, which was similar to the hypothyroid values ($p>0.05$; n=8). Under all conditions tested, the extracellular appearance of HL protein, measured by ELISA, paralleled that of HL activity. At 3h of incubation, HL secreted by hypothyroid cells and GH-treated cells had a similar specific activity compared to HL secreted by euthyroid controls (11.2 ± 2.2, 11.5 ± 3.5 and 12.0 ± 3.0 mU/µg HL protein, respectively: $p>0.05$, n= 5-7). This indicates that the low secretion rate of HL activity by cells from hypothyroid and GH-supplemented rats is not due to secretion of inactive HL, nor to enhanced inactivation of the secreted HL.

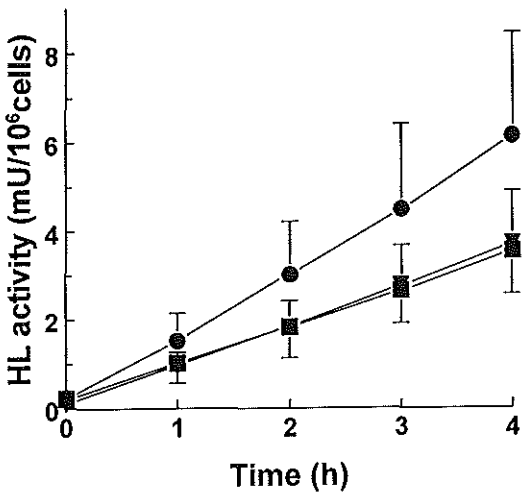

Figure 3 **HL secretion by isolated rat hepatocytes.**
Hepatocytes, freshly isolated from control (●), hypothyroid (▼) and GH-supplemented rats (■) were incubated in the presence of 25 U/ml heparin. At the indicated times, a sample was withdrawn, and the HL activity was determined in the cell-free media. Data represent mean ± S.D. for 7 to 11 experiments.

Translation and post-translational processing of HL.

The effect of GH-substitution in hypothyroid rats on translation and post-translational processing of HL was investigated using pulse-chase experiments with [^{35}S]methionine. With hepatocytes from control, hypothyroid and GH-supplemented rats, 10 min pulse labelling followed by immunoprecipitation of HL yielded an intracellular band of 53 kDa, representing immature, Endo H sensitive HL (30, 31). Incorporation of [^{35}S]methionine into the HL-protein band was significantly lower with cells of hypothyroid and GH-supplemented rats than with control cells, as determined by densitometry (41 ±12 vs. 131 ± 10 in arbitrary units, $p<0.05$, n=5). This indicates that there was less HL de novo synthesis in the hypothyroid and in the GH-supplemented state than in the euthyroid controls.

During the subsequent chase, the intensity of the HL-protein band decreased, while

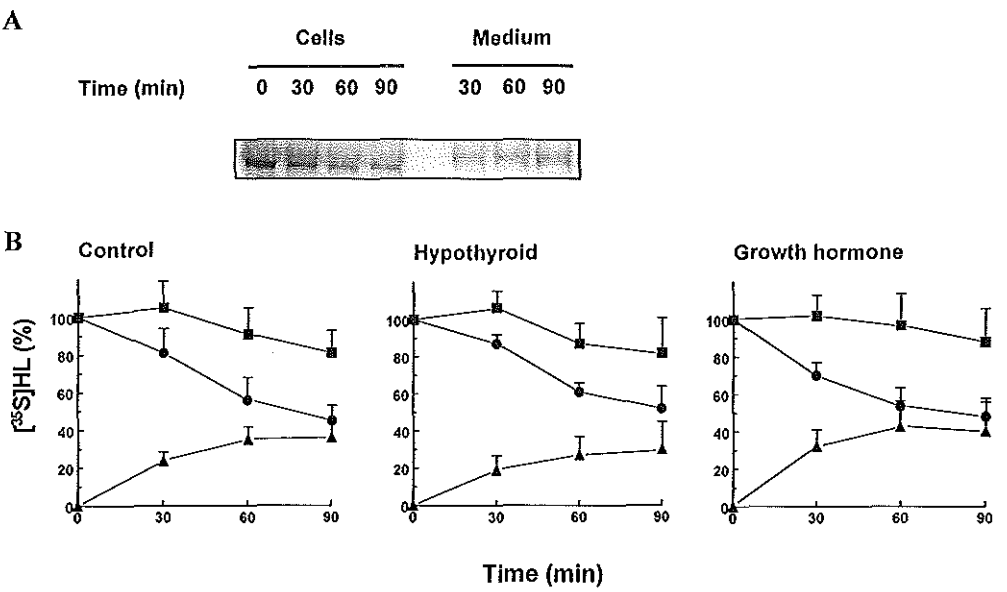

Figure 4 **Secretion of [^{35}S]HL in liver parenchymal cells.**

Cells isolated from control rats and hypothyroid rats, with or without GH-substitution were pulsed with [^{35}S]methionine for 10 min, and then chased in the presence of 1 mM methionine. At the indicated times, the incubations were stopped on ice and cells and media were separated by centrifugation. From both the cell-lysates and media, HL was immunoprecipitated and analyzed by SDS-PAGE. (A) Representative fluorograph of a pulse-chase experiment with control hepatocytes. (B) The ^{35}S-labelled HL bands were quantified and the relative blackness of the bands were expressed as percentage of the zero min timepoint. Data represent mean ± S.D. (n = 5 to 6) of the intracellular (●), secreted (▲) and total HL-protein (■).

simultaneously a 58 kDa band representing mature, EndoH resistant HL became visible intracellularly as well as extracellularly (Fig.4A). In control cells the total ^{35}S-label in the 53 plus 58 kDa bands gradually fell to about 80% of the initial value during the chase, indicating that newly synthesized HL protein was slowly degraded under these conditions. In cells isolated from hypothyroid rats, whether or not substituted with GH, the maturation of [^{35}S]HL proceeded in a similar way and at a similar rate as in the controls (Fig. 4B). The total amount of [^{35}S]HL slightly fell, but also here no differences with control were apparent. These observations indicate that the reduction in HL secretion by liver cells from hypothyroid and GH-supplemented rats was solely due to a lower translation rate and that post-translational processing was not affected by hypothyroidism or GH-substitution.

DISCUSSION

We studied the effects of hypothyroidism and GH-supplementation on the expression of HL in rats at several levels. In hypothyroid rats, HL mRNA levels (Fig.2), liver HL activity (Table I) and in

vitro HL secretion (Fig.3 and Table II) were all affected to a similar extent, suggesting that the reduction of HL expression was mainly due to a lowered transcription. Earlier findings (16), showing that GH completely restores HL mRNA levels in hypothyroid rat liver were confirmed using RNAse protection assays and isolated parenchymal cells. Remarkably, HL secretion by isolated parenchymal liver cells was not stimulated by in vivo GH administration, suggesting a post-transcriptional block of HL expression. Pulse-chase experiments using [^{35}S]methionine showed that the newly synthesized HL protein was secreted at a similar rate by hepatocytes from control, as well as hypothyroid rats whether supplemented with GH or not (Fig.4). As the specific enzyme activity of HL protein was similar in all conditions, post-translational modification affecting the catalytic activity appears unlikely. Moreover, the electrophoretic mobility of HL on SDS-PAGE was not altered, indicating that no gross post-translational changes had occurred. We conclude that the translation of HL mRNA must have been impaired in the GH-supplemented, hypothyroid rats. From these findings it can be inferred that in the hypothyroid state both the transcription and the translation of HL are reduced in parallel and that GH-substitution only restores HL mRNA levels, but not translational rates. Indeed, the de novo synthesis of HL was found to be lower in hypothyroid cells in the presence or absence of GH-substitution compared to euthyroid controls. Thus, the low HL activity in the hypothyroid state results from regulation at both the transcriptional and the translational level.

The thyroid hormone insufficiency causes HL mRNA levels to drop. The rat HL gene contains putative thyroid-responsive elements (32) suggesting that thyroid hormone itself may affect transcription. However, supplementation of hypothyroid rats with GH alone was sufficient to completely restore HL mRNA levels. GH may upregulate HL transcription, or increase the stability of HL mRNA. In HepG2 cells, HL expression was also found not to be directly affected by thyroid hormone, but rather indirectly, through its effect on other cellular processes (33). So, at least part of the effect of hypothyroidism on HL expression is mediated by the concomitant GH deficiency. We recently reported a similar regulation by GH and thyroid hormone of the LDL-receptor (LDL-R) mRNA in the liver. We found that in hypothyroid rats LDL-R activity and LDL-R mRNA were both lowered. GH-substitution also led to complete restoration of the LDL-R mRNA levels (17). Thus, GH has similar transcriptional effects on both HL and the LDL-R. Interestingly, both proteins are involved in cholesterol metabolism. Whereas LDL-R mediates the uptake of LDL-cholesterol, HL facilitates the selective uptake of HDL-cholesterol (33-37). This suggest that liver cholesterol uptake may play a central role in the parallel regulation of both proteins by thyroid hormone and GH.

Translation of HL mRNA in the hypothyroid state appears to be rate-limited by the lack of thyroid hormone. As a result, the rise in HL mRNA upon GH supplementation is not matched by increased translation, due to the thyroid hormone deficiency. Also in hypophysectomized rats, addition of both GH and thyroid hormone was necessary to normalize HL activities in the liver (38). How hypothyroidism prevents the translation of the HL mRNA induced by GH is not clear. Thyroid hormone may interfere with translation of HL mRNA either directly, or through the induction of a regulatory protein. The regulation by thyroid homone is probably not acute, as addition of thyroid hormone during incubation of parenchymal liver cells did influence neither the HL secretion, nor the [^{35}S]methionine incorporation into HL protein, both in conditions with low (hypothyroidism) and with high (GH-supplementation) HL mRNA levels. Alternatively, the HL mRNA induced by

GH may be somehow distinct from the HL mRNA in control liver, resulting in the marked reduction of translational efficiency. Sequencing of the coding part of HL mRNA, after RT-PCR did not show any differences with the published sequence (not shown). Whether any alterations in the untranslated regions or in the cap structure of the GH-induced HL mRNA occur, is unknown at present. The precise role of thyroid hormone and GH in the translation of HL mRNA needs further research.

Acknowledgments

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CHAPTER 4

Post-transcriptional effects of fenofibrate on HL expression

Bernadette P. Neve, Adrie J.M. Verhoeven, Ina Kalkman and Hans Jansen.

4. POSTTRANSCRIPTIONAL EFFECTS OF FENOFIBRATE ON HL EXPRESSION IN THE RAT

ABSTRACT

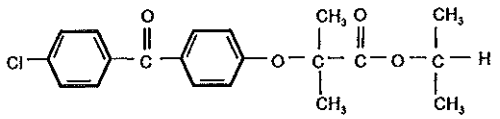
In rats, the fenofibrate-mediated down-regulation of HL expression is mainly induced at the transcriptional level. Besides regulation of HL transcription, fenofibrate has been indicated to affect HL post-transcriptionally. Here, we studied the effects of fenofibrate on expression of HL more closely. In liver homogenates from fenofibrate-treated rats, HL activity was 40% lower than in controls. After normalizing to the amount of DNA, thus partly correcting for the increased liver weight after fenofibrate treatment, the HL activity in liver homogenates was still 25% decreased compared to control. However, the pool of HL activity released after heparin perfusion of the liver was not affected by fenofibrate-treatment. Secretion of HL activity by isolated hepatocytes from fenofibrate-treated rats was inhibited up to 70% compared to control hepatocytes. The HL mRNA level in the liver was 50% decreased after fenofibrate-treatment. In accordance with this, pulse-labelling of hepatocytes with [^{35}S]methionine showed a decreased synthesis of HL in hepatocytes of fenofibrate-treated rats. The processing of pre-synthesed HL was not different from control. Pulse-chase experiments showed that the HL maturation was similar in hepatocytes from fenofibrate-treated and control rats. These results suggest that fenofibrate reduced de novo synthesis of HL mainly at the transcriptional level. Despite the reduced rate of HL synthesis, heparin-releasable HL activity in the liver was not affected. Thus, additional regulation of HL expression may occur at the post-translational level, possibly at the level of HL turnover.

INTRODUCTION

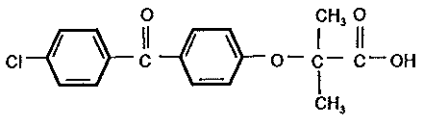
Fenofibrate is a fibric acid-derivative drug (Fig.1), which has proven useful in the treatment of hypertriglyceridaemia and -cholesterolaemia (see for review(1-3)). In humans, fibrates are very effective in lowering plasma triglyceride levels, and thus may correct atherogenic profiles. In rats, the effects of fenofibrate on TG-rich particles is mediated by limiting the availability of substrates for TG synthesis in the liver, and by inducing LPL and reducing Apo CIII. Further, synthesis of Apo AI, AII and AIV in the liver was decreased by fenofibrate-treatment in rats (4). Recently, fibrates have been shown to effect expression of genes involved in intracellular and extracellular lipid metabolism via interaction with the nuclear peroxisome proliferator-activated receptors (PPARs) (4). Specific DNA response elements (PPRE) have been identified for several activated genes. Not for all affected genes a functional PPRE has been established.

Both hepatic lipase (HL) and lipoprotein lipase (LPL) play an important role in plasma triglyceride metabolism by catalysing lipolysis of lipoproteins in liver and peripheral tissues, respectively. In addition, HL mediates uptake of (chylomicron) remnant particles in the liver. Expression of these lipolytic enzymes is differentially affected by fenofibrate. Whereas LPL is strongly induced, treatment with fenofibrate, or other fibrate-analogs, resulted in an small increase

of HL expression in humans (2, 4-7). In normolipidaemic subjects the HL activity was not affected by fenofibrate(8). In contrast, the HL expression in rats is markedly down-regulated (9), which was mainly induced by a decreased transcription after fenofibrate-treatment. Whereas the LPL gene contains a functional PPRE, no such element has yet been identified for human or rat HL gene (4). In addition to transcriptional regulation, fenofibrate may also post-transcriptionally affect HL expression (9). To identify the mechanism of post-transcriptional regulation, we studied the effects of fenofibrate on HL expression in rat liver into more detail. In particular, we isolated hepatocytes from fenofibrate-treated rats and determined the effect on HL synthesis, intracellular processing and transport.



Fenofibrate



Fenofibric acid

Figure 1 **Structure of fenofibrate and of the derived, active fenofibric acid**

MATERIALS AND METHODS

Materials

Fenofibrate was a gift from Dr. B. Staels, standard chow was purchased from Hope Farms (Wilnis, The Netherlands). Benzamidine and amino acids were purchased from Merck (Darmstadt, Germany). Trasylol was from Bayer (Mijdrecht, The Netherlands) and heparin from Leo Pharmaceutical Products (Weesp, The Netherlands). CNBr-Activated Sepharose 4B was obtained from Pharmacia (Uppsala, Sweden). Ham's F10 and methionine-free MEM were from Gibco BRL (Paisle, U.K.). Glycerol tri[1-^{14}C]oleate (50-80 Ci/mmol) and [α-^{32}P]dCTP (3 000 Ci/mmol) were purchased from Amersham (Amersham, U.K.), Tran-^{35}S-label (1 100 Ci/mmol) was from ICN (Costa Mesa, CA, U.S.A.). Broad range markers for SDS-PAGE came from Bio-Rad (Richmond, CA, U.S.A.). Quiabrane was from Qiagen GmbH (Hilden, Germany). All other chemicals were from Sigma (St. Louis, MO, U.S.A.).

Animals

Male Wistar rats (± 250g) were housed under standard conditions (21°C, 12 hour light cycle) and provided with chow and tap-water ad libitum. Control rats were fed a standard chow, whereas treated rats were fed with standard chow containing 0.5% (w/w) fenofibrate for fourteen days.

Liver perfusion and cell isolation

Livers of control and fenofibrate-treated rats were perfused in situ with Berry medium (140 mM NaCl, 5.4 mM KCl, 8.18 mM $MgSO_4$, 0.8 mM Na_2HPO_4, 25 mM $NaHCO_3$, 2.54 mM $CaCl_2$ and 6 mM D-glucose) containing 25 U/ml heparin and 1% BSA. For determination of total HL activity in the liver homogenate, a small lobule was excluded from perfusion, cut and directly frozen in liquid nitrogen. The heparin-perfusate (80 ml) was collected on ice and frozen in

liquid nitrogen for later analysis of HL activity. Thereafter, the liver was flushed with Berry buffer without calcium, and hepatocytes were isolated by recirculating perfusion of the liver in situ with collagenase type I in Berry buffer.

Hepatocyte incubation

Hepatocytes were isolated from control or fenofibrate-treated rats by recirculating liver perfusion with collagenase type I, and non-parenchymal cells were removed by differential centrifugation according to Seglen (11). The cells were washed with Ham's F10 medium containing 25 U/ml of heparin to remove residual extracellularly bound HL. After centrifugation (2 min, 50*g*), 1 ml of packed cells was resuspended in 20 ml of Ham's F10 medium containing 25 U/ml of heparin and 20% heat-inactivated, dialysed bovine serum (10). The cell suspensions were incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 in a shaking water bath. Cell viability ranged from 85 to 95%, as determined by Trypan blue exclusion and remained essentially unaltered during all incubations. At the indicated times, 0.5 ml-samples were collected from the incubations and put on ice. Cells and media were separated by centrifugation (5 s, 10 000*g*, 4°C) and the cell-free media were used for analysis of secreted HL activity.

HL activity

HL activity was determined by a triacylglycerol hydrolase assay at pH 8.5 in 0.6 M NaCl using a gum acacia-stabilized glycerol[^{14}C]trioleate emulsion as substrate (10). Assays were performed for 45 min at 30°C. Enzyme activities are expressed as mU (nmoles of free fatty acids released per min). For determination of HL activity in the liver homogenates, part of the frozen lobules were homogenized at 25 mg wet weight/ml in PBS containing 5 U/ml heparin. The homogenates were centrifuged for 2 min, 10 000*g* at 4°C, and 25 µl of the supernatant was directly used in the enzyme assay. Of the heparin perfusates 10 µl was used in the assay.

Total DNA measurements

The amount of DNA was measured as described by Kapuscinski et al (12, 13) and Lee et al (14). In short, part of the frozen lobule was homogenised in 0.2 M NaOH. After centrifugation, the suspension was neutralized with 0.9 M HCl and incubated with 400 ng/ml 4,6-diamino-2-phenylindole-(HCl)$_2$ in 0.4 M acetate-buffer (pH 7.8) containing 1mM EDTA, 10 µg/ml BSA and 0.2% Na-azide. The fluorescence (excitation 362 nm, emission 450 nm) was read and compared with a standard curve prepared from herring sperm DNA.

HL mRNA analysis

Total RNA was isolated from part of the frozen liver lobule by the method of Chomczynski and Sacchi (15). Of the RNA, 30 µg was separated by electrophoresis in a formamide-containing 1% agarose gel and transferred to Quiabrane membrane. The filter was hybridized over night with a random-primed ^{32}P-labelled HL cDNA probe (generated by PCR of rat HL cDNA using the specific primers HL-1 (5'-GTGGG CATCA AACAG CCC-3', nt 697-714; numbering according to Komaromy and Schotz (16)) and HL-2 (5'-CAGAC ATTGG CCCAC ACTG-3', nt 1292-1274), as described previously (17, 18)). After hybridisation, the filter was washed at 42°C in successively 5 x SSC, 2 x SSC and 0.2 x SSC containing 0.1% SDS. The radioactivity on the membrane was analysed by phosphor imaging using a BI-screen and the GS363 Molecular Imager (Bio-Rad, Richmond, CA, U.S.A.). After stripping, the filter was reprobed with a ^{32}P-labelled GAPDH cDNA probe.

Pulse-chase experiments with [^{35}S]methionine

Freshly isolated hepatocytes were pre-incubated for 30 min in methionine-free MEM containing 25 U/ml of heparin and 20% heat-inactivated, dialysed bovine serum. After a 5-min pulse with 80 µCi/ml of Tran-^{35}S-label, cold methionine was added at a final concentration of 1 mM. After washing twice by centrifugation (2 min, 50*g*, room temperature) and resuspending in Ham's F10 medium, the cells were incubated further in Ham's F10 medium containing 25 U/ml of heparin, 20% serum and 1.3 mM methionine. After the indicated chase times the incubations were stopped on ice. The whole cell suspension was lysed with 1% Triton X-100, 1% sodium deoxycholate, 25 U/ml heparin, 1 mM methionine and the protease inhibitors leupeptin (1 µg/ml), antipain (1 µg/ml), chymostatin (1 µg/ml), pepstatin (1 µg/ml), benzamidine (1 mM), Trasylol (10 IU/ml) and EDTA (1 mM). After a 30-min incubation on ice, the lysates were centrifuged for 10 min at 10 000*g* and 4°C. The post-nuclear supernatants were used for immunoprecipitation.

Immunoprecipitation of HL

HL protein was immunoprecipitated using goat polyclonal anti-HL IgGs immobilized onto Sepharose as described previously (19). The post-nuclear supernatants (1 ml) were incubated overnight at 4°C with 50 µl of a 50%-slurry of the immobilized anti-HL IgGs. The beads were collected by centrifugation, and then washed twice with 1 ml of successively PBS, 1 M NaCl in PBS, 0.2% Tween-20 in PBS, and finally PBS (all at 4°C). The bound proteins were released by a 5-min incubation at 95°C in Laemmli sample buffer without ß-mercaptoethanol. After removal of the beads the proteins were reduced with ß-mercaptoethanol and then resolved by SDS-PAGE on 10% gels. Protein bands were visualized by staining with Coomassie Brilliant Blue, and their molecular masses were estimated using broad range markers run in parallel. The radioactivity in the dried gels was analysed by phosphor imaging using CS-screens and the GS363 Molecular Imager.

Protein de novo synthesis

To determine the overall protein de novo synthesis, 5 µl of the lysates from the 5 min-pulsed cells were spotted onto Whatman 3MM paper. After boiling in 5% TCA, the filters were washed successively with ethanol:ether (1:1) and ether, and the radioactivity in TCA-precipitable material was determined by a 1-hour exposure to a CS-screen followed by analysis with the GS363 Molecular Imager system.

Statistics

Statistical significances ($p<0.05$) were determined either by a Student t-test or by a one-way ANOVA, followed by the Student-Newman-Keuls test (20).

RESULTS

HL activity in the liver

The HL activity in liver homogenates of control rats was 489 ± 98 mU/g wet weight (Table I). After fourteen days of fenofibrate-treatment the HL activity was decreased by 40% to 294 ± 40 mU/g wet weight. The livers of fenofibrate-treated rats were enlarged. The amount of DNA was 1223 ± 159 and 1013 ± 128 µg/g wet weight for control and fenofibrate liver, respectively, which is not significantly different ($p>0.05$, $n=4$). When the HL activity was corrected for the amount of DNA, fenofibrate-treatment resulted in a 25 % lower HL activity in the liver homogenates (Table I). In contrast, the HL activity that was released from the liver by perfusion with 25 U/ml heparin was not affected by fenofibrate-treatment. Thus, the pool of heparin releasable HL in fenofibrate-treated rats remained similar to control, although HL activity per g wet weight in the liver homogenate was decreased.

Table I **HL activity in the liver**

	Homogenates		Perfusates
	mU/ g ww	mU/ µg DNA	mU/total liver
Control	489 ± 98	0.40 ± 0.04	2152 ± 272
Fenofibrate-treated	294 ± 40*	0.30 ± 0.07*	2407 ± 895

From an isolated liver lobule a 10% (w/w) homogenate was prepared to measure the HL activity. Another part of the lobule was used to measure DNA. The remaining liver was perfused with 25 U/ml heparin and 0.1% BSA. The perfusate was collected on ice and analysed for HL activity. Data are mean ± SD for 4 to 5 animals per group. Statistically significant differences from control are marked with an asterisk ($p<0.05$).

HL mRNA levels in the liver

Total RNA was isolated from livers of fenofibrate-treated and control rats. The amount of total RNA/g wet weight was decreased by fenofibrate treatment to 70% of control (Table II). When the amount was expressed per µg DNA, the difference was not statistically significant. HL mRNA was analysed by Northern blotting, and subsequent hybridization with a ^{32}P-labelled specific probe (Fig.2). The amount of HL mRNA was expressed relative to the amount of GAPDH mRNA measured in the same lane. After fenofibrate-treatment, the ratio of HL to GAPDH mRNA was reduced by 50% (Table II), indication a reduced HL mRNA expression.

Table II **HL mRNA in the liver**

	Total RNA		HL mRNA
	µg/g wet weight	ng/µg DNA	relative units
Control	4.7 ± 0.4	4.3 ± 0.3	0.18 ± 0.04
Fenofibrate-treated	3.0 ± 0.3*	3.2 ± 0.3	0.09 ± 0.02*

Total RNA was isolated out of 90 ± 30 mg (wet weight) liver, and quantified by spectrofotometry at 260 nm. The data are mean ± SEM of 4 to 6 animals per group. HL mRNA was determined by Northern blot analysis. The radioactivity on the membrane after hybridization with a ^{32}P-labelled HL and GAPDH cDNA-probe was quantified by phosphor-imaging. Data are expressed as units of HL mRNA relative to the GAPDH mRNA signal, and are mean ± SEM (n=3). Statistically significant differences ($p<0.05$) from control are marked with an asterisk.

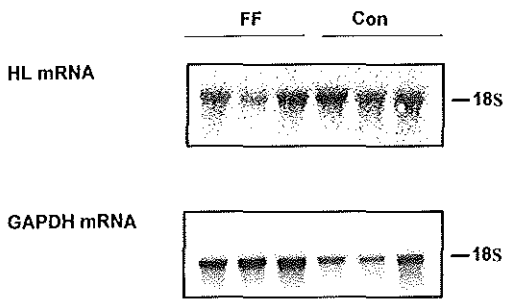


Figure 2 **HL mRNA analysis**

Total RNA was isolated from part of the frozen liver lobule. HL and GAPDH mRNA were determined by Northern blot analysis. The ^{32}P-radioactivity on the membrane was visualized by phosphor imaging. The position of the 18S ribosomal RNA is indicated at the right.

Secretion of HL activity by hepatocytes

Freshly isolated hepatocytes were prepared and 1 ml of packed cells was resuspended in 20 ml medium. With control hepatocytes, this yielded a final cell density of 3.9 ± 0.6 *10^6/ml, whereas with hepatocytes from fenofibrate-treated rats this resulted in 2.9 ± 0.6 *10^6/ml. This may reflect

an increase in cell size induced by fenofibrate treatment.

During in vitro incubation of hepatocytes from control rats in the presence of heparin, HL activity was secreted at a constant rate of 0.81 ± 0.16 mU/h/10^6 cells (Fig.3). After 2 hours, the HL activity in the cell-free medium was 1.60 ± 0.32 mU/10^6 cells (n=3). Secretion of HL activity by hepatocytes isolated from fenofibrate-treated rats was also linear with time, but slower compared to control. The HL activity secreted into the medium after 2h incubation was 0.41 ± 0.28 mU/10^6 cells, which is 25% of control.

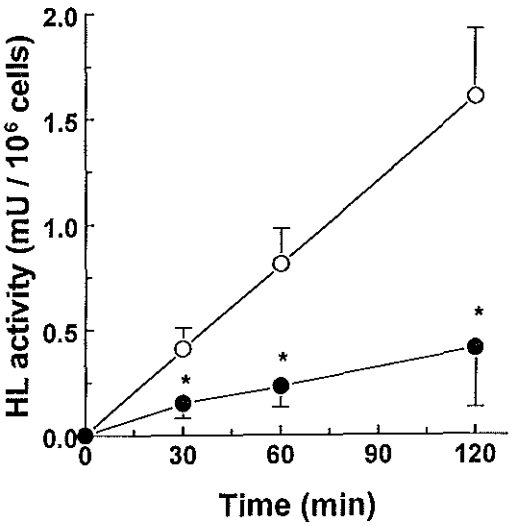


Figure 3

Secretion of HL activity by rat hepatocytes.

Freshly isolated hepatocytes from control (○) and fenofibrate-treated rats (●) were incubated in medium containing 25 U/ml heparin. At the indicated times samples were withdrawn, and the cell-free media were assayed for HL activity. Data represent mean ± SD of 4 independent experiments. Asterisks indicate significant differences from control ($p<0.05$).

Effect of fenofibrate on denovo synthesis and maturation of HL

After a 5-min pulse labelling with [^{35}S]methionine, the immunoprecipitated [^{35}S]HL from the total control cellsuspension was predomantly in the 53 kDa-form, which is Endo H sensitive (21, 22). [^{35}S]HL immunoprecipitated from fenofibrate-treated cells appeared similar to control on SDS-PAGE (Fig. 3). However, in comparison to control, less ^{35}S-radioactivity was incorporated after fenofibrate-treatment (Table III). The radioactivity of [^{35}S]HL per cell was reduced by 55% of control by fenofibrate treatment. ^{35}S-Incorporation into overall proteins, as determined by TCA-precipitation, was decreased by approximately 20%. The differences between control and fenofibrate-treated cells did not reach statistically significance with the low numbers of experiments performed.

During the subsequent chase with control cells, the ^{35}S-labelled 53 kDa protein gradually disappeared (Fig.4). In parallel the mature, Endo H resistant HL-protein of 58 kDa was formed. Total [^{35}S]HL, which was determined by the sum of the radioactivity in the 53 and 58 kDa protein, remained constant during the 45-min of chase, indicating that there was no degradation of HL under these conditions.

In hepatocytes from fenofibrate-treated rats, the disappearance of ^{35}S-radioactivity in the 53 kDa protein, and appearance in the 58 kDa protein during the chase occurred to a similar extent and at a similar rate as in control cells. Apparently the maturation of HL was not altered in fenofibrate-treated rats.

Table III **Protein and HL denovo synthesis**

	Total protein (*10^3)		HL	
	cpm/ml	cpm/10^{6}cells	cpm/ml	cpm/10^6 cells
Control	50.1 ± 11.0	12.7 ± 1.4	14.4 ± 3.2	4.1 ± 1.2
Fenofibrate-treated	29.1 ± 13.6	9.1 ± 4.5	6.0 ± 2.0	1.9 ± 0.7

Hepatocytes isolated from control and fenofibrate-treated rats were pulsed with [^{35}S]methionine for 5 min. Thereafter, the radioactivity in total TCA-precipitable material and immunoprecipitated HL was determined. Data represent the amount of ^{35}S-labelled protein, which was calculated in terms of cpm/ml cell suspension by taking into account the different times of exposure to the phosphor screens. Data are mean ± SEM of 2 to 3 independent hepatocyte isolations per group. Each labelling was performed in duplicate. The power of the performed t-test (<0.10) was too low to find significant differences.

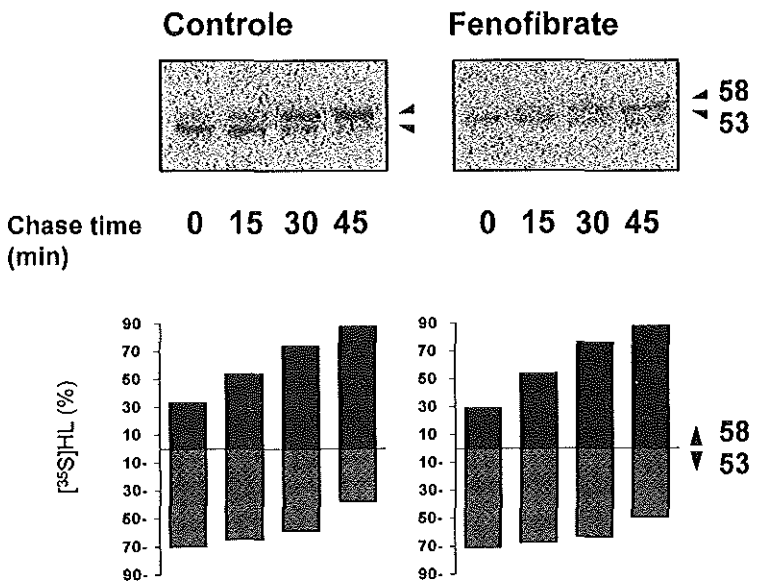


Figure 4 **Maturation of denovo synthesized HL protein**

Pulse-chase experiments were performed with hepatocytes isolated from control rats (Con) and fenofibrate-treated rats (FF). The cells were pulsed for 5 min with [^{35}S]methionine and then chased for the indicated times. Thereafter, HL was immunoprecipitated from the lysate of the whole cell suspension, and analysed by SDS-PAGE and phosphor imaging. The figure represents a typical example of the experiments (n=3). The apparent molecular weights of the HL bands are indicated in kDa. ^{35}S-Radioactivity in the 53 kDa band (downward bars) and 58 kDa band (upwards bars) is depicted underneath the phosphor image. The radioactivity is expressed as percentage of total [^{35}S]HL in the 5-min pulse-labelled cell suspension (53 plus 58 kDa band at chase time zero).

DISCUSSION

Our study confirms previous reports that fenofibrate markedly reduced HL expression in rat liver (9). In rats fed a 0.5% fenofibrate chow diet for 14 days the HL activity in liver homogenates was 40% lower compared to control rats. When the HL activity was expressed per μg DNA the decrease was 25%, indicating that the HL activity expressed per cell was decreased. The cells of fenofibrate-treated rats were enlarged, which is in line with increase in total liver weight reported by Staels et al (9). However, the pool of heparin-releasable HL activity in the liver was similar in control and fenofibrate-treated rats. Hence, the fall in total liver HL activity must be attributed to a decrease in the non-heparin-releasable pool of liver HL. This latter pool may reflect intracellular HL either before being secreted or after internalization. In contrast to the study of Staels et al (9), the heparin-releasable HL activity in the liver of our rats was not affected by fenofibrate. The nature of this difference is unclear, but may reflect differences in nutritional status of the animals in both studies. The rats used here were fed. In fasted rats HL activity is downregulated by catecholamines (22, 23), which might also be the case for fenofibrate-treated rats. Alternatively, a pool of extracellular HL bound to sites sensitive to 25 U/ml heparin-concentrations, but not to levels used by others may exist.

In parallel with the 50% reduction in HL mRNA, secretion of HL activity by hepatocytes from fenofibrate-treated rats was decreased up to 70% compared to control. In addition, pulse-labelling experiments showed that the incorporation of [^{35}S]methionine in newly synthesized HL was reduced by 55% in the hepatocytes of fenofibrate-treated rats. The secretion of HL activity by hepatocytes of fenofibrate-treated rats was linear with time. Pulse-chase experiments showed that HL maturation in fenofibrate-treated rats was similar to controls. Hence, maturation and secretion of de novo synthesized HL appear not to be affected by fenofibrate-treatment under the condition used.

Despite the reduction in HL mRNA, synthesis and secretion, the total HL activity in the liver was only slightly reduced, which was completely attributable to the fall in the heparin-non releasable pool. These data agree with previous studies showing that the HL expression is regulated by fenofibrate at transcriptional level, but also at the post-translational level (9). Our data suggest that the turnover of secreted HL in fenofibrate-treated rats is markedly reduced in respect to control animals. Since this effect of fenofibrate was not observed previously in fasted rats, the turnover of HL may not be a restrictive factor during fasting. This is probably the first time that regulation of HL expression is attributed to 'decreased turnover'. And it may well be an additional site of post-transcriptional regulation of HL expression in the rat liver, besides the translation (18), maturation (23) and binding (24, 25) of HL.

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CHAPTER 5

Secretion and intracellular processing of hepatic lipase

CHAPTER 5.1

Secretion and intracellular activation of human hepatic lipase requires proper oligosaccharide processing.

Adrie J.M. Verhoeven, Bernadette P. Neve and Hans Jansen

5.1 SECRETION AND INTRACELLULAR ACTIVATION OF HUMAN HEPATIC LIPASE REQUIRES PROPER OLIGOSACCHARIDE PROCESSING.

ABSTRACT

Human hepatic lipase (HL) is a glycoprotein with four N-linked oligosaccharide side chains. The importance of glycosylation in the intracellular activation and secretion of HL was studied in HepG2 cells using various inhibitors of N-glycosylation, oligosaccharide processing and vesicular transport. Secretion of HL was inhibited by tunicamycin, castanospermine, N-methyldeoxynojirimycin, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), monensin and brefeldin A, but not by 1-deoxymannojirimycin. Secretion of α_1-antitrypsin, an unrelated N-glycoprotein, was inhibited by tunicamycin, monensin and brefeldin A but not by castanospermine, N-methyldeoxynojirimycin and CCCP. Intracellular HL activity was decreased with tunicamycin, castanospermine, N-methyldeoxynojirimycin and CCCP, but increased with monensin and brefeldin A. In the absence of protein de novo synthesis, HL activity secreted into the medium was 8.5 fold higher than the HL activity that disappeared from the cells, suggesting a secretion-coupled activation of human HL. In cells pre-treated with monensin or brefeldin A, this factor ranged from 1.1 to 1.9, indicating that the apparent activation of HL had already occurred. We conclude that oligosaccharide processing by glucosidases in the endoplasmic reticulum is necessary for transport of human HL, but not α_1-antitrypsin, from the endoplasmic reticulum to the Golgi, where HL acquires triglyceridase 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 hydrolyzes 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 may act as a ligand protein for remnant binding to the liver (9). By contributing to reverse cholesterol transport and by reducing the number of atherogenic remnants in the circulation, HL is thought to protect against the development of premature atherosclerosis. Indeed, a low HL activity appears to be associated with an increased atherosclerotic risk (10, 11). In line with this, aortic accumulation of cholesterol was markedly reduced in cholesterol-fed transgenic mice that over-express human HL (12).

HL is a glycoprotein bearing two (rat) to four (human) asparagine-linked glycans (13-15). For the synthesis and secretion of fully active HL by rat hepatocytes, N-glycosylation is a prerequisite (16, 17). This was confirmed by expression studies using HL cDNA constructs in which the glycosylation sites had been removed by site-directed mutagenesis (14, 15). These studies demonstrated that occupation of Asn56/57 in human and rat HL is both necessary and sufficient for

secretion of catalytically active lipase (14). When glycosylation is prevented, either by tunicamycin or by site-directed mutagenesis, inactive HL protein accumulates intracellularly (14, 18). When expressed in Xenopus oocytes, however, a small portion of the non-glycosylated rat HL protein was found to be secreted and catalytically active (19). This observation argues against the possibility that N-glycosylation per se is necessary for catalytic activity. Rather, N-glycosylation may be required for 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 (17, 20). 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 cis/medial Golgi and glycosyltransferases in medial/trans Golgi (21). Notably, trimming of the terminal glucose residues by the RER glucosidases appears to be crucial for the acquisition of catalytic activity and secretion of rat HL protein (17, 20). Once the glucose residues have been removed, activation and subsequent secretion continues independently of further oligosaccharide processing. Glucose trimming of N-glycoproteins has been implicated in the RER quality control system for newly synthesized proteins, which prevents malfolded proteins from leaving the RER (22, 23). Whether the presence of terminal glucoses itself prevents the acquisition of catalytic activity of rat HL, or primarily interferes with transport to the Golgi where subsequently activation may occur, is unknown.

In the present study, we examined the role of N-linked glycosylation in the secretion and activation of human HL by HepG2 cells. The four putative N-glycosylation sites identified in the HL cDNA (24) all appear to be occupied in HL secreted by these cells (14, 25). First, we studied the importance of oligosaccharide processing in secretion and intracellular activation of human HL using inhibitors of glucose trimming in the RER (castanospermine and N-methyldeoxynojirimycin) and mannose trimming in the cis-Golgi (1-deoxymannojirimycin) (26, 27). Secondly, we determined in which intracellular compartment HL protein is apparently activated, by using inhibitors that primarily affect vesicular transport in the secretory pathway. CCCP and monensin inhibit transport of glycoproteins from the RER to the Golgi (28, 29) and from medial- to trans-Golgi (21), respectively. Brefeldin A blocks the transport of proteins into post-Golgi compartments and induces the redistribution of the Golgi into the RER (30, 31). The effects of the inhibitors on expression of HL were compared to that of α_1-antitrypsin, an unrelated N-glycoprotein. The data show that proper oligosaccharide processing by RER glucosidases is essential for secretion of fully active HL but not for secretion of α_1-antitrypsin. Glucose trimming appears to be necessary for the translocation of HL protein to the Golgi compartment, where the protein apparently acquires its triglyceridase activity.

MATERIAL AND METHODS

Materials

Castanospermine, 1-deoxymannojirimycin, (+)-brefeldin A and cycloheximide were from Boehringer Mannheim (Mannheim, Germany), whereas N-methyldeoxynojirimycin and Endo-H were from Genzyme (Boston, MA, U.S.A.). Monensin and CCCP were purchased from Calbiochem (La Jolla, CA, U.S.A.). Heparin was obtained from Leo

Pharmaceuticals (Weesp, The Netherlands). Trasylol was from Boehringer Mannheim (Mannheim, Germany); other protease inhibitors and tunicamycin were from Sigma (St. Louis, MO, U.S.A.). Media, fetal bovine serum and Tran^{35}S-label (1 100 Ci/mmole) were obtained from ICN (Costa Mesa, CA, U.S.A.). Culture plastics and EIA code 3590 plates were from Costar (Cambridge, MA, U.S.A.). Glycerol tri[1-^{14}C]oleate (50-80 mCi/mmole) was from Amersham (Amersham, UK). Rabbit antibodies against human α_1-antitrypsin and horse-radish peroxidase-conjugated rabbit anti-goat IgGs were from Dakopatts (Glostrup, Denmark). Zysorbin was from Zymed Laboratories (San Fransisco, CA, U.S.A.)

HepG2 cell culture and incubation

Human hepatoma cells HepG2 were grown as monolayer cultures in Dulbecco's minimum essential medium supplemented with 10 % fetal bovine serum (25). 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 6-wells plates. At confluency, the medium was replaced by 1 ml of fresh medium containing 25 U/ml of heparin, and the incubations were started with the addition of inhibitors. Brefeldin A, CCCP and monensin were added from 1000-fold stock solutions in ethanol, whereas tunicamycin was added from a 1000-fold stock in dimethyl sulfoxide; other inhibitors were added from 100-fold stocks in PBS. The cell cultures were incubated for the indicated times 37 °C in a humidified atmosphere of 95 % O_2/5 % CO_2. At the end of the incubation, the plates were placed on ice and all subsequent handlings were at 4 °C. When the cells were to be re-incubated, however, the plates were not allowed to cool down below room temperature. The medium was collected for analysis of secreted HL. The cell layer was washed twice with PBS and then, the cells were released from the plates by a 5-min incubation in PBS containing 2.5 mM EDTA. After centrifugation (15 sec, 10 000*g*), the cells were resuspended in 250 µl of a 40 mM NH_4OH buffer, pH 8.1 (32) containing 25 U/ml heparin and a cocktail of protease inhibitors (1 mM EDTA, 10 U/ml Trasylol, 0.1 mM benzamidine and 2 µg/ml each of leupeptin, antipain, chymostatin and pepstatin). After 30 min on ice, the lysates were sonified (15 sec, 14 µ, MSE Soniprep 150) and centrifuged for 10 min at 10 000*g*. The supernatants were used for analysis of intracellular HL.

Hepatic lipase activity

HL activity was determined by a triacylglycerol hydrolase assay at pH 8.5 in 0.6 M NaCl using a gum acacia stabilized glycerol [^{14}C]trioleate emulsion as substrate (17). Assays were performed for 2 h at 30 °C. Activities were expressed as mU (nmoles of free fatty acids released per min). In a total assay volume of 125 µl, release of free fatty acids was linear with time and sample volume up to 50 µl for the cell-free media and 10 µl for the cell lysates.

In immuno-inhibition assays, 40 µl of the cell-free media or 10 µl of the cell lysates were pre-incubated for 3 h on ice in a total volume of 50 µl with 50 µg goat anti-human HL IgGs. This antibody was raised against partly purified human HL from postheparin plasma as previously described (33). After centrifugation (10 min, 10 000*g*, 4 °C), 75 µl of substrate was added to the supernatant, and the residual immunoresistant triglyceridase activity was determined.

Hepatic lipase 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 postheparin plasma as described by Martin et al. (24) and by Persoon et al. (33), respectively. From the antisera partly purified IgG fractions were prepared by 50% ammoniumsulfate precipitation and elution through a human albumin-Sepharose column. Polystyrene 96-well EIA plates were coated with the rabbit anti-HL IgGs. After blocking with 1% bovine serum albumin in PBS, the wells were incubated successively with: (i) sample; (ii) 3 µg/ml of goat anti-HL IgGs in PBS, and (iii) peroxidase-conjugated rabbit anti-goat IgGs at a 1:10 000 dilution in PBS. Finally, the presence of peroxidase was detected with 3,3',5,5'-tetramethylbenzidine (Merck, Darmstadt, Germany) as substrate. Color development was stopped with H_2SO_4 (4 M, final concentration), and the absorbance at 450 nm 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 mU/ml, whereas HL protein mass was determined at 23.6 µg/ml by reading this plasma against a standard curve of purified HL. The ELISA enabled accurate measurements of the amount of human HL in the range of 5 to 500 ng/ml.

Alpha-1 antitrypsin

Synthesis and secretion of α_1-antitrypsin was measured by 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 another 3h. Thereafter, the plates were put on ice. The medium was collected into vials containing cold methionine (final concentration 1 mM) and the cocktail of protease inhibitors described above. After washing twice with cold PBS, the cells were lysed in cold PBS containing 1% Triton X-100, 1% sodium deoxycholate, 0.25% SDS, 1 mM methionine, 25 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 10 000 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 of 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% Triton X-100, 0.25% SDS, 0.25% sodium deoxycholate and 1 mM PMSF, and twice in PBS. Washed pellets were resuspended in Laemmli's sample buffer, immunoprecipitated proteins were released by boiling for 5 min and separated by SDS-PAGE in 7.5% gels. The ^{35}S-labelled proteins were visualized, and their radioactivity was determined, by exposure of the dried gels to a phosphor screen (BioRad GS-393 Molecular Imager System, Richmond, CA, U.S.A.).

In some experiments, immunoprecipitated proteins were released by boiling for 5 min in a 50 mM NaP_i buffer, pH 6.0, containing 0.25% SDS. After removal of the bacterial membranes (5 min, 10 000*g*, 4 °C), the eluted proteins were incubated overnight with 40 mU/ml Endo-H at 37 °C in 50 mM NaP_i, 0.1% SDS (34). The digestion was stopped by addition of Laemmli's sample buffer, and after boiling the samples were analysed by SDS-PAGE and phosphor imaging, as described earlier. Endo-H sensitivity was indicated by an increase in electrophoretic mobility.

Protein de novo synthesis

Incorporation of [^{35}S]methionine into TCA-precipitable material was taken as a measure for overall protein de novo synthesis. HepG2 cells were incubated with Tran^{35}S-label, and cell-free media and cell lysates were prepared as described earlier. Five-μl aliquots were spotted in duplicate onto Whatman 3MM filters (Whatman, Maidstone, UK), and TCA-precipitation was performed as previously described (17). The radioactivity on the filters was measured using the Molecular Imaging System.

Statistics

Statistical significances were determined by two-way 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 (Fig. 1A) while intracellular activity remained virtually constant throughout the 24 h incubation period (Fig. 1B). The lipase activity present in the extracellular medium was largely sensitive to immuno-inhibition with anti-human HL IgGs (Fig. 1C), indicating that the extracellular activity represents HL. Of the lipase activity measured in the cell lysates, however, approximately 65% was resistant to immuno-inhibition suggesting that HL represented only a small fraction of total intracellular lipase activity. When cycloheximide was added at the start of the incubation, secretion of HL activity was hardly affected during the first 3 h (Fig. 1A), although inhibition of protein de novo synthesis occurred almost instantaneously (Fig. 1D). Simultaneously, intracellular lipase activity decreased (Fig. 1B) indicating that pre-existing HL was secreted from the cells during the incubation with cycloheximide. Between 3 and 6 h after addition of cycloheximide, secretion of HL

activity became completely blocked, while intracellular lipase activity continued to fall throughout the incubation. After 22 h, intracellular lipase activity was no longer sensitive to immunoinhibition with anti-HL (Fig. 1C). Both the immunosensitive and immunoresistant fractions of intracellular lipase were reduced by the prolonged cycloheximide treatment of the cells. Comparison of the data in Fig. 1A and B shows that in the absence of protein de novo synthesis much more HL activity appeared in the extracellular medium than was lost from the cells (0.5 *vs* 0.05 mU/well at 6 h incubation). Hence, intracellular HL is apparently activated during the secretory process.

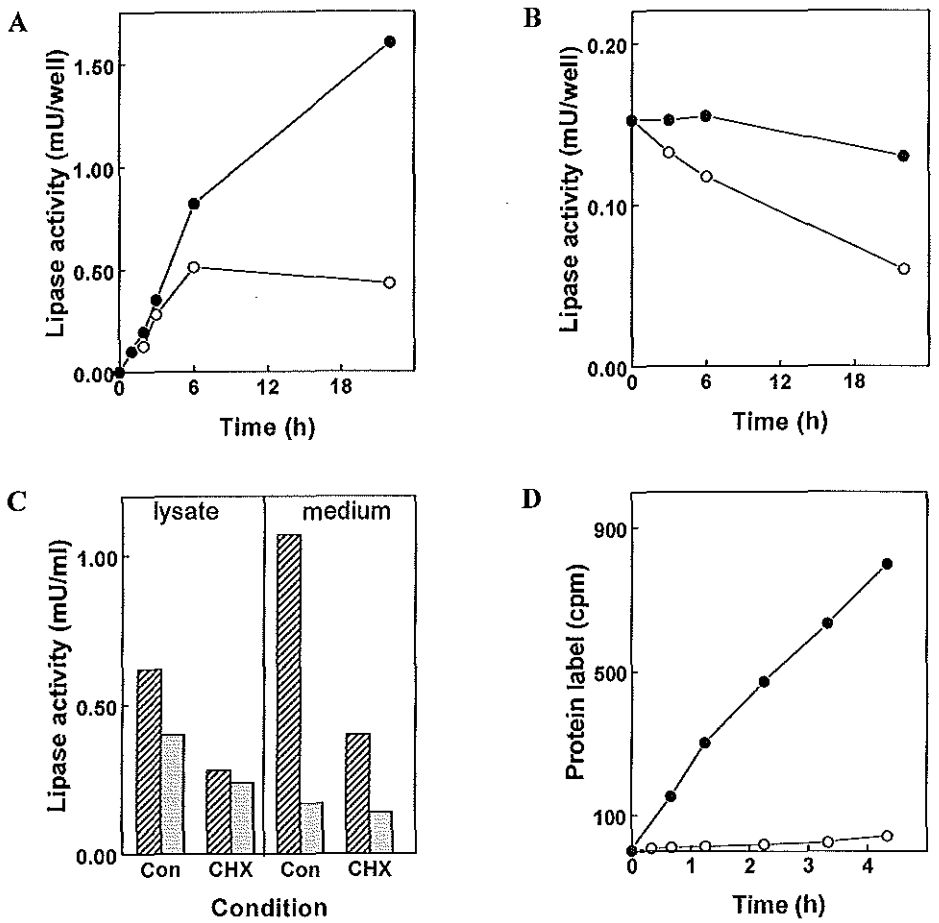
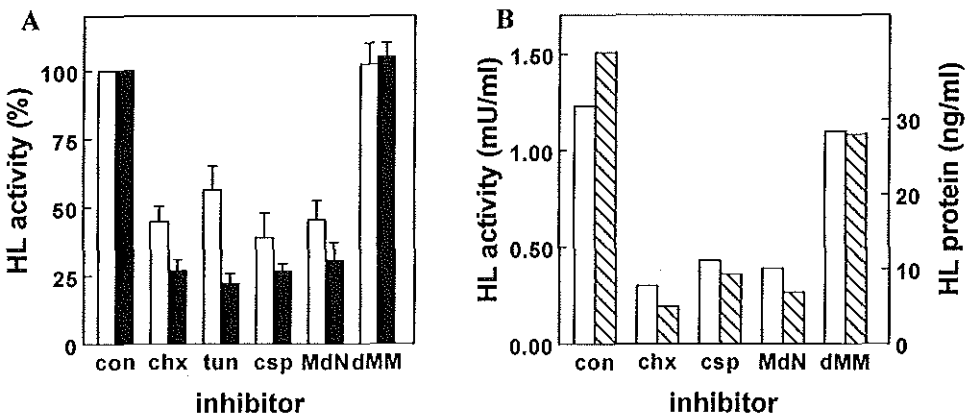


Figure 1 **Synthesis and secretion of hepatic lipase activity by HepG2.**

HepG2 cells were incubated in the presence of 25 U/ml of heparin without (●) or with 20 µg/ml of cycloheximide (○). At the times indicated, the medium and cells were harvested and triglyceridase activity was measured in the medium (A) and cell lysates (B). (C); Cell lysates and cell-free media were prepared from HepG2 cells that had been incubated for 22 h without (con) or with cycloheximide (chx), and triglyceridase activity was measured before (left bars) and after (right bars) incubation with goat anti-HL IgG's. (D); Effect of cycloheximide treatment on [^{35}S]methionine incorporation into TCA-precipitable material. The data points are averages of ^{35}S-radioactivity precipitated in duplicate from aliquots of cell lysates plus cell-free media; note the difference in time scale between the panels A, B and D. All data are representative for 2 similar experiments.

Effect of N-glycosylation inhibitors on secretion.

In the presence of tunicamycin, secretion of HL activity into the medium was inhibited to a similar extent as with cycloheximide, both after 6-8 h and 18-22 h of incubation (Fig. 2A). With the RER glucosidase inhibitors CSP and MdN, secretion of HL activity was inhibited to a similar extent as with tunicamycin and cycloheximide. Secretion of HL protein was reduced in parallel to that of HL activity (Fig. 2B). With the mannosidase inhibitor dMM, secretion of both HL activity (Fig. 2A) and protein (Fig. 2B) continued almost unaffected.



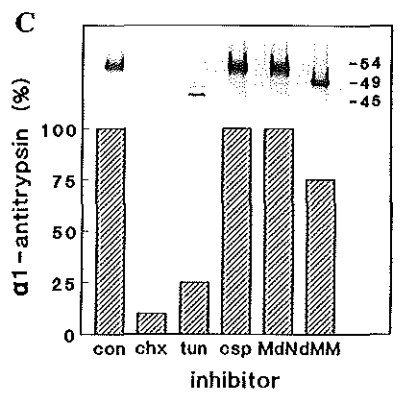


Figure 2 **Effect of glycosylation inhibitors on secretion of HL and α_1-antitrypsin.**

HepG2 cells were incubated in the absence (con) or presence of 20 µg/ml of cycloheximide (chx), 10 µg/ml of tunicamycin (tun), 100 µg/ml of castanospermine (csp), 1 mM N-methyldeoxynojirimycin (MdN) or 1 mM deoxymannojirimycin (dMM). The extracellular appearance of HL (A, B) and α_1-antitrypsin (C) was measured as outlined in the Method section. (A) HL activities in the extracellular medium were determined after 6 to 8 h (open bars) and 18 to 22 h (closed bars) of incubation; data (means ± SEM; n = 6-7) are expressed as percentage of control, which was 1.02 ± 0.10 and 2.49 ± 0.37 mU/well after 6 to8 h and 18 to 22 h, respectively. Except for dMM, the HL activity in the media containing glycosylation inhibitors was significantly different from controls at both time points ($p<0.05$). (B) HL activity and amount of HL protein, as determined by ELISA, both measured in the same extracellular media after 6 h incubation. (C) α_1-Antitrypsin was immunoprecipitated from 6-h incubation media also containing Tran^{35}S-label, and the radiolabelled proteins were analyzed by SDS-PAGE and phosphor imaging. Part of the image is shown at the top; the molecular size of the radioactive bands is indicated in kDa. The radioactivity in each band (in arbitrary units) was expressed relative to the control incubation. The data in panels B and C are representative for 2 similar experiments.

HepG2 cells secreted α_1-antitrypsin into the extracellular medium, which on SDS-PAGE migrated as a 54 kDa protein band (Fig. 2C). In the presence of tunicamycin, secretion was markedly reduced. The small amount of α_1-antitrypsin in the extracellular medium migrated at an apparent molecular mass of 45 kDa which corresponds to the nonglycosylated protein. In the presence of CSP or MdN, however, the cells secreted similar amounts of α_1-antitrypsin as control cells. On SDS-PAGE, the protein was indistinguishable from α_1-antitrypsin secreted by control cells; the bands migrated at the 54 kDa position of the mature protein (Fig. 2C), and migration was also not affected by Endo H treatment (not shown). Secretion of α_1-antitrypsin was hardly affected by incubation of the cells with dMM, but the electrophoretic mobility of the protein was higher than in controls. The α_1-antitrypsin migrated as a sharp band at approximately 49 kDa (Fig. 2C), which decreased to 45 kDa upon Endo-H treatment (not shown). This protein therefore corresponds to the high-mannose type form of α_1-antitrypsin. The most conspicuous difference between secretion of HL and α_1-antitrypsin by the HepG2 cells was that HL was highly sensitive to the RER glucosidase inhibitors CSP and MdN, while α_1-antitrypsin was not.

Intracellular effects of the N-glycosylation inhibitors

In cells treated for 6-8 h with either tunicamycin, CSP or MdN, intracellular lipase activity was approximately 75-80% of control cells, which is slightly higher than in the cycloheximide-treated cells (Fig. 3A). Immuno-inhibition assays using anti-human HL IgGs showed that the fall in intracellular lipase activity was almost completely due to a decrease in the immunosensitive part (not shown). With tunicamycin, overall protein de novo synthesis was reduced to 86 ± 10% of control compared to 11 ± 4% with cycloheximide (mean ± SEM, n = 4). With CSP and MdN, protein de novo synthesis was only slightly reduced to 93 ± 6% and 97 ± 3% of control, respectively (mean ± SEM, n = 3). Upon prolonged incubation, the intracellular lipase activity did not fall any further except for the cycloheximide-treated cells. In cells treated with dMM, intracellular lipase activity was maintained at control levels throughout the entire incubation (Fig. 3A). Under all conditions tested, the amount of HL protein in the cell lysates remained below the detection limit of the ELISA. Hence, there is no evidence for intracellular accumulation of (inactive) HL protein in the cells under conditions where secretion of HL is abolished.

Intracellular levels of α_1-antitrypsin were not affected by treating the cells with either tunicamycin, CSP, MdN or dMM. However, the electrophoretic mobility of the respective proteins differed markedly from control (Fig. 3B). α_1-Antitrypsin from control cell lysates migrated predominantly as a 49 kDa, Endo-H sensitive band and a fainter, Endo-H resistant band at 54 kDa (Fig. 3B). Treatment of the HepG2 cells with tunicamycin resulted in the intracellular accumulation of α_1-antitrypsin mainly with a molecular mass of 45 kDa (not shown). In CSP- and MdN-treated cells, α_1-antitrypsin migrated predominantly as a 51 kDa, Endo-H sensitive band with the 49 kDa and 54 kDa band also seen in control cells as relatively minor components (Fig. 3B). Finally, α_1-antitrypsin from dMM-treated cells migrated as a 49 kDa, Endo-H sensitive band.

Effect of CCCP, monensin and brefeldin A.

With increasing concentrations of CCCP, secretion of HL activity into the extracellular medium gradually fell to about 30% of control at 10 μM (Fig. 4A). The amount of HL protein fell in

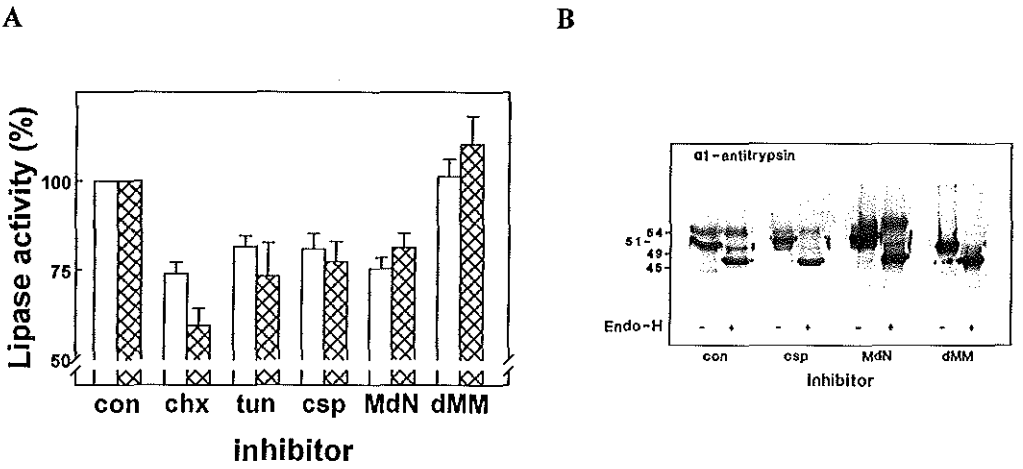


Figure 3 **Effect of glycosylation inhibitors on intracellular HL and α_1-antitrypsin.**

Experiments were performed as described for Fig. 2, and lipase activity (**A**) and radiolabelled α_1-antitrypsin (**B**) were determined in cell lysates prepared after 6 to 8 h (**A**; open bars, and **B**) and 18 to 22 h (**A**; filled bars) of incubation. Data (mean ± SEM, n = 5-6) on intracellular lipase activity were expressed as percentage of control, which was 0.24 ± 0.05 and 0.18 ± 0.03 mU/well, respectively. Except for dMM, the HL activity in the media containing glycosylation inhibitors was significantly different from controls at both time points ($p<0.05$). (**B**) immunoprecipitates prepared from the cell lysates were treated without (-) or with (+) Endo-H before electrophoretic separation; the molecular mass of the radioactive bands (in kDa) is indicated at the left. The data are representative for 2 similar experiments.

parallel. In contrast, 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 and 10 μM CCCP was indistinguishable from the protein secreted by control cells (not shown). Simultaneously, CCCP reduced the lipase activity present in the cell lysate in a concentration-dependent manner (Fig. 4B). With 5 μM CCCP, intracellular lipase activity was similar to the activity in cycloheximide-treated cells. Under all conditions, the amount of intracellular HL protein remained below the detection limit of the ELISA. The amount of intracellular α_1-antitrypsin was not affected by CCCP up to 5 μM (Fig. 4B), and parallelled the effect on overall protein synthesis. Intracellular α_1-antitrypsin consisted of the 49 kDa, Endo H sensitive and the 54 kDa, Endo H resistant protein band also seen in control cells (cf. Fig. 3B); the relative amount of the Endo H resistant band diminished when CCCP was increased from 5 to 10 μM. The 51 kDa protein band present in CSP- and MdN-treated cells was not observed in CCCP-treated cells.

When HepG2 cells were incubated with 10 μM monensin or 0.2 μg/ml BFA, secretion of HL activity was reduced to levels well below that observed with cycloheximide (Table I). The amount of HL protein in the extracellular medium was reduced in parallel. Simultaneously, secretion of newly synthesized α_1-antitrypsin was almost completely blocked by monensin and BFA (not shown). In contrast to cycloheximide, the intracellular lipase activity markedly increased upon

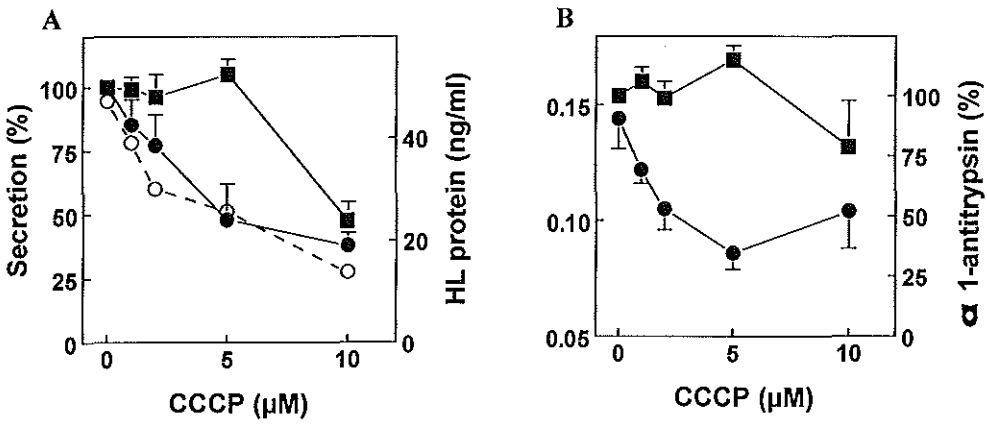


Figure 4 **Effect of CCCP on expression of HL and α_1-antitrypsin.**

HepG2 cells were incubated for 6 h in the presence of different concentrations of CCCP. Thereafter, the lipase activity (•) and amount of HL protein (○) and ^{35}S-labelled α_1-antitrypsin (■) were measured in the extracellular medium (A) and cell lysates (B). Data represent mean ± SEM for 3 independent experiments, except for the amount of HL protein which was measured in 1 experiment. Data on HL activity are expressed as a percentage of control (0.66 ± 0.08 mU/ml); similarly, data on radio-activity in α_1-antitrypsin (expressed in arbitrary units) are given as percentage of control.

Table I **Effect of monensin and brefeldin-A on expression of HL.**

Treatment:	none	cycloheximide (20 μg/ml)	monensin (10 μM)	brefeldin-A (0.2 μg/ml)
Secretion				
HL activity (mU/ml)	0.92 ± 0.09	0.37 ± 0.03[a]	0.27 ± 0.02[a]	0.19 ± 0.02[a]
HL protein (ng/ml)	13.1	< 5	< 5	< 5
Intracellular lipase				
Activity (mU/ml)	1.16 ± 0.18	0.89 ± 0.11[a]	1.54 ± 0.15[a]	1.95 ± 0.12[a]
HL protein (ng/ml)	< 5	< 5	6.4	8.8
Overall protein synthesis (% of control)	100	11.2 ± 3.6[a]	97.8 ± 3.8	74.7 ± 11.3[a]

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 [^{35}S]methionine incorporation into TCA-precipitable material. Data are expressed as mean ± SEM for 3-5 independent experiments. [a] : statistically significant difference from controls ($p < 0.05$).

incubation with monensin or BFA (Table I). Immunoinhibition with anti-HL IgGs showed that this increase occurred exclusively in the immunosensitive fraction (not shown). In addition, the amount of HL protein was also elevated to levels well above the detection limit of the ELISA (Table I). Overall protein de novo synthesis was not affected by monensin, whereas it was reduced approximately 25% by BFA. With both agents, α_1-antitrypsin accumulated intracellularly as a 49 kDa protein; the 54 kDa, Endo H resistant form was absent. The 49 kDa protein from monensin-treated cells was completely Endo H sensitive whereas the protein from BFA-treated cells was partly Endo H resistant (not shown).

Combined effect of brefeldin A 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 reduce intracellular lipase activity in HepG2 cells (Table II). Instead, intracellular lipase activity increased upon incubation with BFA whether or not CSP or MdN were present. By ELISA, similar results were found for intracellular HL protein (not shown). The BFA-induced inhibition of HL secretion (Table II) 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 both in the absence and presence of BFA (not shown). This observation suggests that the glucosidase inhibitors were still effective in the presence of BFA. Nevertheless, their inhibitory effect on intracellular HL activity was overcome by co-incubation with BFA.

Table II

Effect of co-incubation of glycosylation inhibitors and brefeldin A on HL expression.

	intracellular lipase (mU/well)		extracellular HL (mU/well)	
Treatment	-BFA	+BFA	-BFA	+BFA
none	0.23 ± 0.05	0.36 ± 0.08	1.28 ± 0.15	0.26 ± 0.06
tunicamycin (10 μg/ml)	0.18 ± 0.07[a]	0.33 ± 0.10	0.60 ± 0.18[a]	0.26 ± 0.09
castanospermine (100 μg/ml)	0.17 ± 0.06[a]	0.32 ± 0.09	0.47 ± 0.07[a]	0.20 ± 0.10
methyldeoxynojirimycin (1 mM)	0.17 ± 0.06[a]	0.36 ± 0.09	0.65 ± 0.18[a]	0.22 ± 0.08

HepG2 cells were incubated for 6-8 h in the presence of heparin without or with brefeldin-A (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. Data are expressed as mean ± SEM for 3-5 independent experiments. [a]: statistically significant difference from the controls incubated without glycosylation inhibitor ($p < 0.05$).

Activation of HL during the secretion process.

We noted a discrepancy between the HL activity secreted by HepG2 cells in the absence of protein de novo synthesis and the concurrent disappearance of lipase activity from the cells (Fig. 1A and B). To examine this in more detail, we first incubated cells for 6-8 h without or with BFA or monensin in the presence of heparin. After removing the extracellular medium, the cells were re-incubated overnight but now in the presence of cycloheximide. As shown in figure 5, the intracellular lipase activity of the control cells fell by about 0.06 mU/well while the extracellular HL activity increased to 0.50 mU/well despite the absence of protein de novo synthesis, suggesting that HL is activated during the secretion process. In seven similar experiments, this apparent activation was by a factor 7.8 ± 0.8 (mean ± SEM). Inhibition of secretion by BFA and monensin was at least partly reversible. Upon their removal from the medium, secretion of HL activity resumed even in the presence of cycloheximide. Now, the HL activity secreted by the BFA-pretreated cells in the presence of cycloheximide was similar to the concomitant fall in intracellular lipase activity. In six such experiments the apparent activation factor was 1.3 ± 0.2 for BFA-treated cells, whereas 1.9 was found in an experiment with monensin-treated cells. Hence, the apparent activation of HL that normally occurs during the secretory process has already taken place in BFA- and monensin-treated cells.

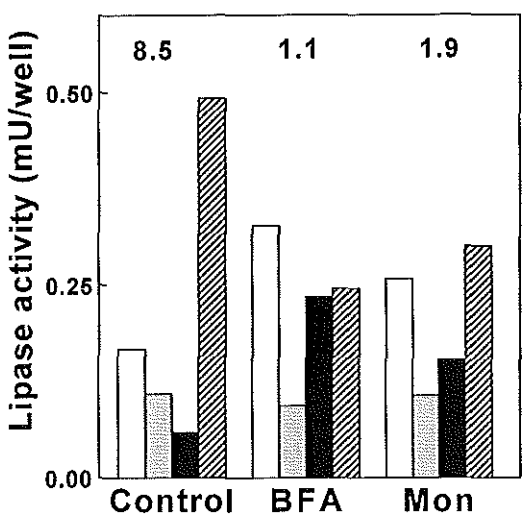


Figure 5 **Activation of HL during secretion in the absence of protein de novo synthesis.**

HepG2 cells were pre-incubated for 6 h with 25 U/ml heparin in the absence (control) or presence of 0.2 μg/ml brefeldin A (BFA) or 10 μM monensin (Mon). Per treatment group, 2 wells were immediately harvested for measurement of intracellular lipase activity (left, open bars). Two other wells were washed free of inhibitor with PBS (room temperature), and incubation was continued overnight in fresh medium containing heparin and 20 μg/ml of cycloheximide to prevent additional HL de novo synthesis. Thereafter, media and cells were harvested. Lipase activity was determined in the cell lysates (middle, shaded bars) and the difference with the activity measured in the cells before the overnight incubation with cycloheximide was calculated (middle, dark bars). This difference was compared with the HL activity that was secreted into the extracellular medium during the overnight incubation with cycloheximide (right, shaded bars). The data for the duplicate incubations were averaged. The numbers above the bars indicate the ratio of secreted activity to the decrease in intracellular activity, and reflect the apparent activation of HL during secretion.

DISCUSSION

Previous studies using CHO cells transfected with HL cDNA constructs have demonstrated that N-linked glycosylation of rat and human HL is essential for secretion of a catalytically active protein by these cells (14, 15). Removal of the N-glycosylation sites from rat HL cDNA results in the secretion of a small amount of fully active enzyme in the Xenopus oocyte system, indicating that N-glycosylation is not necessary for catalytic activity per se, but rather for proper intracellular trafficking of newly synthesized protein (19). Here, we show for the first time that secretion and intracellular activation of endogenous HL by HepG2 cells not only require N-glycosylation but also glucose trimming of the N-linked oligosaccharide chains by RER glucosidases. In this respect human HL behaves similarly to rat HL (17, 20), but differently from a number of other N-glycoproteins including α_1-antitrypsin (Fig. 2) (34, 35). Glucose trimming by RER glucosidases I and II has been implicated in the quality control system of the secretory pathway, which prevents malfolded or unassembled glycoproteins from leaving the RER for the Golgi (22, 23). In this model, glycoproteins bearing terminal glucose residues associate with the molecular chaperone calnexin, which assists in the folding process (22, 36, 37). Our observations suggest that the 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 fraction 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 HL, secretion of α_1-antitrypsin was unaffected by CSP or MdN. In the presence of these inhibitors, maturation of α_1-antitrypsin proceeded through a distinct intracellular intermediate of 51 kDa (Fig. 3B) (34). Despite inhibition of the RER glucosidases, α_1-antitrypsin appeared to be normally deglucosylated and further processed to an Endo H resistant form. In HepG2 cells, removal of glucosylated mannose from oligosaccharides has been shown to occur through an endo-α-D-mannosidase present in the Golgi, thereby providing an alternate processing route for α_1-antitrypsin and several other N-glycoproteins (35, 38). Utilization of this alternate route requires that these glycoproteins are 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 terminal glucose residues prevents HL protein but not α_1-antitrypsin from leaving the RER. Perhaps, α_1-antitrypsin folds into a transport-competent form without the help of calnexins. Compared to α_1-antitrypsin, the maturation of HL was also much more sensitive to inhibition by CCCP (Fig. 4), an H^+-ionophore and mitochondrial uncoupler. This observation may indicate that folding of HL into a transport-competent structure requires some additional pH or ATP dependent steps. Alternatively, proper folding of HL may require assembly into an oligomeric form in contrast to the monomeric α_1-antitrypsin. In this respect, it is noteworthy that human and rat HL behave like multimeric proteins on gel-filtration (39, 40).

Our data suggest that human HL acquires catalytic activity during maturation in the secretory pathway. In the HepG2 cells, the enzyme activity of HL increased 5-10 fold upon secretion, which is similar to the activation seen with rat HL (20). Several lines of evidence suggest that this apparent activation occurs in the cis- to medial-Golgi region. First, when HL protein is retained within the RER, either by treating the cells with the glucosidase inhibitors CSP or MdN (Fig. 2), or with CCCP

(Fig. 4), intracellular HL activity decreases compared to untreated cells although protein de novo synthesis is hardly affected. Secondly, the apparent activation has already occurred in cells treated with monensin which inhibits intra-Golgi transport (Fig. 6). 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 activation of HL is completely reversed (Table II). How HL is modified in the cis-to-medial Golgi remains unknown. Unfortunately, our anti-human HL preparations did not allow reliable immunoprecipation from HepG2 cell lysates and hence, analysis of the glycosylation state of HL in monensin- or BFA-treated cells could not be performed. It is unlikely, however, that trimming of mannose residues by Golgi mannosidase I itself is the mechanism of activation, since in situ inhibition of this process by dMM has no effect on the intracellular activation of HL.

The effects of the glucosidase and vesicle transport 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 activation occurs. For LPL, which is closely related to HL, glucose trimming in the RER is also necessary for the acquisition of lipase activity (41-43). Whereas HL is fully active as a monomer (44), LPL must form a homodimer in order to become catalytically active towards emulsified substrates (45-47). A recent study showed that LPL could dimerize in the RER without acquiring catalytic activity (48, 49). Similar to HL, glucose trimming in the RER may therefore be a prerequisite for translocation of LPL to the Golgi, and hence for secretion of an active protein (42, 48). More detailed studies are necessary to elucidate the activating principle for HL as well as LPL protein within the Golgi.

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CHAPTER 5.2

Intracellular activation of hepatic lipase in rat hepatocytes requires transport to the Golgi compartment.

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5.2 INTRACELLULAR ACTIVATION OF HEPATIC LIPASE IN RAT HEPATOCYTES REQUIRES TRANSPORT TO THE GOLGI COMPARTMENT.

SUMMARY

Hepatic lipase (HL) is an N-glycoprotein that acquires triglyceridase activity somewhere during maturation and secretion. To determine where HL is activated, the effect of drugs that interfere with maturation and intracellular transport of HL protein was studied using freshly isolated rat hepatocytes. Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), castanospermine, monensin and colchicin all inhibited secretion of HL without affecting its specific enzyme activity. Incubation with CCCP and castanospermine reduced intracellular HL activity and HL protein, and decreased the specific enzyme activity by 25-50%. With monensin and colchicin, intracellular HL accumulated and the specific enzyme activity was increased 2-fold to values similar to secreted HL. Pulse-labelling with [^{35}S]methionine demonstrated that HL activity varied in parallel with the 58 kDa, endoglycosidase H-resistant HL protein in the secretion media, and in lysates of control, CCCP- and colchicin-treated cells. In monensin-treated cells, the increase in specific enzyme activity coincided with production of a 53 kDa, partly endoglycosidase H-resistant form of HL. Deoxymannojirimycin-treated cells secreted a 53 kDa, endoglycosidase H-sensitive HL with a specific enzyme activity similar to control HL. We conclude that activation of HL (i) occurs after transport from the endoplasmic reticulum to the Golgi compartment, and (ii) does not involve the development of endoglycosidase H resistance.

INTRODUCTION

Hepatic lipase (HL) is an extracellular enzyme present in the liver of most vertebrates. The enzyme is synthesized and secreted by liver parenchymal cells, and subsequently bound in the space of Disse, where it plays an important role in plasma lipoprotein metabolism (1-3). HL hydrolyzes 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) is facilitated. In addition, HL may act as a ligand protein for remnant binding to the liver (9). In humans, a low HL activity is associated with an increased atherosclerotic risk (10, 11). When expressed in transgenic mice, human HL was shown to markedly reduce the accumulation of aortic cholesterol (12). HL may protect against development of premature atherosclerosis by contributing to reverse cholesterol transport and by reducing the number of atherogenic remnants in the circulation. Expression of HL in the liver is under hormonal and dietary control, which may be exerted at the level of synthesis, intracellular processing, secretion, extracellular binding and internalization of HL.

When studying the post-translational control of HL expression in suspensions of freshly isolated rat hepatocytes, we noted that newly synthesized HL acquires catalytic activity towards triacylglycerols somewhere along the secretory pathway (13). First, the specific enzyme activity of

intracellular HL was 3-5 fold lower than that of secreted HL. Secondly, HL activity secreted by hepatocytes in the absence of protein de novo synthesis was 5-fold higher than was accounted for by the fall in the intracellular HL activity. Such an apparent activation was also observed for human HL in the HepG2 hepatoma cell line (Chapter 5.1). HL is a glycoprotein bearing two (rat) to four (human) asparagine-linked glycans (14-16). For the synthesis and secretion of fully active HL, N-glycosylation is a prerequisite (17, 18). When glycosylation is prevented, either by tunicamycin or by site-directed mutagenesis, inactive HL protein accumulates intracellularly (15, 19). Along the secretory pathway, the N-linked oligosaccharide chains are extensively processed. In rat hepatocytes treated with castanospermine, a selective RER glucosidase inhibitor preventing secretion of newly synthesized HL, inactive HL was present intracellularly; upon removal of the inhibitor, the HL protein acquired catalytic activity and was secreted (13). These observations show that newly synthesized HL protein becomes catalytically active during oligosaccharide processing after the terminal glucose residues have been removed by the glucosidases in the RER, and suggest that activation may be intimately linked to the glycosylation state of the HL protein.

The presence of terminal glucoses on HL protein itself may prevent the acquisition of catalytic activity. However, the glucose residues on N-glycoproteins have recently been implicated in the protein folding and quality control system of the RER, which prevents malfolded proteins from reaching the Golgi (20, 21). It is possible therefore, that glucose trimming is only required for transport of the newly synthesized HL out of the RER and that activation occurs subsequently in a distal compartment of the secretory pathway. In line with this, inhibition of the Golgi mannosidase I with 1-deoxymannojirimycin has no effect on either activation or secretion of HL in rat hepatocytes (13, 18). This suggests that once the glucose residues have been removed, activation and subsequent secretion proceed independently of further oligosaccharide processing.

If glucose trimming in the RER is necessary for activation of HL protein itself rather than for transport of newly synthesized HL protein out of the RER, one would expect that inhibition of the transport process leads to the intracellular accumulation of active HL protein. The present study was performed to test this possibility. We determined in which intracellular compartment HL protein is activated, by using inhibitors that primarily affect vesicular transport in the secretory pathway. CCCP, monensin and colchicin inhibit transport of glycoproteins from the RER to the Golgi (22, 23), from medial- to trans-Golgi (24) and between the Golgi and the plasmamembrane (25, 26), respectively. Our data show that active HL accumulates in monensin- and in colchicin-treated hepatocytes, but not in cells treated with CCCP. Hence, glucose trimming alone does not activate HL but is necessary for translocation of HL protein to the Golgi compartment, where the protein apparently acquires its triglyceridase activity.

EXPERIMENTAL PROCEDURES

Materials

Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) and monensin were purchased from Calbiochem (La Jolla, CA, U.S.A.), whereas colchicin was from Merck (Darmstadt, Germany). Castanospermine and 1-deoxymannojirimycin were from Boehringer Mannheim (Germany). Endo H was from Genzyme (Boston, MA, U.S.A.). Protease inhibitors were from Sigma (St. Louis, MI, U.S.A.), except for Trasylol which was from Bayer (Mijdrecht, Holland). Tran^{35}S-label (1100 Ci/mmole) was obtained from ICN (Costa Mesa, Ca, U.S.A.), and glycerol

tri[1-^{14}C]oleate (50-80 mCi/mmole) was from Amersham (Amersham, UK). Ham's F10 and methionine-free MEM were purchased from Gibco BRL (Breda, Holland), whereas bovine serum was from BioTrading (Wilnis, Holland). Heparin was from Leo Pharmaceuticals (Weesp, Holland). Goat and rabbit anti-HL antisera were raised against rat HL purified from liver heparin perfusates according to Jensen & Bensadoun (27); from the antisera partly purified IgG fractions were prepared by precipitation in 50% ammonium sulphate followed by 17% Na_2SO_4, as described previously (13). Alkaline phosphatase-conjugated goat anti-rabbit IgG was obtained from Tago (Burlingame, CA, U.S.A.), and *p*-nitrophenol phosphate was from Merck. Broad-range protein size markers were from BioRad (Richmond, CA, U.S.A.). All other chemicals were from Sigma. Polystyrene 96-well EIA plates (code 3590) were from Costar (Cambridge, MA, U.S.A.).

Hepatocyte isolation and incubation.

Hepatocytes were isolated from male Wistar rats (200-250 g body weight) by collagenase perfusion: non-parenchymal cells were removed by differential centrifugation (28). Cell viability was determined by Trypan blue exclusion and ranged from 85 to 90%. The cells were suspended at a density of 4x106 cells/ml in MEM containing 25 U/ml of heparin and 20% of dialysed, heat-inactivated bovine serum (29). Cell suspensions were incubated at 37°C under an atmosphere of 5% CO_2/95% O_2 in a shaking water bath. The incubations were started with the addition of inhibitors. CCCP, monensin and colchicin were added from 1000-fold stock solutions in ethanol; other inhibitors were added from 100-fold stocks in PBS. At the times indicated, samples of the cell suspension were collected on ice. The cells were separated from the medium by centrifugation for 5 s at 10 000*g*. The cells were washed once in PBS and then resuspended at 15x106 cells/ml in a 40 mM NH_4OH buffer, pH 8.1 (30) containing 25 U/ml heparin and a cocktail of protease inhibitors (1 mM EDTA, 10 U/ml Trasylol, 0.1 mM benzamidine and 2 µg/ml each of leupeptin, antipain, chymostatin and pepstatin). After 30 min on ice, the lysates were sonicated for 15 s on ice (MSE Soniprep 150, amplitude 14 μ) and centrifuged for 10 min at 10 000*g* and 4°C. The supernatants were used for analysis of intracellular HL. Cell-free media and lysates were rapidly frozen in liquid nitrogen and stored at -80°C until use.

Hepatic lipase activity.

HL activity was determined by a triacylglycerol hydrolase assay at pH 8.5 in 0.6 M NaCl using a gum acacia stabilized glycerol [^{14}C]trioleate emulsion as substrate (18). Assays were performed for 30 min at 30°C. Activities were expressed as mU (nmoles of free fatty acids released per min). In a total assay volume of 125 µl, release of free fatty acids was linear with time and sample volume up to 50 µl for the cell-free media and 10 µl for the cell lysates.

In immuno-inhibition assays, 40 µl of the cell-free media or 10 µl of the cell lysates were pre-incubated for 1 h on ice in a total volume of 50 µl with either 100 µg goat non-immune IgGs or anti-rat HL IgGs. Thereafter, 75 µl of substrate was added to the supernatant, and the residual immunoresistant triglyceridase activity was determined. The lipase activity in the extracellular media was completely inhibited by anti-HL IgGs whereas 85 to 95% of the lipase activity in the cell lysates was sensitive to immuno-inhibition.

Hepatic lipase mass.

The amount of HL protein was determined by a solid-phase ELISA in which the antigen was sandwiched between goat and rabbit polyclonal anti-HL IgGs. EIA plate wells were coated with 20 µg goat anti-HL IgGs. After blocking with 1% BSA in PBS, the wells were incubated successively with: (i) sample, either 50 µl of cell-free medium or 5 µl of cell lysate; (ii) 3 µg/ml of rabbit anti-HL IgGs in PBS, and (iii) alkaline phosphatase-conjugated goat anti-rabbit IgG at a 1:1500 dilution in PBS. Finally, the presence of alkaline phosphatase was detected with *p*-nitrophenol phosphate as substrate. Color development was stopped with NaOH (1 M, final concentration), and the absorbance at 405 nm was measured in a Molecular Devices microplate reader. Absorbances were read against a standard curve prepared for each plate by serial dilutions of rat HL partly purified from liver heparin perfusates by affinity chromatography on Sepharose-heparin; HL activity was eluted from the column by a linear salt-gradient and the peak fractions were pooled. After adding BSA to a final concentration of 1%, aliquots were frozen in liquid nitrogen and stored at -80°C until use.

Pulse-labelling with [^{35}S]methionine.

Freshly isolated rat hepatocytes were incubated in methionine-free MEM in the presence of inhibitors, as described above. After 1 h, 80 µCi of Tran^{35}S-label was added per ml of cell suspension, and the incubation was continued for another 2 hours. The incubations were stopped on ice, and the cells and media were separated by centrifugation (5 s,

10 000*g*). The cell-free medium was collected in vials containing cold methionine (final concentration 1 mM) and the cocktail of protease inhibitors described above. After washing twice with cold PBS, the cells were lysed in cold PBS containing 1% Triton X-100, 1% sodium deoxycholate, 0.25% SDS, 1 mM methionine, 25 U/ml heparin, 10 mM HEPES (pH 7.4) and the cocktail of protease inhibitors. After 30 min on ice, the lysates were centrifuged for 10 min at 10 000*g* and 4°C, and the supernatants were used for further analysis.

Immunoprecipitations.
HL protein was immunoprecipitated from cell-free media and cell lysates by overnight incubation at 4°C with 50 µl of a 50% slurry of goat anti-HL IgGs immobilized onto Sepharose (13). The beads were collected by centrifugation (20 s, 10 000*g*), and washed twice in successively: (i) 1% Triton X-100 in PBS, (ii) 1 M NaCl in PBS, and (iii) PBS (all containing 1 mM PMSF). Finally, the beads were washed once in 50 mM NaP_i, pH 6.0. The immunoprecipitated proteins were released by boiling for 5 min in 50 mM NaP_i, pH 6.0 containing 0.5% SDS, and the beads were removed by centrifugation. The eluate was diluted in 50 mM NaP_i, pH 6.0 to reduce the concentration of SDS to 0.2%. Part of the eluate was incubated overnight at 37°C in the presence or absence of 40 mU/ml of Endo H. After addition of Laemmli's sample buffer and boiling for 5 min, the proteins were separated by SDS-PAGE using 7.5% gels. The gel was Coomassie-stained for estimation of molecular sizes. The ^{35}S-labelled proteins were visualized, and their radioactivity was determined, by exposure of the dried gels to a phosphor screen (BioRad GS-393 Molecular Imager System, Richmond, CA, U.S.A.). Endo-H sensitivity was indicated by an increase in electrophoretic mobility.

Overall protein de novo synthesis.
Incorporation of [^{35}S]methionine into TCA-precipitable material was taken as a measure for overall protein de novo synthesis. Incubations were performed as described above. Of the cell-free media and lysates, 5-µl aliquots were spotted in duplicate onto Whatman 3MM filters (Whatman, Maidstone, UK), and TCA-precipitation was performed as previously described (18). The radioactivity on the filters was measured using the Molecular Imager System. The duplicate measurements, which never differed by more than 5%, were averaged. The data were corrected for the TCA-precipitable material in the media and lysates of a control cell suspension that was put on ice before addition of Tran^{35}S-label.

Statistics.
All data are expressed as means ± S.D. Differences were tested statistically by two-way ANOVA followed by the Student-Newman-Keuls test, and considered significant at $p<0.05$ (31).

RESULTS

Effect of CCCP on HL synthesis and secretion.

When freshly isolated rat hepatocytes were incubated for 3 h in the presence of heparin, HL activity in the cell lysates remained almost constant at 4.3 ± 1.3 mU/ml (n=6), which equals 0.27 ± 0.08 mU/10^6 cells. During this incubation, HL activity in the extracellular medium increased from 0.4 ± 0.1 mU/ml to 9.1 ± 2.6 mU/ml, which corresponds to 2.3 ± 0.6 mU/10^6 cells. In the presence of increasing concentrations of CCCP, the extracellular appearance of HL activity gradually fell (Fig. 1A). Complete inhibition was obtained with 20 µM CCCP and above. Intracellular HL activity decreased to 63 ± 10% (n=3) of controls when cells were incubated with 10 µM CCCP; a further increase in the CCCP concentration did not have an additional effect on intracellular HL activity To study the effect of CCCP on de novo synthesis of HL protein, [^{35}S]methionine was included during the last hour of incubation. Figure 1B shows that CCCP induced a dose-dependent reduction in the ^{35}S-radioactivity of HL protein immunoprecipitated from the media plus lysate (53+58 kDa bands; see later). This paralleled the effect of the inhibitor on incorporation of ^{35}S-radioactivity

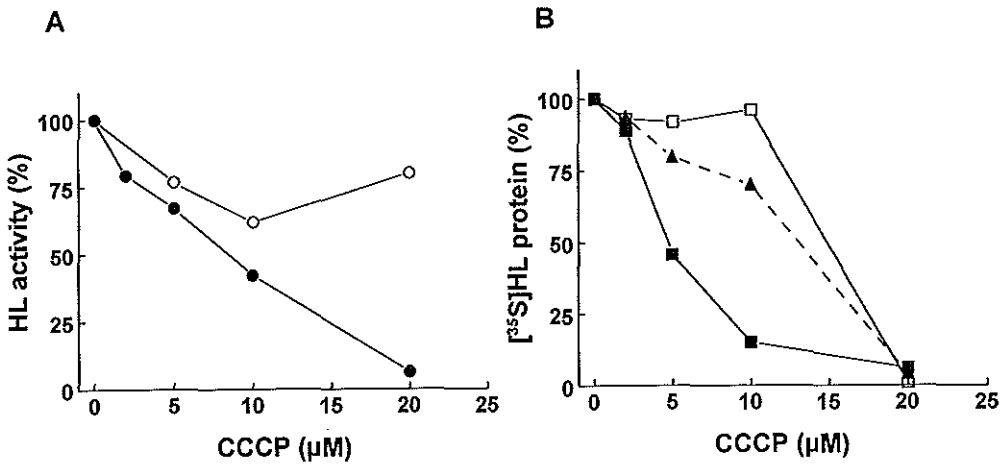

Figure 1 **Effect of CCCP on synthesis and secretion of HL.**

Freshly isolated rat hepatocytes were incubated for 2 h in the presence of different concentrations of CCCP. At the end of the incubation, the HL activity in the cell-free medium (●) and cell lysates (○) was measured (A). Data are expressed as percentage of the activity found for the control incubation, which was 7.2 and 4.3 mU/ml in the cell-free medium and cell lysate, respectively. In parallel incubations, 80 μCi/ml of Tran^{35}S-label was added after 1 h of pre-incubation with CCCP (B). The incubation was continued for an additional hour and then cell-free media (■) and cell lysates (□) were prepared. HL protein was immunoprecipitated by overnight incubation with goat anti-HL IgGs coupled to Sepharose. The immunoprecipitated proteins were separated by SDS-PAGE and the radioactivity in the immunoreactive bands at the 53-58 kDa position in the gels were quantified by phosphor imaging. The sum of the radioactivity in the bands from the lysate and cell-free medium was taken as a measure for total synthesis of HL protein (▲). Data are expressed as percentage of the radioactivity present in the corresponding bands from control media and lysate. The results are representative for 2 similar experiments.

into TCA-precipitable material, and hence on overall protein synthesis (not shown). With 20 μM CCCP and above, HL and overall protein synthesis were completely blocked. When incubated with CCCP up till 10 μM, [^{35}S]HL in immunoprecipitates from the cell lysates was hardly affected, whereas [^{35}S]HL in the extracellular media was highly sensitive to inhibition. With 10 μM of CCCP, where HL synthesis was reduced by approximately 30%, the newly synthesized HL protein was no longer secreted into the extracellular medium but remained in the cells, in agreement with CCCP being an inhibitor of the RER-to-Golgi transport. Therefore, CCCP was used at a final concentration of 10 μM in further studies.

The effect of 10 μM CCCP on HL expression was compared with that of 100 μg/ml of castanospermine, which inhibits RER-to-Golgi transport of N-glycoproteins by interfering with oligosaccharides processing (Table I). After 3 h of incubation, both HL activity and HL protein were reduced in the extracellular medium of CCCP-treated cells by approximately 85% compared to controls, whereas with castanospermine, both parameters were decreased in parallel by 65%. The specific enzyme activity of secreted HL was about 45 mU/μg, which was not significantly affected

by either treatment. Under the conditions used, the specific enzyme activity of HL in the control cell lysates was only 50% of that in the cell-free media. Upon treating the cells with CCCP, the amount of intracellular HL protein was hardly affected although simultaneously, HL activity decreased by approximately 35% (Table I). Hence, the specific enzyme activity of intracellular HL was 25% lower in CCCP-treated cells than in control cells. In the presence of CSP, both HL protein and HL activity in the cells were significantly reduced compared to controls, but the effect on HL activity was stronger than on HL protein. As a result, the specific enzyme activity of residual HL fell by approximately 50%, which was significantly more than with CCCP-treatment ($p<0.05$; n=4). In parallel incubations, the effect of the inhibitors on protein de novo synthesis was determined. Incorporation of [^{35}S]methionine into total TCA-precipitable material was reduced to 52.5 ± 24.4% and 71.2 ± 8.1% of control by CCCP and CSP, respectively (n= 3).

Table I **Effect of castanospermine and CCCP on intracellular and extracellular HL.**

	HL activity		HL protein		specific activity	
	mU/ml	%	µg/ml	%	mU/µg	%
Cell-free media						
control	10.4 ± 1.9	100	0.20 ± 0.06	100	42.8 ± 8.4	100
CCCP	1.4 ± 1.1^{+}	14	0.03 ± 0.02^{+}	15	40.1 ± 15.4	94
CSP	3.4 ± 0.6^{+}	33	0.07 ± 0.02^{+}	37	44.5 ± 12.8	104
Cell lysates						
control	4.8 ± 2.1	100	0.22 ± 0.07	100	23.1 ± 10.5	100
CCCP	3.0 ± 0.2*	63	0.21 ± 0.10	94	17.7 ± 1.3*	76
CSP	1.8 ± 0.5*	36	0.13 ± 0.04*	61	10.9 ± 3.4*	47

Freshly isolated rat hepatocytes were incubated for 3 h in the presence of heparin without further additions (control), or with 10 µM CCCP or 100 µg/ml castanospermine (CSP). Then, cell-free media and cell lysates were assayed for HL activity and HL protein. Data are expressed as mean ± SD for 3-5 independent experiments.* and $^{+}$: statistically significant difference from control lysates and media, respectively ($p<0.05$).

Effect of monensin and colchicin.

During the first 2 h of incubation, the rat hepatocytes secreted HL activity into the medium at a constant rate (Fig. 2A). Upon addition of 50 µM monensin, secretion of HL activity was instantaneously and almost completely inhibited. After 2 h, HL activity in the extracellular medium was 14 ± 4% (n = 4) of parallel controls. Simultaneously, the intracellular HL activity increased linearly with time to 250 ± 44% (n = 4) of control cells (Fig. 2B). Under these conditions, [^{35}S]methionine incorporation into total protein was 44.2 ± 13.1% of control (n = 3). Similar results were obtained when 50 µM colchicin was added to the cell suspension at the start of the incubation. The secretion rate of HL activity was reduced to 45 ± 9% (Fig. 2A), whereas intracellular HL activity gradually increased to 236 ± 34% of controls (n = 4) after 2 h of incubation (Fig. 2B). Protein de novo synthesis was reduced to 71.7 ± 16.6% of control (n = 3). Immuno-inhibition assays

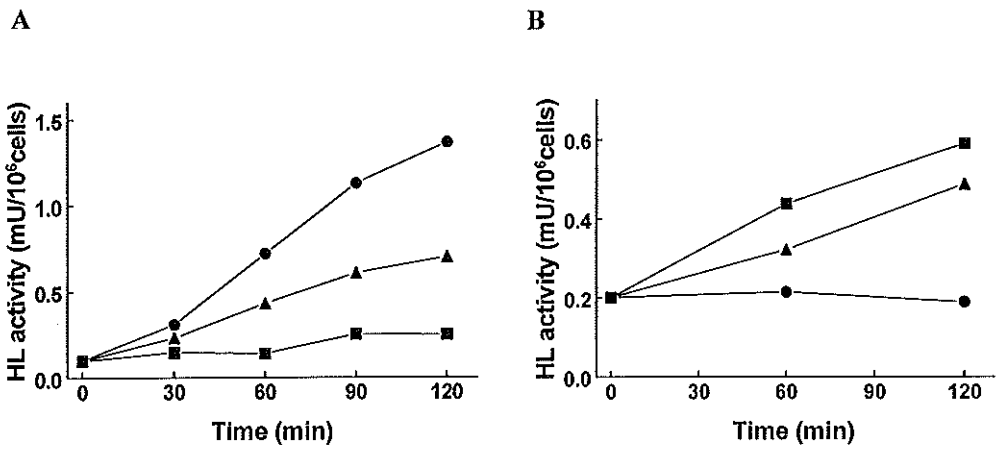

Figure 2

Effect of monensin and colchicin on extracellular and intracellular HL activity.

Freshly isolated rat hepatocytes were incubated in the absence (●) or presence of 50 μM monensin (■) or 50 μM colchicin (▲). At the indicated times, aliquots of the cell suspension were collected from the incubation, and HL activity was measured in the cell-free media (A) and cell lysates (B). Data are representative for 2 similar experiments.

using anti-HL IgGs confirmed that HL was responsible for the observed changes in intracellular and secreted triglyceridase activity (data not shown).

After 2 h of incubation with monensin or colchicin, the amount of HL protein in the extracellular medium was reduced in parallel with HL activity, as was also observed for castanospermine and CCCP (Fig. 3A). In the cell lysates, the amount of HL protein increased to 136 ± 21% and 137 ± 22% of control values (n = 4) with monensin and colchicin, respectively (Fig. 3B). These increases in intracellular HL protein were significantly less than the concomitant change in intracellular HL activity ($p<0.05$; n = 4). As a result, the specific enzyme activity of intracellular HL was increased with both inhibitors.

Specific triglyceridase activity of HL.

The effects of the different agents on the specific enzyme activity of HL are summarized in figure 3C. Under all conditions, the specific enzyme activity of secreted HL remained constant at approximately 45 mU/μg HL. For intracellular HL, the specific enzyme activity was much lower than for secreted HL, which was further reduced by 25 % and 50% upon treating the cells with CCCP and castanospermine, respectively. In monensin- and colchicin-treated cells, the specific activity was increased to levels close to that of secreted HL. In the presence of 1 mM 1-deoxymannojirimycin, an inhibitor of Golgi mannosidase I, neither secretion of HL (Fig. 3A) nor intracellular HL (Fig. 3B) were altered. Hence, the specific enzyme activity of intracellular and secreted HL was not affected by incubation with 1-deoxymannojirimycin (Fig. 3C), although the

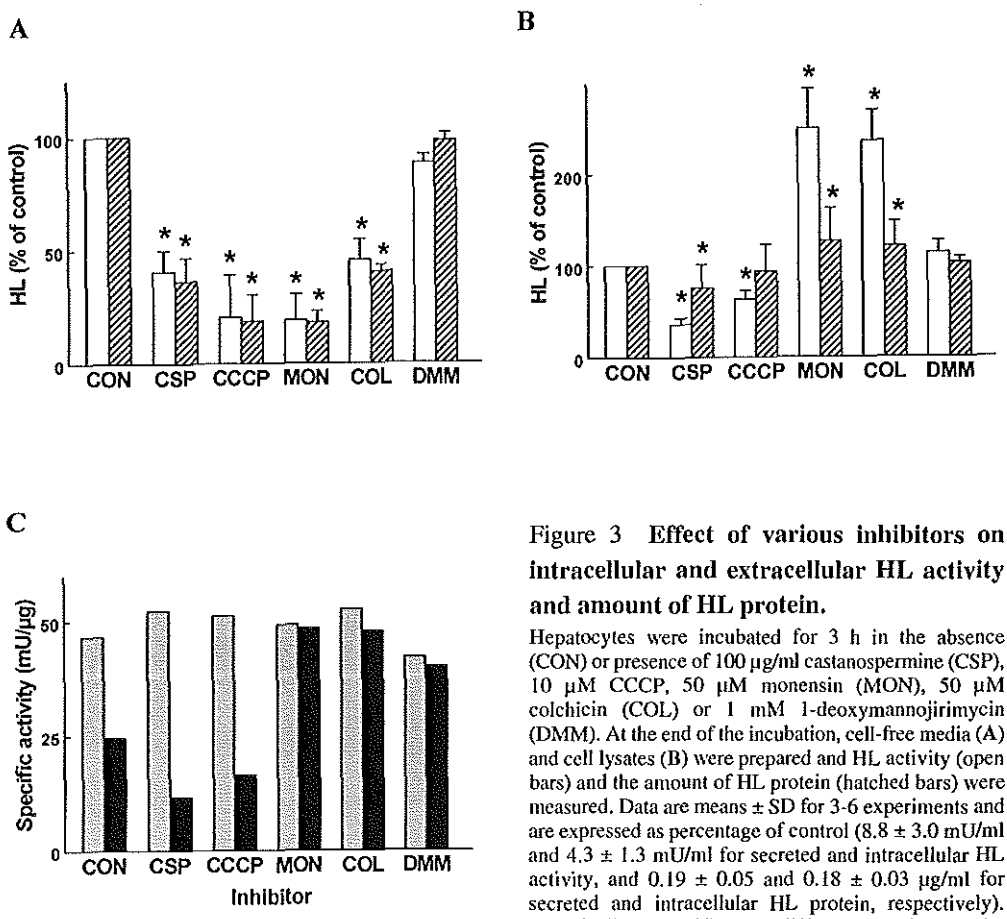

Figure 3 **Effect of various inhibitors on intracellular and extracellular HL activity and amount of HL protein.**

Hepatocytes were incubated for 3 h in the absence (CON) or presence of 100 µg/ml castanospermine (CSP), 10 µM CCCP, 50 µM monensin (MON), 50 µM colchicin (COL) or 1 mM 1-deoxymannojirimycin (DMM). At the end of the incubation, cell-free media (A) and cell lysates (B) were prepared and HL activity (open bars) and the amount of HL protein (hatched bars) were measured. Data are means ± SD for 3-6 experiments and are expressed as percentage of control (8.8 ± 3.0 mU/ml and 4.3 ± 1.3 mU/ml for secreted and intracellular HL activity, and 0.19 ± 0.05 and 0.18 ± 0.03 µg/ml for secreted and intracellular HL protein, respectively). Statistically significant differences from the corresponding controls are indicated by asterisks ($p < 0.05$). (C): Specific enzyme activity of HL in the extracellular medium (gray bars) and cell lysate (closed bars) was calculated from the mean HL activity and HL protein found for each condition in the medium and cell lysate, respectively.

glycosylation state of mature HL was altered from the complex into the high-mannose type (see later). Taken together, these observations suggest that the catalytic activity of HL increases after its transport from the RER to the Golgi compartment.

Immunoprecipitation of ^{35}S-labelled HL.

Hepatocytes were incubated for 3 h with the various inhibitors, and [^{35}S]methionine was present during the last 2 h. HL protein was immunoprecipitated from the cell lysates and cell-free media, and then analyzed by SDS-PAGE and phosphor imaging. [^{35}S]HL from control media migrated as

a single band of approximately 58 kDa (Fig. 4A). The ^{35}S-labelled HL secreted by CCCP- and colchicin-treated cells also migrated at the position of 58 kDa, although the radioactivity in this band was reduced to 35 and 17% of control, respectively. In the presence of monensin, secretion of [^{35}S]HL was decreased to 3 % of control, and the radioactive band migrated at a slightly higher mobility than the 58 kDa band in the other secretion media (Fig. 4A). [^{35}S]HL immunoprecipitated from control lysates appeared as two bands on SDS-PAGE, a band of approximately 53 kDa, and a band of 58 kDa that co-migrated with [^{35}S]HL from the cell-free media (Fig. 4B). The total ^{35}S-radioactivity in HL immunoprecipitated from CCCP-treated cells was similar to control cells, whereas in monensin- and colchicin-treated cells total ^{35}S-labelled HL was 2- to 2.5 fold higher. Total ^{35}S-radioactivity in HL from media plus cell lysates was reduced by approximately 50% with all four inhibitors compared to control, which agrees to the observed partial inhibition of protein de novo synthesis. In lysates prepared from CCCP-treated cells, the radioactivity of the 53 kDa band was increased whereas that of the 58 kDa band was increased (Fig. 4B). In colchicin-treated cells, the ^{35}S-label in the 53 kDa and 58 kDa bands were increased in parallel. In contrast, the accumulation of [^{35}S]HL radioactivity in the monensin-treated cells only occurred in the 53 kDa

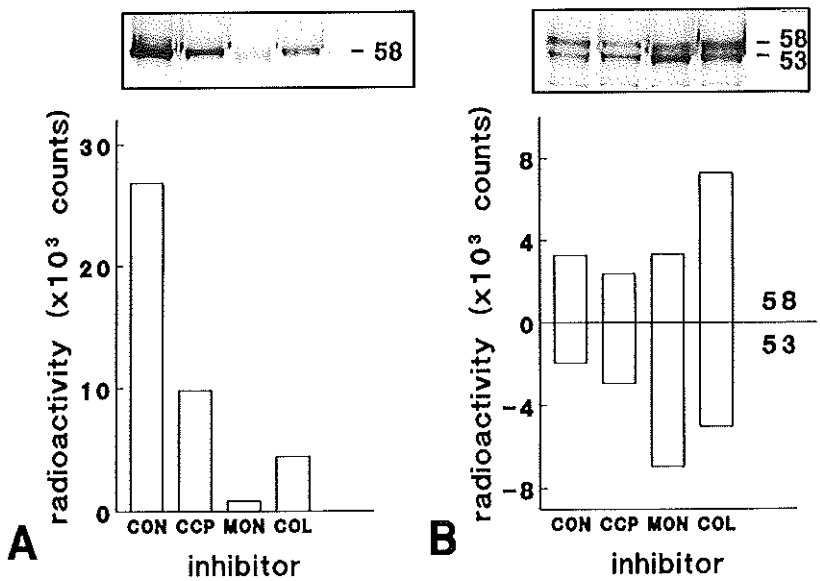

Figure 4 **Effect of various inhibitors on the electrophoretic mobility of HL.**

Cells were incubated for 3 h in the absence (CON) or presence of 10 μM CCCP (CCP), 50 μM monensin (MON) or 50 μM colchicin (COL). During the last 2 h of the incubation, 80 μCi/ml of Tran^{35}S-label was present. At the end of the incubation, HL protein was immunoprecipitated from the cell-free media and cell lysates, and then analysed by SDS-PAGE and phospor imaging. The radioactivity in the immunoreactive bands was quantified. (A) and (B) show part of the phosphor image and the quantitative results for the cell-free media and cell lysates, respectively. The positions of the 58 kDa and 53 kDa bands are indicated. (B) Quantitative data for the 58 kDa and 53 kDa bands are given as upward and downward bars, respectively. The results are representative for 3 similar experiments.

band; in addition, the largest band migrated at a mobility that was slightly higher than the 58 kDa band found in the other cells. Comparison of the data in figure 4 with the effect of the inhibitors on intracellular and secreted HL activity indicated that HL activity varied in parallel with the expression of the 58 kDa protein form, except for the monensin-treated cells, where intracellular HL activity increased in parallel with the 53 kDa protein form.

Sensitivity to Endoglycosidase H.

The electrophoretic mobility of [^{35}S]HL secreted in the absence or presence of CCCP, monensin or colchicin was not affected by overnight incubation with Endo H (Fig. 5A). Hence, secreted HL was completely Endo H resistant. As a reference, we used [^{35}S]HL that was secreted by cells in the presence of 1-deoxymannojirimycin, which prevents the maturation of the N-glycans from high-mannose to complex-type. This HL migrated as a single 53 kDa band, the mobility of which was almost completely shifted to 47 kDa corresponding to deglycosylated HL upon digestion with Endo H (Fig. 5A). Of the two HL bands immunoprecipitated from control cell lysates, the 58 kDa band was Endo H resistant whereas the 53 kDa band was sensitive to Endo H (Fig. 5B). Upon digestion, the 53 kDa band was completely shifted to a higher mobility, partly to the position of 47 kDa, and partly to the position of a 51 kDa band. The mobility of the latter band was not altered upon prolonged incubation with extra Endo H (not shown), and may reflect HL with one Endo H resistant and one Endo H sensitive oligosaccharide. Similar results were obtained with [^{35}S]HL in lysates from CCCP- and colchicin-treated cells. With monensin-treated cells, however, part of the [^{35}S]HL migrating at the 53 kDa position was not shifted to a higher mobility upon digestion with Endo H and thus appeared to be Endo H resistant. These data suggest that the accumulation of intracellular HL activity observed in monensin-treated cells coincides with the production of a 53 kDa, mainly Endo H resistant form of HL.

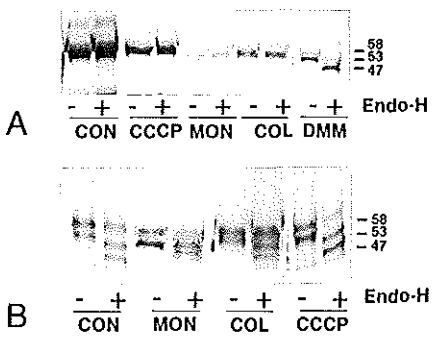

Figure 5 **Effect of various inhibitors on the Endo H sensitivity of HL.**

Experiments were performed as described in the legends to figure 4, except that prior to electrophoretic separation, the immunoprecipitated proteins were incubated overnight at 37 °C without (-) or with (+) Endo H. As a positive control for Endo H activity, a secretion medium from a 3 h incubation of rat hepatocytes with 1 mM 1-deoxymannojirimycin (DMM) was included. (A) and (B) Phosphor images of the gels obtained with cell lysates and cell-free media, respectively. The positions of the 58 kDa and 53 kDa HL protein forms, as well as the 47 kDa, deglycosylated form are indicated. Data are representative for 2 similar experiments.

DISCUSSION

The data presented here confirm our previous report that newly synthesized HL protein is apparently activated during maturation and secretion in rat hepatocytes (13). This was also observed for human HL in human HepG2 cells (Chapter 5.1). This activation was prevented by treating the cells with inhibitors of RER glucosidases, which interfere with proper oligosaccharide processing of the newly synthesized HL (13). Upon incubation with castanospermine the specific enzyme activity of intracellular HL decreased, which may suggest that activation is closely coupled to glucose trimming in the RER. However, we show here that a fall in the specific activity of intracellular HL was also induced with CCCP, which interferes with transport of glycoproteins out of the RER to the Golgi, but leaves oligosaccharide processing in the RER essentially unaffected. Hence, glucose trimming alone appears not to be sufficient for activation of HL, but is necessary for transport of HL out of the RER; HL then matures into a catalytically active protein in a vesicular compartment distal from the RER. The RER glucosidases have been recently proposed to assist in the folding of newly synthesized glycoproteins thereby making them transport-competent (20). In contrast to CCCP and castanospermine, a marked increase in the specific enzyme activity of intracellular HL was induced by treating the cells with monensin, which interferes with intra-Golgi vesicular transport (24), as well as with colchicin, which interferes with post-Golgi transport in rat hepatocytes (25, 26). This finding clearly demonstrates that HL has acquired full catalytic activity when accumulating in the Golgi compartment. Our data are best explained by the model that newly synthesized HL must reach the Golgi before it is activated.

Lipoprotein lipase, which is closely related to hepatic lipase, has also been shown to acquire catalytic activity after the glucose residues of the glycan side chains have been removed. Studies with CCCP and monensin in mouse brown fat adipocytes (32) and 3T3-L1 adipocytes (33) led to the conclusion that LPL is activated after transport of the protein from the RER into the Golgi. This was supported by the observation that incubation of adipocytes with brefeldin-A, which induces the fusion of the RER with the Golgi compartment, results in the intracellular accumulation of fully active LPL (32). In our studies using freshly isolated rat hepatocytes, maturation and secretion of HL was not affected by BFA (data not shown), possibly due to the rapid detoxification of the drug by these cells (34). In HepG2 cells, BFA induced the intracellular accumulation of catalytically active HL (Chapter 5.1); moreover, the inactive HL that accumulated in castanospermine-treated HepG2 cells was converted to fully active HL upon co-incubation with BFA (Chapter 5.1). These combined data suggest that both HL and LPL require transport to the Golgi-compartment to become catalytically active. In contrast, Ben-Zeev et al. (35) reported that LPL accumulated intracellularly as a fully active enzyme when expressed as a hybrid with a C-terminal KDEL sequence. As this sequence was thought to function as an RER retention signal, the authors concluded that activation of LPL does occur before the protein reaches the Golgi-compartment. Recent studies have demonstrated, however, that the KDEL sequence functions rather as a retrieval signal; KDEL-bearing proteins are cycled back into the RER from the Golgi or even beyond (36-38). Therefore, the catalytically active LPL-KDEL hybrid that accumulates in the RER may have been activated in the Golgi before being cycled back into the RER.

Our data indicate that catalytic activity co-varies with the presence of the 58 kDa, Endo H

resistant form of HL in CCCP and colchicin-treated cells, and with the 53 kDa, mainly Endo H resistant form in monensin-treated cells. N-glycoproteins are processed from an Endo H sensitive into an Endo H resistant form upon trimming of mannose residues by Golgi mannosidases. These observations suggest therefore, that the activation of HL is closely linked to, or occurs only after, some of the mannoses on the glycan chains have been trimmed off by the Golgi mannosidases. However, cells incubated with 1-deoxymannojirimycin secrete a 53 kDa, fully Endo H sensitive form of HL whose specific enzyme activity is virtually identical to that of control HL (Fig 3; (18)). Therefore, trimming of the oligosaccharides on HL by the Golgi mannosidases is not crucial for the acquisition of triglyceridase activity. Which modification of HL is associated with the activation process remains unknown at present. The necessity to exit the RER in order to become activated is not unique to lipases, but has recently also been reported for two membrane-bound N-glycoproteins, the macrophage mannose receptor (39) and the trans-Golgi network protease furin (40). The modification that causes activation of these proteins has also not been identified, but was shown not to depend on oligosaccharide processing. It may involve a rather subtle covalent or non-covalent modification not detected by the rather course methods of SDS-PAGE and Endo H digestion. Alternatively, transport of the newly synthesized proteins out of the RER will move them away from the numerous RER chaperones that may block their full activation.

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CHAPTER 6

General Discussion

6 GENERAL DISCUSSION

Post-transcriptional regulation of HL

The studies described in this thesis focussed on regulation of HL expression in liver at the post-transcriptional level. In chapter 2 we describe the effects of catecholamines on the synthesis and processing of HL. In rat liver parenchymal cells an Endo H sensitive, 53 kDa precursor of HL is synthesized. This protein matures into an Endo H resistant 58 kDa HL, which is active and rapidly secreted. As we were interested in the post-transcriptional regulation of HL expression, we studied the short-term effects of adrenaline. In freshly isolated rat hepatocytes, the de novo synthesis of the 53 kDa Endo H sensitive HL precursor form was unaffected by adrenaline. Thus, HL expression was mainly modulated post-translationally. HL transcription and/or translation may be affected after long-term exposure to adrenaline, but this was not determined here. Pulse-chase experiments showed that adrenaline inhibited the secretion of HL by retarding the maturation of the 53 kDa HL precursor into the active 58 kDa protein. Further, adrenaline stimulated degradation of newly synthesized HL protein. The effect of adrenaline on HL secretion was mediated by the α_{1B}-adrenergic receptor pathway. Although α_{1B}-adrenoceptors signal via an increase in intracellular cAMP and calcium, only calcium mobilization could mimic the effects of adrenaline. In particular, calcium levels in the ER seemed to mediate the altered maturation of HL in the presence of adrenaline. The sequence of events that leads to degradation of the HL protein is still unclear. However, incubation with adrenaline and the protease inhibitor ALLN resulted in a relative accumulation of the 53 kDa HL, suggesting that mainly the 53 kDa protein is degraded in the presence of adrenaline. The specific enzyme activity of the secreted HL was similar in adrenaline-treated and control cells, and the apparent molecular weight of the secreted HL on SDS-PAGE seemed unaltered, indicating that the post-transcriptional regulation is not mediated by gross changes in the state of glycosylation. Our data suggest that adrenaline primarily affects exit of HL from the ER, and that the stimulated degradation of HL occurs by an ALLN sensitive proteases, which may reside in the ER (1-4).

Besides regulation at the level of exit from the ER, we have identified two other sites of post-transcriptional regulation of HL expression. First, the translation efficiency of HL mRNA may be (indirectly) modulated by thyroid hormone. Studies with hypothyroid rats showed that substitution with growth hormone normalizes HL mRNA levels, but fail to restore HL de novo synthesis. Apparently, translation of HL mRNA is rate-limiting in a hypothyroid state. Second, HL turnover may be affected by fenofibrate. The HL activity in liver homogenates of fenofibrate-treated rats was only 25% reduced, despite a 75% fall in HL secretion by isolated hepatocytes. The turnover of HL may be decreased by changes in HL binding characteristics, internalization and/or subsequent intracellular degradation.

In figure 1, the modulation of HL expression by adrenaline, thyroid hormone, growth hormone and fenofibrate are summarized. Although we hypothesized that N-glycosylation of HL may be involved in (post-)translational regulation (Chapter 1.1.4), we have not encountered any condition of altered HL expression that co-incides with gross changes in glycosylation of secreted HL. Moreover, we have not encountered any condition in which the specific enzyme activity of secreted HL was altered.

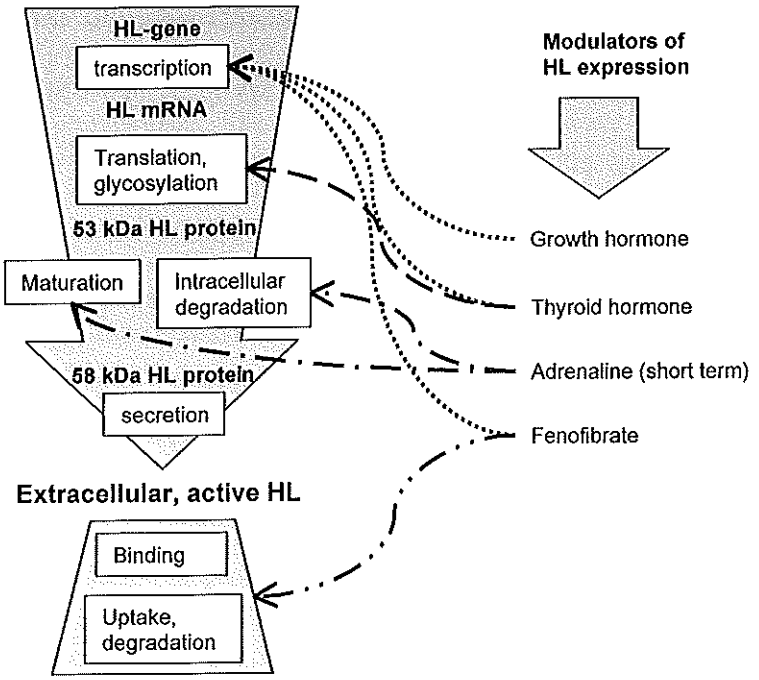

Figure 1 **Modulators of hepatic lipase expression.**
Representation of sequential processes, which determine the expression of extracellular HL activity. The site of action of the studied modulators are schematically represented by arrows.

In vivo, HL expression is determined by many factors. One aspect is the efficacy of adrenaline, which is modulated in the rat. The effect of adrenaline on HL secretion by freshly isolated rat hepatocytes is mediated via the α_{1B}-adrenergic receptor pathway (Chapter 2.2). In rats, second messenger signalling of the α_1-adrenoceptor pathway changes during aging. Both the cAMP generation and the increase of intracellular calcium, which mediates the effects on HL expression, are lowered during aging (5, 6). Other hormones may also interfere with the signalling induced by catecholamines. For example, EGF abolishes the effect of adrenaline on HL secretion in rat hepatocytes, probably by disrupting signalling (7). Further, changes in thyroid status were reported to be associated with a reduction of α-adrenergic receptors in rats (8).

Besides catecholamines, which lower HL expression, the positive effects of insulin on HL may contribute to the daily changes associated with feeding and fasting. However, only chronic insulin administration to rats increases HL activity in liver (9). Incubation of freshly isolated rat hepatocytes with insulin had no clear effects, both an increase and slight decrease in secretion of HL activity were reported (10, 11). In humans, insuline may increase HL expression, as in non-insulin-dependent diabetic patients HL positively correlates with insulin levels (12-14). In addition, in normolipidemic male patients with coronary artery disease (REGRESS), which were divided in

quartiles based on plasma insuline, HL activity was increased at higher insuline concentrations. In the carriers of a novel mutation in the human HL promotor region, C-480T was no correlation between HL activity and insuline (15).

Whether HL activity in humans also follows a cycle of high and low activity during fasting and feeding is not clear. There is little day-to-day variations in fasting HL activity levels (13). Schlierf et al (16) reported median values of 368 mU/ml for postprandial and 261 mU/ml for fasting HL activity. The difference in HL activity was not statistically significant, which might be due to the large variances between individuals. Whether in humans prolonged exposure to adrenaline, for instance during mental stress, causes decreased HL synthesis and secretion is not known. Adrenaline may contribute to the development and/or complications of arteriosclerosis (17). Post-translational inhibition of HL expression by adrenaline may contribute to the correlation between adrenaline and arteriosclerosis.

Regulation of HL in liver has many similar features as regulation of LPL expression in white adipose tissue, but differs on several essential points. The down-regulating effect of adrenaline on HL is mediated by α_1-adrenoceptors (Chapter 2), and that on LPL is mediated by β-adrenergic receptors (18). We showed that adrenaline had no short term effects on HL translation and synthesis (Chapter 2.1), whereas adrenaline acutely affects the translation of LPL mRNA (19). Adrenaline increases the intracellular degradation of HL (Chapter 2.1), but decreases the degradation of LPL (20). Further, opposite regulation of HL and LPL expression was described for thyroid hormone. Down-regulation of HL during hypothyroidism involves impaired transcription, which resulted from a concomitant fall in growth hormone. Hypothyroidism may impair the translation of HL mRNA (Chapter 3). On the other hand, LPL mRNA translation is stimulated during hypothyroidism (21). For LPL the involvement of a translation repressor was suggested in acute down-regulation of LPL expression by adrenaline and in up-regulation of LPL mRNA translation during hypothyroidism (19, 21).

Glycosylation of HL

We have studied several conditions where HL expression is regulated post-transcriptionally, but have not encountered a condition where regulation is primarily at the level of N-glycosylation. For human and rat HL, we and others have established that the N-glycosylation is essential for maturation and activation of the de novo synthesized HL protein. In chapter 5, we have presented evidence that glucose trimming of HL at the ER is necessary for the translocation from the ER to the Golgi, whereafter the protein acquires its triglyceridase activity in the Golgi and is secreted into the extracellular medium. Once arrived in the Golgi, further processing of the glycan chains is not important for secretion of an active protein. No changes in specific activity of the secreted HL protein were found, suggesting that alternative processing of the glycosylation chain, which change the specific enzyme activity are unlikely.

The importance of the intracellular pathway is underlined by the *cld/cld* syndrome in mouse. In these mice functional expression of LPL and HL is deficient, and both proteins remain intracellularly, mainly in the rough ER (22-24). Several groups have hypothesized that impaired transport or maturation of HL and LPL may originate from a dysfunctional, lipase specific

chaperone. Accumulation of LPL and HL in the ER of the *cld/cld* mouse cells may result from a missing chaperone that normally assists transport out of the ER into the Golgi. Alternatively, an ER-chaperone might fail to release LPL and HL.

A human counterpart of the mouse *cld/cld* syndrome might exist. A family with combined reduction of LPL and HL has been described by Auwerx et al (25). The presence of both low HL and low LPL activity has also been reported for some isolated cases (26, 27). In the REGRESS population, from the 812 normolipidemic patients, suffering from coronary artery disease we found 60 individuals (7.4%), which have both LPL and HL activities amount to less than 50% of matched controls. Furthermore, 6 out of 35 patients admitted to the cardiology department of the academic hospital in Rotterdam for angiography had both low LPL and HL activity (42 ± 13, 160 ± 39 mU/ml). Combined lipase reduction in patients may originate from different complex factors. Patients may have accumulated mutations in both LPL and HL genes. However, the number of HL mutations, which interfere with HL activity described so far is low (28, 29). The patients found with combined lipase reduction appears to be much higher than would be expected statistically. This suggests the involvement of common factors, presently unknown, which reduce expression of HL in liver and LPL in extra hepatic tissues. Such a factor could be a dysfunctional chaperone as suggested for the mouse *cld/cld* syndrome. In fact, the patient with both low HL and LPL activity, which was described by Fager et al (27) appears to have normal LPL synthesis, but LPL is not effectively transported from the ER to the Golgi.

Model for intracellular events in regulated HL secretion

We have shown that post-translational regulation of HL expression by adrenaline involves Ca^{2+}-mediated retardation of HL maturation and subsequent degradation (Chapter 2). The degradation may occur in the ER or early Golgi. In HepG2 cells (Chapter 5.1) we showed that ER-to-Golgi transport of HL protein was very sensitive to inhibition by CCCP, suggesting the involvement of ATP-dependent association with ER chaperones. In addition, we showed in pulse-chase experiments with rat hepatocytes that CCCP causes accumulation of the 53 kDa HL without degradation of HL protein (Chapter 2.3). In rat hepatocytes activation occurs only after transport from the ER to the Golgi (Chapter 5.1). HL enzyme activity may be masked by binding to an ER-located chaperone. Both LPL and pancreatic lipase require a cofactor for catalytic activity (Chapter 1.1.2), which may prevent lipase from being active within the intracellular pathway. As HL does not depend on a cofactor for its enzymatic activity, a chaperone shielding HL activity may protect against breakdown of potential intracellular substrates of HL. Interactions of HL with ER chaperones may be an important regulatory step in the folding and secretion of active HL. Glycosylation, ATP and ER-Ca^{2+} may affect the association of HL with chaperones. In figure 2, the sequential events in HL secretion, which may explain our observations are depicted. Unbroken arrows represent modulation of HL, which may not be sensitive to regulation. Once in the Golgi, secretion of active HL seems not to be regulated, and than only the inhibitors of vesicular transport, like monensin and colchicine are able to prevent the secretion. The processes indicated by dash-dotted arrows are expected to be regulated. Three models may explain the observed data. First, the adrenaline-, thapsigargin- or EGTA-mediated decrease of ER-Ca^{2+} may primarily stimulate degradation (arrow A). Thus, leaving less 53 kDa protein available for maturation. The degradation would occur only after stimulation

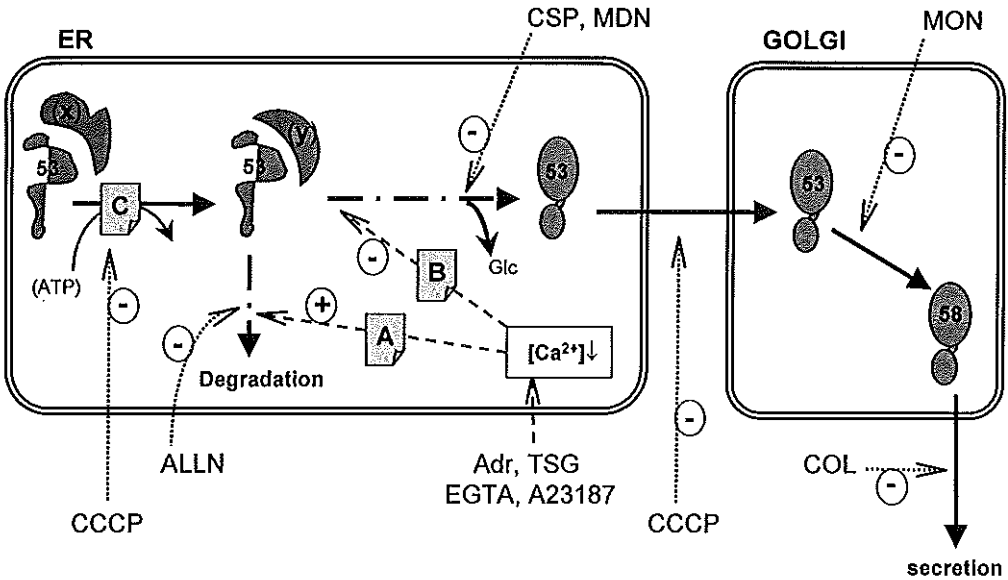

Figure 2 **Hypothesis on the regulation of hepatic lipase secretion.**

Schematic representation of transport, processing and regulation of intracellular HL protein. Endo H sensitive, immature 53 kDa HL is present in the ER and early Golgi. In the Golgi HL is modulated towards a mature, Endo H resistant 58 kDa protein and is secreted as an active enzyme. Unbroken arrows represent modulation of HL, which may not be sensitive to regulation. Dash-dotted lines are expected to be regulated in vivo. The arrows marked A, B, C represent events, which are part of the models discussed in the text. The (x) and (y) represent chaperones, which may be involved in folding and maturation of HL. Adr, adrenaline; TSG, thapsigargin; CSP, castanospermine; MDN, N-methyldeoxynojirimycin; MON, monensin; COL, colchicin; Glc, glucose.

with adrenaline, thapsigargin, A23187 and EGTA, which agrees with our experiments, which showed that there was no degradation in control cells. In addition, the presence of CCCP failed to induce degradation of the 53 kDa HL. In this model, inhibiting the formation of transportable 53 kDa protein (with glucoses trimmed off the glycosylation chains) by castanospermine or N-methyldeoxynojirimycin would not necessarily stimulate HL degradation. However, castanospermine may cause degradation of HL protein (30). Further, when the adrenaline-mediated degradation was inhibited by ALLN, the 53 kDa protein relatively accumulated, but failed to restore HL maturation of the 53 kDa form to mature 58 kDa HL. We therefore propose a second model.

In this model, decreased levels of ER-Ca^{2+} may inhibit normal maturation and/or folding (arrow B), whereafter more 53 kDa protein is available to the ALLN-sensitive degradation. In line with this castanospermine and N-methyldeoxynojirimycin would induce degradation of HL protein. CCCP incubations would then, either inhibit the formation of degradation-sensitive 53 kDa HL (arrow C), or induce the accumulation of a 53 kDa HL form that is no longer sensitive to proteases. The formation of degradation-sensitive 53 kDa HL may be ATP dependent, and thus affected by CCCP. An argument against this model is the finding that there was no degradation of intracellular HL in control cells.

The third model may combine both mechanisms (arrows A+B): The adrenaline-, thapsigargin- or EGTA-mediated decrease of ER-Ca^{2+} may inhibit normal maturation and/or folding, whereafter more 53 kDa protein is available to the stimulated, ALLN-sensitive degradation process. The lowered ER-Ca^{2+} may affect Ca^{2+}-sensitive proteases and chaperones (depicted as (y) in Fig.2) involved in the intracellular pathway of HL. Discrimination in favour of one hypothesis may become possible when more is known on the interaction of HL with intracellular chaperones/proteases.

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SUMMARY

Summary

We studied hepatic lipase (HL) expression in rat hepatocytes under conditions where regulation at the post-transcriptional level has been implicated. Although we hypothesized that N-glycosylation of HL may be involved in this regulation (Chapter 1.1.4), we have not encountered a condition where regulation is primarily at the level of N-glycosylation. Instead, the oligosaccharide processing can be used to follow the intracellular transport of HL, as the process is essential for HL folding and subsequent maturation and secretion.

REGULATION OF HEPATIC LIPASE EXPRESSION AT THE LEVEL OF MATURATION AND SECRETION

Modulation of hepatic lipase expression by adrenaline in rat hepatocytes (Chapter 2)

In rats the daily changes in HL activity in the liver follow the diurnal rhythem of the catecholamines associated with feeding and fasting. We have studied the mechanism by which adrenaline lowers HL secretion in suspensions of freshly isolated rat hepatocytes. Adrenaline acutely inhibited the secretion of HL activity (Chapter 2.1). The cells secreted significantly less HL activity in the presence of adrenaline than with cycloheximide, which completely blocked protein de novo synthesis. The specific enzyme activity of secreted HL was not affected. Intracellularly, HL activity was lowered by adrenaline-treatment. Pulse-labelling with [^{35}S]methionine showed that the de novo synthesis of the 53 kDa EndoH-sensitive HL precursor form was unaffected by adrenaline. During subsequent chase of the control cells, the 53 kDa form was converted to a 58 kDa EndoH resistant HL protein, which was rapidly secreted into the medium. In the presence of adrenaline, formation of the 58 kDa protein was markedly reduced, whereas the 53 kDa protein disappeared at a similar rate as in controls. This suggests that part of the HL protein was degraded. In contrast to adrenaline, inhibition of HL secretion by colchicine was accompanied by an intracellular accumulation of HL activity and of the 58 kDa protein. We concluded that adrenaline inhibits HL secretion posttranslationally by retarding the maturation of the 53 kDa HL precursor into an active 58 kDa protein, possibly by stimulating degradation of newly synthesized HL protein.

Adrenaline acutely inhibited the secretion of HL activity through activation of the α_1-adrenergic pathway. Incubation in the presence of propranolol, a selective β-antagonist, did not alter the effect of adrenaline. In our isolated rat hepatocytes the acute effect of adrenaline was abolished partly by the α_{1A}-antagonist WB4101, but completely by chloroethylclonidine, a selective α_{1B}-antagonist (Chapter 2.2). The effect of adrenaline was mimicked by the non-selective α_1-agonist phenylephrine, but not by the selective α_{1A}-agonist methoxamine. Thus, adrenaline inhibited the secretion of HL via α_{1B}-adrenergic receptors. This adrenoceptor subtype was recently described to signal through both Ca^{2+}_i and cAMP. However, the effect of adrenaline on HL expression is mediated via Ca^{2+} rather than cAMP, as similar inhibition of HL secretion was induced by elevating Ca^{2+}_i with vasopressin, angiotensin or A23187, but not by elevating cAMP with forskolin or addition of 8-Br-cAMP.

Pulse-chase experiments with [^{35}S]methionine showed that adrenaline, thapsigargin and A23187, all mediating an increase of intracellular calcium, retarded the processing of the 53 kDa, high mannose precursor to the mature 58 kDa HL protein, and stimulated intracellular degradation

(Chapter 2.2). However, lowering of intracellular Ca^{2+} by treating the cells with EGTA also reduced HL secretion by a similar mechanism. Thus, both high and low intracellular Ca^{2+} have similar post-translational effects on HL. This contradiction may be explained by Ca^{2+}-levels in the endoplasmic reticulum (ER). Besides altering the intracellular Ca^{2+}, all the agents tested lower ER-Ca^{2+}.

As described above, adrenaline acts post-translationally, by retarding the maturation of the 53 kDa protein to the 58 kDa HL protein, and by stimulating intracellular degradation. The lysosomal degradation inhibitors leupeptin and chloroquine did not influence the effect of adrenaline on the secreted and intracellular HL activity (Chapter 2.3). Of several protease inhibitors tested, only the cysteine-protease inhibitor N-acetyl-leucine-leucine-norleucinal (ALLN) partly reversed the adrenaline-induced degradation. In the presence of ALLN, the adrenaline-induced intracellular decrease of the immature 53 kDa form was partly prevented, but maturation to the 58 kDa form was not restored. The 53 kDa protein that accumulated intracellularly seemed to have no triglyceridase-activity. These results suggest that the adrenaline-induced degradation was a result of impaired maturation, and exposure to ALLN-sensitive proteases.

REGULATION OF HL EXPRESSION AT OTHER POST-TRANSCRIPTIONAL LEVELS

Modulation of hepatic lipase expression by growth hormone in hypothyroid rats (Chapter 3)
During hypothyroidism hepatic lipase (HL) activity is decreased. The low HL may be due to thyroid hormone insufficiency or to the concomitant fall in growth hormone (GH) activity. We studied HL expression in hepatocytes freshly isolated from hypothyroid rats with and without additional GH-substitution. In all animals HL mRNA was detected by RT-PCR in the hepatocytes, but not in the non-parenchymal cells. In hypothyroid cells HL mRNA levels were reduced by 40%, and the in vitro secretion of HL-activity and HL-protein was decreased by about 50%. In cells from GH-substituted hypothyroid rats, HL mRNA level was normalised, but the secretion of HL remained 50% lower than control. The specific enzyme activity of secreted HL was similar under all conditions. The discrepancy between HL mRNA and HL secretion in GH-supplemented hypothyroid rats may be due to (post)translational effects. Therefore, we studied the HL synthesis and maturation in hepatocytes from hypothyroid rats with or without GH-substitution. Pulse-labelling experiments with [^{35}S]methionine showed that the incorporation of [^{35}S]methionine into HL protein was lower both in hypothyroid cells and in GH-supplemented hypothyroid cells than in control cells. During the subsequent chase, the intracellular processing and transport of newly synthesized HL protein in the hepatocytes from hypothyroid rats, whether or not supplemented with GH, was similar to control cells. We conclude that in livers of hypothyroid, GH-substituted rats translation of HL mRNA is inhibited despite restoration of HL mRNA levels.

Modulation of HL expression in fenofibrate-treated rats (Chapter 4)
In rats, the fenofibrate-mediated down-regulation of HL expression is mainly induced at the transcriptional level. Besides regulation of HL transcription, fenofibrate has been indicated to affect HL post-transcriptionally. Here, we studied the effects of fenofibrate on expression of HL more

closely. In the fenofibrate-treated rats, HL activity in liver homogenates was 40% lower compared to control rats. Corrected for the increase in cell volume after fenofibrate treatment, the HL activity in the liver homogenate was 25% lower than control. However, the heparin-releasable pool of HL activity was similar to control after fenofibrate treatment.

Secretion of HL activity by freshly isolated hepatocytes from fenofibrate-treated rats was inhibited up to 70% compared to control hepatocytes. Pulse-labelling experiments with [^{35}S]methionine showed very low synthesis of HL in the fenofibrate hepatocytes, which is in accordance to the decreased mRNA levels. Further, pulse-chase experiments showed that HL maturation in hepatocytes from fenofibrate-treated rats was similar to controls. These results suggest that fenofibrate decreased HL synthesis mainly at the transcriptional level. Despite the low rate of HL synthesis, HL activity bound to the liver on heparin-releasable sites was hardly affected. Our data therefore indicate that HL expression is post-translational regulated by changes in HL turnover. Post-translational regulation by fenofibrate may take place at the level of HL binding, internalization and/or subsequent degradation, rather than at the level of maturation of newly synthesized HL.

N-GLYCOSYLATION OF HEPATIC LIPASE

We found no evidence for direct regulation at the level of N-glycosylation and oligosaccharide processing in any of the above described cases. However, N-linked glycosylation of HL has been shown to be essential for secretion of a catalytically active protein. Hence, we closely examined the role of N-linked glycosylation in intracellular processing and activation of HL in rat hepatocytes and in the human hepatoma cell line, HepG2.

Secretion and intracellular processing of hepatic lipase (Chapter 5)
In chapter 5.1, we describe our studies with human HepG2 cells. In the presence of cycloheximide, which completely blocks the de novo protein synthesis, processing of the pre-existing HL in the cells proceeded normally. However, much more HL activity appeared in the extracellular medium than was lost from the cells. Thus, intracellular HL is apparently activated during the secretory process. Preventing N-glycosylation with tunicamycin or blocking the trimming of the terminal glucose residues in the ER by castanospermine (CSP) or N-methyldeoxynojirimycin (MdN) resulted in an inhibition of HL secretion similar to cycloheximide. Intracellular HL activity was lost, whereas the amount of HL protein remained below the detection limit of our ELISA. The dependence of HL secretion on proper glucose trimming in the ER contrasts sharply with the secretion of α_1-antitrypsin, an unrelated N-glycoprotein, which was unaffected during incubation with CSP or MdN. Interfering at a later stage of the oligosaccharide-chain processing, by incubation of the cells with deoxymannojirimycin did not affect the secretion of HL. The effect of CSP and MdN on intracellular HL was mimicked by incubation with CCCP, which blocks transport of ER vesicles to Golgi. In contrast, the secretion of α_1-antitrypsin was also unaffected by CCCP incubation. Blocking vesicle transport between the Golgi compartments with monensin, or merging the ER and Golgi compartment with brefeldin A (BFA) resulted in an intracellular accumulation of HL activity. The apparent activation of intracellular HL in the presence of BFA also occurred

after incubation with either CSP or MdN, which alone caused a fall in intracellular HL activity. From these results we conclude that, glucose trimming at the ER of human HL is primarily necessary for the translocation of newly synthesized protein from the ER to the Golgi, whereafter the protein acquires its triglyceridase activity and is secreted into the extracellular medium. Which process in the Golgi compartment is responsible for the apparent activation of HL protein remains unknown.

In chapter 5.2, we describe our studies of the intracellular processing of hepatic lipase in suspension of freshly isolated rat hepatocytes. Analogous to our studies in HepG2 cells, we examined the role of N-linked glycosylation in the secretion and activation of rat HL. Secretion of a catalytically active HL is depending on proper N-linked glycosylation. In particular, trimming of the terminal glucose residues in the ER appears to be crucial for gaining activity. Incubation of the hepatocytes with tunicamycin, castanospermine (CSP) or N-methyldeoxynojirimycin (MdN) resulted in similar inhibition of HL secretion than with cycloheximide. Although HL denovo synthesis was virtually unaffected, intracellular active HL protein could not be detected. Interfering at a later stage of the oligosaccharide-chain processing, by incubation with deoxymannojirimycin failed to affect the secretion of active HL. Incubation with CCCP, blocking transport of ER vesicles to Golgi, resulted in a similar fall in intracellular HL as observed with CSP and NdM. Blocking vesicular transport either between the Golgi compartments with monensin, or between the Golgi and the plasma membrane with colchicine resulted in an increase of intracellular HL activity. Unfortunately, brefeldin A was rapidly inactivated by the hepatocytes and could not be used to disrupt the ER/Golgi compartments. We conclude that the HL protein acquires its triglyceridase activity in the Golgi, whereafter it is secreted into the extracellular medium. Which process in the Golgi compartment is responsible for the apparent activation of HL protein remains unknown.

Hypothesis on the regulation of hepatic lipase secretion (Chapter 6)

In the discussion, a model explaining our observations on the intracellular pathway of HL is proposed. After synthesis of the Endo H sensitive, 53 kDa HL precursor, exit from the ER requires proper glucose trimming. Transport from the ER to Golgi is essential for maturation to the 58 kDa, Endo H resitant protein and for gaining catalytic activity. Regulation by adrenaline affects the rate and extent of exit from the ER. The effect of adrenaline is probably mediated by lowering calcium levels in the ER, which may retard HL processing to a transport-competent form, and simultaneously enhances degradation of the 53 kDa HL protein.

SAMENVATTING

Samenvatting

Lever lipase (HL) is een enzym dat wordt gesynthetiseerd door de parenchym cellen van de lever en dat vervolgens bindt in de ruimte van Disse in de lever. HL speelt een belangrijke rol in het lipiden metabolisme. HL hydrolyseert fosfolipiden en triglyceriden in 'high-' en 'intermediate'-'density' lipoproteïnen. Verder is HL betrokken by de verwijdering van 'remnant'-partikels in de lever, en draagt het bij aan het omgekeerde cholesterol transport. Door deze activiteiten draagt HL verlaging mogelijk bij tot vervroegde atherosclerosis. Bij hartpatienten wordt inderdaad een inverse relatie gevonden tussen de lever lipase activiteit en de mate van coronaire vaatvernauwing.

De expressie van HL activiteit wordt gereguleerd door dieet-veranderingen en verschillende hormonen. De regulatie kan plaats vinden op verschillende niveau's. Diverse studies hebben vastgesteld dat er regulatie op het niveau van HL mRNA synthese (transcriptie) is, maar ook daarna is er regulatie (post-transcriptioneel). By de synthese van het HL eiwit wordt het voorzien van suikerketens aan de twee(rat) of vier (humaan) specifieke asparagines (N-glycosylering). Additie en daarop-volgende modificatie van deze ketens zijn essentieel voor de secretie van een actief lever lipase, en spelen mogelijk een rol bij de post-transcriptionele regulatie. Verscheidene condities waarbij er mogelijk sprake is van post-transcriptionele regulatie van HL expressie werden nader bestudeerd.

REGULATIE VAN MATURATIE EN SECRETIE VAN LEVER LIPASE IN RATTE PARENCHYM CELLEN.

Modulatie van lever lipase expressie door adrenaline (Hoofdstuk 2).

In de rat worden dagelijkse veranderingen in HL activiteit in de lever veroorzaakt door veranderingen in catecholamines, die optreden tijdens het ritme van voeden en vasten. We hebben het mechanisme bestudeerd waarmee adrenaline de secretie van actief HL remt in vers geïsoleerde lever cellen. Adrenaline verlaagde de secretie van HL acuut (Hoofdstuk 2.1). In de incubaties met adrenaline was de secretie van HL activiteit lager dan in condities met cycloheximide, waar de eiwit de novo synthese volledig was geremd. De specifieke enzym activiteit was niet veranderd. Ook intracellulair was de activiteit van HL gedaald door de incubatie met adrenaline. Pulse-chase experimenten met [^{35}S]methionine lieten zien dat de synthese van de 53 kDa, Endo H gevoelige HL precursor vorm niet beïnvloed werd door adrenaline. Tijdens de daarop volgende chase werd in controle cellen het 53 kDa eiwit omgevormd tot het 58 kDa Endo-resistente HL, dat snel in het medium werd gesecreteerd. Wanneer adrenaline aanwezig was tijdens de chase, was de vorming van het 58 kDa eiwit aanmerkelijk trager. Het 53 kDa eiwit verdween echter even snel als in de controle cellen. Dit suggereert dat een deel van het nieuw gevormde HL eiwit werd afgebroken onder invloed van adrenaline. In tegenstelling tot adrenaline, ging remming van HL secretie door colchicine samen met een intracellulaire ophoping van actief HL en het 58 kDa HL-eiwit. We concluderen dat adrenaline de secretie van HL post-translationeel remt door de maturatie van het 53 kDa eiwit naar 58 kDa eiwit te vertragen, mogelijk door de degradatie van het nieuw gevormde eiwit te stimuleren.

De acute remming van de HL secretie door adrenaline werd gemediëerd door activatie van de α_1-adrenerge cascade. Propranolol, een selectieve β-antagonist, had geen invloed op het effect van adrenaline. In onze geïsoleerde ratte lever cellen was het acute effect van adrenaline gedeeltelijk

op te heffen door incubatie met de α_{1A}-antagonist WB4101, en voledig op te heffen met chloroethylclonidine, een selectieve α_{1B}-remmer (Hoofdstuk 2.2). Gelijke effecten als met adrenaline werden verkregen door incubatie met de niet-selectieve α_1-agonist phenylephrine, maar niet met de selectieve α_{1A}-agonist methoxamine. De inhibitie van HL secretie door adrenaline verliep dus via α_{1B}-adrenerge receptoren. Voor dit subtype receptoren is beschreven dat ze signaleren via zowel intracellulair calcium als cAMP. Het effect van adrenaline wordt voornamelijk via calcium gemediëerd, omdat soortgelijke remming van HL ook wordt geïnduceerd door verhoging van intracellulair calcium met vasopressin, angiotensin, thapsigargin of de calcium ionophore A23187, maar niet door cAMP verhoging met forskolin of 8-Br-cAMP.

In pulse-chase experimenten met [^{35}S]methionine vertraagden zowel adrenaline, A23187 als thapsigargin (die alle een verhoging van intracellulair calcium teweeg brengen) de processing van het 53 kDa, premature eiwit naar het mature, 58 kDa eiwit. Verder was de intracellulaire degradatie van nieuw gevormd HL gestimuleerd (Hooftstuk 2.2). Doch een verlaging van intracellulair calcium door incubatie van de cellen met EGTA verlaagde de HL secretie door eenzelfde mechanisme. Dus, zowel hoog als laag intracellulair calcium heeft dezelfde post-translationele effecten op de HL expressie. Deze tegenstelling zou verklaard kunnen worden door dat al deze stoffen een verlaging van calcium niveaus in het endoplasmatische reticulum (ER) induceren.

Zoals juist beschreven werkt adrenaline post-transcriptioneel, door vertraging van de maturatie van het 53 kDa eiwit naar het 58 kDa eiwit en stimulatie van de intracellulaire afbraak van HL. Remmers van de lysosomale afbraak waren niet in staat het effect van adrenaline op de gesecreteerde en intracellulaire HL te beinvloeden (Hoofdstuk 2.3). Van de gebruikte protease-remmers was alleen de calpain I-protease remmer N-acetyl-leucine-leucine-norleucinal (ALLN) in staat om de adrenaline-geïnduceerde degradatie gedeeltelijk op te heffen. Als ALLN aanwezig was, verdween er minder 53 kDa prematuur eiwit, maar de maturatie naar het 58 kDa eiwit werd niet hersteld. De intracellulaire ophoping van het 53 kDa eiwit ging niet gepaard met een verhoging van triglyceridase activiteit. Deze resultaten suggereren dat de adrenaline-gemedieerde afbraak van HL het resultaat is van verstoorde maturatie en de blootstelling aan calcium-gevoelige cysteïne proteases in het ER.

REGULATIE VAN DE HL EXPRESSIE OP ANDERE POSTTRANSCRIPTIONELE NIVEAUS

Modulatie van lever lipase expressie door groeihormoon tijdens hypothyroïdie (Hoofdstuk 3)
Tijdens hypothyroïdie is de HL activiteit in de lever verlaagd. Deze lage activiteit wordt mogelijk veroorzaakt door de verlaging van het schildklier hormoon of door de daarbij optredende verlaging van groeihormoon (GH) activiteit. We hebben de HL expressie bestudeerd in lever cellen geisoleerd uit hypothyroïde ratten met en zonder substitutie van groeihormoon (Hoofdstuk 3). In alle ratten kon HL mRNA met behulp van RT-PCR worden aangetoond in parenchym cellen, maar niet in de andere cellen van de lever. In hypothyroïde cellen was het HL mRNA 40% verlaagd, de in vitro secretie van HL activiteit en HL eiwit was ongeveer 50% verlaagd. In cellen van

GH-gesubstitueerde hypothyroïde ratten was de hoeveelheid HL mRNA genormaliseerd, maar de secretie van HL bleef verlaagd. De specifieke enzym activiteit van het gesecreteerde HL was in alle gevallen gelijk. De discrepantie tussen HL mRNA en HL secretie in de GH-supplementeerde ratten zou een gevolg kunnen zijn van (post)translationele effecten. Daarom werd ook de HL synthese en maturatie bestudeerd met behulp van pulse-chase experimenten. De inbouw van [^{35}S]methionine in HL in hypothyroïde cellen en GH-gesupplementeerde cellen was lager dan in controle cellen. Tijdens de chase verliep de intracellulaire processing en het transport van nieuw gesynthetiseerd HL eiwit in parenchym cellen van hypothyroïde ratten, met of zonder GH-substitutie gelijk aan die van controle cellen. We concluderen dat in levers van GH-gesupplementeerde hypothyroïde ratten de translatie van HL mRNA achterblijft ondanks het herstel van HL mRNA niveaus.

Modulatie van lever lipase expressie in fenofibrate-behandelde ratten (Hoofdstuk 4)
Verlaging van HL expressie door fenofibraat is voornamelijk transcriptioneel, maar er zijn ook post-transcriptionele effecten van fenofibrate op HL beschreven. Het effect van fenofibrate op de HL expressie in de ratte lever werd nader bestudeerd. In fenofibraat-behandelde ratten was de HL activiteit in het lever homogenaat verlaagd tot 60% van controle levers. Ook als we corrigeerden voor het toegenomen cell volume, bleek de HL activiteit 25% lager dan in controle. De HL activiteit in de heparine-perfusaten was echter gelijk aan die van de controle.

De secretie van HL activiteit door lever parenchym cellen uit fenofibraat behandelde ratten was 70% geremd ten opzichte van controle cellen. Ook de incorporatie van [^{35}S]methionine in pulse-chase experimenten in de fenofibraat-cellen was verlaagd. De lage synthese van HL komt overeen met de verlaging van de hoeveelheid HL mRNA. Maturatie van het gesynthetiseerde HL eiwit in fenofibraat-cellen verschilde niet van dat in controle cellen. Dus, ondanks de verlaagde HL synthese, had fenofibraat geen effect op de HL activiteit die in de lever gebonden was op heparin-gevoelige plaatsen. Dit wijst erop dat er post-translationele regulatie plaats vindt op niveau van HL turnover. Post-translationele regulatie door fenofibrate kan plaats vinden op het niveau van HL binding, internalisatie en/of degradatie, maar vindt waarschijnlijk niet plaats op het niveau van maturatie van nieuw gesynthetiseerd HL.

N-GLYCOSYLERING VAN LEVER LIPASE

In de condities die we hebben bestudeerd, werden geen indicaties gevonden voor de betrokkenheid van N-glycosylering in de post-transcriptionele regulatie van HL. Dit ondanks eerdere bevindingen dat N-glycosylering, het verankeren en modificeren van suikerketens, voor zowel humaan als ratte HL essentieel is voor de secretie van actief HL. We hebben we de rol van N-glycosylering in de secretie en activering van HL nader bestudeerd in een humane lever cellijn (HepG2) en in vers geïsoleerde ratte lever cellen.

Secretie en intracellulaire processing van lever lipase (Hoofdstuk 5)

In hoofdstuk 5.1 hebben we de intracellulaire processing en activatie van HL in HepG2 cellen nader bestudeerd. Naast glycosylering blijkt juiste processing van de suikerketens, in het bijzonder afsplitsing van de eindstandige glucoses in het ER, cruciaal voor het verkrijgen van activiteit. In aanwezigheid van cycloheximide, waarbij de totale denovo synthese is geblokkeerd, verloopt de processing van het in de cel aanwezige HL onveranderd. Toch verschijnt er meer HL activiteit in het medium dan er verdwijnt uit de cellen. Er vindt dus activering van HL plaats. Het remmen van de N-glycosylering met tunicamycine, of het afsplitsen van de eindstandige glucose residuen in het ER met castanospermine (CSP) of N-methyldeoxynojirimycin (MdN) had eenzelfde remming van HL secretie tot gevolg als incubatie met cycloheximide. De intracellulaire HL activiteit was verdwenen, de totale hoeveelheid HL eiwit bleef onder de detectie limiet van de ELISA. De afhankelijkheid van de HL secretie voor de glucose trimming in het ER is totaal verschillend met de secretie van α_1-antitrypsine. Dit niet gerelateerde glycoprotein werd niet beïnvloed door incubatie met CSP of MdN. Ingrijpen tijdens de latere modulering van de suikerketen, door middel van een incubatie van de cellen met deoxymannojirimycine had geen invloed op de secretie van HL. De effecten van CSP en MdN incubatie op de intracellulaire HL activiteit waren te vergelijken met die van CCCP incubatie, waarbij het transport van ER-vesicles naar het Golgi was geremd. In tegenstelling tot HL verliep de secretie van α_1-antitrypsine onveranderd tijdens CCCP incubatie. Het blokkeren van het vesiculair transport tussen de verschillende Golgi compartimenten met behulp van monensin, of het fuseren van het ER en Golgi geïnduceerd door brefeldin A (BFA) had een ophoping van intracellulaire HL activiteit tot gevolg. De activatie van het intracellulaire HL in aanwezigheid van BFA gebeurde ook na een incubatie met CSP of MdN, hoewel die alleen een verlaging van intracellulaire HL activiteit veroorzaakten. Uit deze gegevens kunnen we concluderen dat glucose trimming voornamelijk belangrijk is voor het transport van nieuw gevormd HL van ER naar Golgi. Daarna verkrijgt het eiwit zijn triglyceridase activiteit in het Golgi en wordt vervolgens gesecreteerd. Welke processen leiden tot de activatie van HL in het Golgi blijft nog onbekend.

In hoofdstuk 5.2 hebben we de intracellulaire processing van HL in suspensies van vers geïsoleerde ratte hepatocyten bestudeerd. Analoog aan de studies in HepG2 cellen, bekeken we de rol van de glycosylering in de activatie en secretie van HL eiwit. De secretie van een katalytisch actief enzym hangt af van de juiste processing van de suikerketens. Voornamelijk het trimmen van de eindstandige glucose residuen in het ER is essentieel voor het verkrijgen van activiteit. Incubatie van lever cellen met tunicamycin, CSP or MdN had eenzelfde effect op de secretie van HL als cycloheximide. Ondanks een nauwelijks veranderde de novo synthese van HL kon er intracellulair geen actief HL eiwit worden aangetoond, mogelijk was er dus eiwit afgebroken. Interfereren later tijdens de processing van de suikerketens met deoxymannojirimycine had geen effect op de secretie van actief HL. Incubatie met CCCP, waardoor het ER-Golgi transport wordt geremd verlaagde de HL activiteit hetzelfde als incubatie met CSP of MdN. Het blokkeren van het transport tussen de verschillende Golgi-compartimenten met monensin, of tussen het Golgi-compartement en het plasmamembraan met colchicine, zorgde voor een intracellulaire ophoping van katalytisch actief HL. BFA werd helaas snel geïnactiveerd door de lever cellen, waardoor het niet bruikbaar was om ER en Golgi te doen versmelten. We kunnen concluderen dat zowel het humane als ratte HL eiwit actief wordt in het Golgi, waarna er secretie plaats vind. Hoe de activatie van HL in het Golgi tot

stand komt blijft nog onopgehelderd.

Hypothese over de regulatie van lever lipase secretie (Hoofdstuk 6)
In de discussie hebben we een model voorgesteld, dat onze bevindingen op het gebied van de intracellulaire processen die de HL secretie beinvloeden zou kunnen verklaren. Na de synthese van het Endo H gevoelige, premature eiwit van 53 kDa, is het transport uit het ER afhankelijk van de afsplitsing van de eindstandige glucoses. Essentiëel voor de vorming van het mature, Endo H resistente 58 kDa HL eiwit is het transport vanaf het ER naar het Golgi-compartiment. Dat is ook essentiëel voor het verkrijgen van enzym activiteit. Adrenaline beïnvloedt de snelheid waarmee HL het ER verlaat en de hoeveelheid die het ER verlaat. De effecten van adrenaline worden waarschijnlijk gemediëerd door een verlaging van calcium niveaus in het ER. Daardoor kan de vorming van transportabel HL geremd en de afbraak van het premature 53 kDa eiwit gestimuleerd zijn.

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Groetjes
Bernadette

CURRICULUM VITAE

Bernadette Paulina Neve werd op 16 november 1966 geboren te Walsoorden in de gemeente Hontenisse. Na het behalen van het MAVO diploma aan de St. Joseph Mavo (1983), werd het VWO diploma aan de Jansenius Scholengemeenschap te Hulst behaald (1986). In aansluiting hierop begon zij haar studie Medische Biologie aan de Rijksuniversiteit Utrecht. De eerste afstudeer-stage werd uitgevoerd bij het Hubrecht Laboratorium te Utrecht (het Nederlands Instituut voor Ontwikkelingsbiologie) onder leiding van dr. C.L. Mummery en drs. J. Schoorlemmer. Een tweede stage werd gedaan bij het Centrum voor Biomembranen en Lipid Enzymologie (CBLE) te Utrecht onder leiding van drs. B.C. Ossendorp en prof. dr. K.W.A. Wirtz. Een derde stage werd gedaan onder de leiding van dr. W. Stoorvogel bij de afdeling Celbiologie van de faculteit Geneeskunde te Utrecht. Het doctoraal examen werd in 1992 afgelegd. Vanaf mei 1992 werkte zij als Assistent-In-Opleiding bij afdeling Biochemie (Cardiovasculair Onderzoeksinstituut EUR) van de faculteit Geneeskunde en Gezondheidswetenschappen aan de Erasmus Universiteit te Rotterdam. Onder leiding van prof. dr. H. Jansen en dr. A.J.M Verhoeven werd het in dit proefschrift beschreven onderzoek uitgevoerd.

Hepatic Lipase:
Regulation at the post-transcriptional level

Lever lipase:
Regulatie op het post-transcriptionele niveau

Proefschrift

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus Prof. Dr. P.W.C. Akkermans M.A.
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaats
vinden op vrijdag 15 mei 1998 te 13.30 uur

door
Bernadette Paulina Neve
geboren te Hontenisse

STELLINGEN

1 De acute effecten van adrenaline op de expressie van lever lipase in de rat vinden plaats op een post-transcriptioneel niveau.
- *dit proefschrift*

2 In studies naar de expressie van lever lipase dient ook de afbraak van het enzyme te worden betrokken.
- *dit proefschrift*

3 Intracellulaire activatie van lever lipase vindt plaats na transport van het eiwit naar het Golgi-compartiment.
- *dit proefschrift*

4 'Molecular Chaperones' zijn niet per definitie betrokken bij de goede vouwing van eiwitten.
- *R.J. Ellis (1997), Biochem. Biophys. Res. Commun. 238, p687-692*

5 Chocolade is als rode wijn;
beiden hebben de potentie euforie op te wekken
en kunnen bij matig gebruik goed zijn voor hart en vaten
- *E. di Tomaso et al (1996), Nature 382, p677-678*
- *A.L. Waterhouse et al (1996), The Lancet 348, p834*
- *G.J. Soleas et al (1997), J. Clin. Lab. Anal. 11, p287-313*

6 Ook in het intracellulaire, membraan-gemedieerde eiwit transport speelt cholesterol een essentiële rol.
-*K. Simons et al (1997), Nature 387, p 569-572*

7 Het JoJo-effect dat vaak optreedt tijdens diëten is niet beperkt tot het lichaamsgewicht, maar geldt evenzeer voor het enthousiasme over een dieet.

8 De basis van toepasbare wetenschap ligt bij fundamentele kennis.

9 Proefschriften bij het oud papier kunnen zeer demotiverend werken.

10 Soms denkt men dat een Doelenaar van Lillo komt.

Rotterdam, 15 mei 1998