Aspects of Hepatic Lipase Expression

Relation to cholesterol homeostasis

Aspecten van lever lipase expressie

Relatie tot cholesterol homeostase

Proefschrift

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Abbreviations

ABCA-1	ATP Binding cassette transporter A-1
ACTH	Adrenocorticotropic hormone
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine 3'-5'- monophosphate
CAD	Coronary artery disease
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
CM	Chylomicron
CMrem	Chylomicron remnant
FC	Free cholesterol
HB_2	High density lipoprotein binding protein 2
HBP	High density lipoprotein binding protein
hCG	Human chorionic gonadotropin
HDL	High density lipoprotein
HL	Hepatic lipase
HSPG's	Heparan sulfate proteoglycans
IDL	Intermediate density lipoprotein
LCAT	Lecithin cholesterol acyl transferase
LDL	Low density lipoprotein
LIPC	Lipase C (HL gene)
LPL	Lipoprotein lipase
LRP	LDL receptor related protein
mRNA	messenger RNA
PL	Pancreatic lipase
PLTP	Phospholipid transfer protein
PMS	Pregnant mare serum
SR-BI	Scavenger receptor class B type I

Chapter 1

General Introduction

1.1. Short introduction to hepatic lipase

Hepatic lipase (HL; E.C. 3.1.1.34) is a glycosylated lipolytic enzyme with triglyceridase, phospholipase A1 and to some extent esterase activity. It has an apparent molecular weight of about 65 kDa in humans and 58 kDa in rat. HL is synthesized and secreted by the parenchymal liver cells and is extracellularly bound to the microvilli in the space of Disse [1-3] from where it is displaced upon heparin injection. In steroidogenic organs, as adrenal glands and gonads, an enzyme with similar characteristics is also detected [4, 5]. HL plays an important role in lipoprotein metabolism mainly HDL. It has been hypothesized that the enzyme promotes the reverse cholesterol transport by mediating the HDL unesterified cholesterol [6] and/or cholesteryl ester uptake by the liver [7]. A similar role for HL has been suggested in steroid hormone producing tissues, where HDL-cholesterol (ester) may be used for hormone synthesis. More recently it was suggested that HL cooperates with SR-BI (HDL receptor) in the cholesterol uptake [8]. In this process not only the lipolytic activity of HL may be involved but the protein also can have a bridging function between lipoprotein particles and cell surface receptors (see for review [9, 10]). Due to the involvement of hepatic lipase in lipoprotein metabolism it may be an important modulator of the intracellular lipid metabolism [11]. HL has been correlated with coronary vascular disease. However, the role of HL in the development of atherosclerosis is controversial [11]. The atherogenic character of HL activity is mainly ascribed to the capacity to produce pro-atherogenic lipoproteins such small dense LDL particles. An inverse correlation with plasma HDL-cholesterol levels, and an increased activity in atherogenic conditions such as diabetes mellitus type II and familial hypercholesterolemia has been described. Nevertheless, HL deficiency was not associated with a decreased risk to develop atherosclerosis. By hydrolyzing triglycerides and phospholipids in remnant lipoproteins and IDL, hepatic lipase contributes to a more efficient catabolism of these atherogenic particles. Moreover, it mediates the reverse cholesterol transport by stimulating HDL-cholesterol uptake and consequently the formation of lipid-poor HDL particles. In this way, HL would have anti-atherogenic properties. Finally, the atherogenicity of HL can be influenced by the presence of other genetic factors (e.g. CETP polymorphism) and ethnicity.

In this chapter, several characteristics of hepatic lipase will be discussed in more detail.

1.2. Characterization of hepatic lipase

1.2.1. Lipase gene family

Hepatic lipase (HL) together with lipoprotein lipase (LPL), pancreatic lipase (PL) and *Drosophila* yolk proteins (vitellogenins) were identified as members of the lipase gene family [12-14]. Later, PL-related proteins 1 and 2 that form a subfamily of pancreatic lipase were also found to belong to the lipase gene family [15, 16]. Recently, two other enzymes phosphatidylserine phospholipase A1 (PPL A1) [17, 18] and endothelial lipase (EL) [19, 20] were shown to belong to the same gene family. All members of this lipase gene family are water-soluble enzymes that hydrolyze ester bonds of substrates as triglycerides, phospholipids and cholesteryl esters. HL, LPL and PL share a high degree of sequence homology, structural similarities and lipolytic functions. HL is more closely related to LPL than to PL [14]. The active site consensus Gly-X-Ser-X-Gly (X=any aminoacid) in the amino-terminal domain is highly conserved. Site-directed mutagenesis studies of HL and LPL and computer modeling studies based on the crystal structure of PL [21] predicted the same protein folding, a common three-dimensional structure with conservation of the disulfide bridges [22, 23]. The N-terminal domain includes the active site and a lid domain covering the active site determines the substrate specificity [24]. The C-terminal domain is involved in lipid binding [25, 26]. For HL and LPL the C-terminal is also involved in heparin binding [27, 28].

1.2.2. Genomic organization and protein structure of HL

The HL gene (LIPC) is localized on chromosome 15 (q15-q22) in humans [29, 30] and on chromosome 9 in mice [31]. It has 9 exons separated by introns over a length of 60 Kb [32]. The promoter region of HL is found at –550 to +129 of the gene [33, 34]. Several genetic polymorphism of hepatic lipase were described; four of them are present in the 5'- region: A-763G, T-710C, C-514T and G-250A [35, 36]. The -514 C->T variant was first described as C-480T by Jansen and collaborators [37] and we will henceforth use this nomenclature. All four polymorphisms are linked and were described to be correlated with a lowering in enzyme activity and consequently involved in clinical disorders. This topic will be addressed later in this chapter. At 43 and 77 nucleotides upstream of the translation initiation codon were described two transcription start sites [32, 33]. Each exon has a length of 118-234 bp. Exon 1, codes for the signal peptide [32]. Exon 4 codes for the hydrophobic sequence corresponding to the lipid-binding site. Exon 5 codes for the segment containing the catalytic triad [13, 38, 39] and exon 6 and 9 code for sequences rich in basic amino acids involved in the binding domain to the cell surface [32]. Regions rich in basic amino acids are potential heparin binding sites [40-42].

The catalytic triad consists of Ser^{147}-Asp^{176}-His^{263} [14]. In HL the Ser^{147} residue is essential for enzyme activity [39] and the 22 amino acids that form the lid around the catalytic site are responsible for substrate specificity [24], mainly for the phospholipase activity. The C-terminal domain is also involved in the substrate specificity and is linked to the N-terminal domain by a span region [40-42]. The gene is expressed in most species, but has been mainly studied in humans, rats, mice and rabbits. However, the enzyme in this last species, even containing similar mRNA compared with other species, has very little enzyme activity. Contrary to human and rat, mouse has a circulating hepatic lipase activity. Rat and human HL are highly homologous. Human HL is a 476 amino acid mature protein predicting a polypeptide molecular weight of about 53 kDa. However due to N-linked glycosylation the protein has an apparent molecular weight of 65 kDa [30, 43-45]. For the same reason, the rat enzyme exhibits an apparent molecular weight of 58 kDa [46]. Human hepatic lipase has four putative N-linked glycosylation sites (Asn^{20}, Asn^{57}, Asn^{340} and Asn^{376}), the Asn^{57} is necessary for the secretion of active HL [47, 48]. In rat the Asn^{20} and Asn^{340} sites are absent [46]. Moreover, intracellular glycosylation is necessary for maturation and secretion of a fully active human and rat enzyme [45, 49, 50].

Besides mono- di- and triacylglycerol hydrolase activity, HL also has a phospholipase A1 and esterase activity, thus displaying a quite wide range of substrate specificity [51]. HL does not need a cofactor for enzyme activity, however HL activity can be influenced by apolipoproteins. Sindelar and collaborators [52] demonstrated that apoA-IV strongly stimulated the catalytic rate and alters substrate specificity of the enzyme towards human HDL_2 and VLDL. Hime [53] showed that Apo A-I enhances HL-mediated phospholipid hydrolysis in reconstituted HDL containing apo A-II. These results suggested that circulating lipoproteins containing apo A-I/A-II and /or apo A-IV are potential substrates for the enzyme in vivo. In vitro, HL is active at high salt concentration and at alkaline pH. Several studies using LPL-HL chimeras and specific antibodies against the C-terminus suggested that the catalytic site and the lipid binding domain do not necessarily cooperate. For esterase activity the lipid binding site is not required since the aqueous substrate is small enough to reach directly the catalytic site [39, 41].

The structure of the catalytic subunit of HL was approached using several techniques. Gel filtration techniques proposed a tetrameric and a monomeric form for rat [54, 55] and human HL [56], respectively. Studies on LPL-HL chimeric molecules suggested that HL may work as a homodimer [40-42]. Additionally, using radiation inactivation which allows to determine the smallest functional catalytic unit it was suggested that human HL produced by CHO cells is

functional as a homodimer [57]. In this model, the HL monomers are arranged in a head-to-tail manner with the C-terminal domain of one subunit near to the N-terminal of the opposing subunit. Another study proposed that rat HL is functional as an oligomer / dimer [58]. However, using radiation inactivation, we found that rat HL purified from liver and adrenal glands functions as a monomer and a dimer, respectively [59, chapter 2.3].

1.2.3. Localization and regulation

After secretion of hepatic lipase by hepatocytes, the enzyme is extracellularly anchored to microvilli of the parenchymal liver cells from where it can be displaced by heparin. Based on immunofluorescence and electron microscopy studies the enzyme was shown to be almost exclusively located at the luminal side of the parenchymal liver cells [3, 5]. In zona fasciculata of adrenal glands and corpora lutea of ovaries, HL is also extracellularly located and heparin releasable. The extracellular location is crucial for the functionality of hepatic lipase facilitating the binding, lipid hydrolysis and subsequent uptake of the lipid moiety of lipoproteins by the cells.

The gene expression of hepatic lipase can be regulated at the transcriptional level. Several regulatory elements were identified. The rat 5' sequence contains a CAAT box, specific sequences found in genes expressed in liver and GC-rich motifs. Putative regulatory elements involved in thyroid hormone (TRE), glucocorticoid (GRE) and estrogen (ERE), sterol (SRE) and cAMP (CRE) signalling pathways are also present [32, 33, 60, 61]. Additionally, in humans an E-box was also described which may be physiologically important [62]. Furthermore, negative (-2 kb to -555bp and +29 to +129) and positive regulatory elements (-85bp to -16bp) were linked to a down- and up-regulation of the transcription of the human gene, respectively [34, 61, 63-65]. The presence of these putative regulatory elements in the HL gene can explain the regulation of HL by hormones, drugs and nutrients.

In rat liver and hepatoma cell lines, HL activity is decreased by estrogens [66]. In contrast, androgens lead to an increase of HL activity [67]. The enzyme activity is lower in women than in men, however it increases after menopause suggesting regulation with estrogens. Indeed, in clinical studies of post-menopausal women, estrogen replacement therapy leads to a decrease in lipase activity. The decrease of HL is due to repression of its promoter activity suggesting regulation at the transcriptional level [68].

Corticotrophin and corticosteroids as, dexamethasone and triamcinolone were shown to reduce the hepatic enzyme activity in liver, but corticotrophin has the opposite effect in rat adrenal glands [69]. In humans corticotrophin also leads to a decrease of HL activity in liver [70].

This suggests that corticosteroids are able to redistribute lipase activity or influence its expression in different tissues. Other hormones such as thyroid hormones (T3 and T4) stimulate HL activity but not mRNA or protein levels suggesting regulation at post-translational level [71].

Cathecholamines such as adrenaline are also involved in the regulation of HL. They lower enzyme expression suggesting a post-trancriptional and post-translational regulation [72].

The effect of insulin on HL is still controversial and whether it involves regulation at transcriptional or post-transcriptional level remains to be elucidated. In Diabetes type I, post-heparin plasma HL activity is decreased and increases after insulin administration. In Diabetes type II the enzyme activity was shown to be increased [73]. Moreover, in rat hepatocytes insulin stimulated secretion of HL [74].

Heparin releases hepatic lipase from its binding site, but it also stimulates the enzyme activity. In vitro, heparin stimulated lipase activity secretion in medium of isolated hepatocytes and hepatoma cell lines. This observation may be due to up-regulation of gene expression, increase in secretion or stabilization of the enzyme preventing eventual reendocytosis and degradation [75, 76].

Furthermore, hypolipidemia-inducing drugs as fibrates and statins also affect lipase activity. Statins were shown to decrease HL activity [77, 78]. The effect of statins on HL is probably at transcriptional level.

Finally, diets rich in fish oil, saturated fats and cholesterol were reported to decrease HL activity [79, 80].

1.2.4. Hepatic lipase in steroidogenic tissues

Adrenal glands, ovaries and testes contain a lipase activity similar to that found in liver [4, 81, 82]. In these organs this enzyme is also denominated liver- or *l*-type lipase [83]. The lipase shows similar extracellular localization and catalytic activity present in the liver [5]. It is also heparin releasable [4] and is recognized and inhibited by antibodies raised against rat liver HL [84]. In humans adrenals from Cushing's syndrome patients two different forms of mRNA for HL are present. The full-length transcript is similar to that found in liver and in the second transcript exon 3 is absent. Whether this last mRNA is translated into a functional protein or whether it is also present in normal adrenals is not known yet (A.J.M. Verhoeven, personal communication). In rat adrenals and ovaries full-length HL mRNA could not be found [85, 86] suggesting that the enzyme is not locally synthesized and presumably originates from the liver and is transported to these organs. However, HL activity is almost undetectable in the

circulation. The origin of the lipase found in extrahepatic tissues is uncertain. In rat adrenal glands and ovaries the HL gene is transcribed into a truncated mRNA which does not yield the mature protein [85]. However, a shorter (40-45 kDa) intracellular protein was found to immunoreact with antibodies raised against HL [85] and to be transiently upregulated by hCG administration (Chapter 2.1.). Furthermore, this truncated mRNA lacking the 2 first exons of the HL gene has an alternative 5' sequence [85].

In rat steroidogenic tissues, the enzyme is almost exclusively localized in the steroidogenic most active compartment as zona fasciculata in adrenal cortex and corpora lutea in ovaries [5, 82, 87]. In adrenal glands, the lipase activity follows the pattern of serum corticosterone produced after administration of adrenocorticotropic hormone (ACTH) [88]. The lipase activity in rat ovaries is increased during (pseudo) pregnancy and lactation and it follows the pattern of serum progesterone secreted by ovaries during the estrous cycle [83, 89, 90]. It was observed that in ovaries the release of HL activity by heparin impaired the secretion of progesterone [90]. In line with this, ovaries of HL knockout female mice (HL -/-) produce less progesterone. This can explain the decreased ovulation and reduced litter size observed in these mice [91]. The localization, the induction of lipase activity during steroidogenesis and its role in lipoprotein metabolism strongly suggest that hepatic lipase is involved in the cholesterol homeostasis in adrenal glands and ovaries (see also chapter 1.3.4.).

1.3. Role of hepatic lipase in cholesterol homeostasis

1.3.1. Intracellular cholesterol homeostasis

Mammalian cells use cholesterol to maintain integrity of membranes and as a regulator of several functions in/of the cell. The cell relies on different sources to maintain its metabolically active cholesterol pool: de novo cholesterol synthesis from acetate, intracellular CE stores and plasma lipoprotein-cholesterol. The intracellular unesterified cholesterol (UC) pool directly available for cell demand is in dynamic equilibrium with the different cholesterol sources and with the UC pool present in the plasma membrane (see for review [92]). The plasma membrane UC may also originate from circulating lipoproteins. Since HL may stimulate the uptake of cholesterol from lipoproteins, it is likely to be involved in intracellular cholesterol homeostasis. In line with this, inhibition of HL induced de novo cholesterol synthesis in rat liver [93], and an increase in HDL receptor, scavenger receptor class B type I (SR-BI) expression in rat adrenals [94]. Similarly, HL deficiency in mice also stimulated SR-BI expression [95]. Furthermore, treatment with statins (HMG-CoA reductase inhibitors) resulted

in increase of LDL receptor numbers and subsequently in increase of cholesterol uptake by the liver but in a decrease of HL activity [77]. The results here described suggest that when HL is not available to supply cholesterol, the cell compensates this by stimulation of an alternative route, e.g. lipoprotein receptor mediated uptake. In this concept, lipase is reduced when the cholesterol content in the cell is already elevated. The intracellular cholesterol content depends on the expression of several genes involved in the cholesterol homeostasis, which are regulated at the transcriptional level via transcription factors as SREBP. LIPC is one of these genes. Recently, we found that overexpression of SREBP-2 in HepG2 cells decreased the promoter activity of HL (unpublished data). Taken together, we propose that besides playing a role in the lipoprotein homeostasis in the bloodstream, HL is also crucial for the maintenance of the cholesterol levels in cells.

Apart from hepatic lipase, other factors are involved in the uptake of cholesterol and in its intracellular redistribution. Caveolin-1, a plasma membrane-embedded structural protein [96] present in free cholesterol and sphingolipids rich microdomains, was suggested to be associated with lipoprotein receptors as SR-BI [97, 98]. However, caveolin-1 may be more important for the plasma membrane integrity [99-101] and subsequently for efficient cholesterol flux to/from the cell than for a direct effect on cholesterol level.

1.3.2. HL and lipoprotein metabolism

In plasma, cholesterol, triglycerides and phospholipids are transported in the form of lipoprotein molecules in which lipids are bound to apolipoproteins. In Figure 1 plasma lipoprotein metabolism is shown (see for review [102]). In the enterocyte, dietary lipids and bile acid are incorporated into triglyceride-rich lipoproteins as chylomicron (CM) and secreted into circulation. After lipolysis by lipoprotein lipase (LPL) which decreases their triglyceride core they are converted into chylomicron remnants (CMrem). The CMrem migrates to the liver to be taken up by remnant receptors that recognize their apolipoprotein E and afterwards undergoes lysosomal digestion.

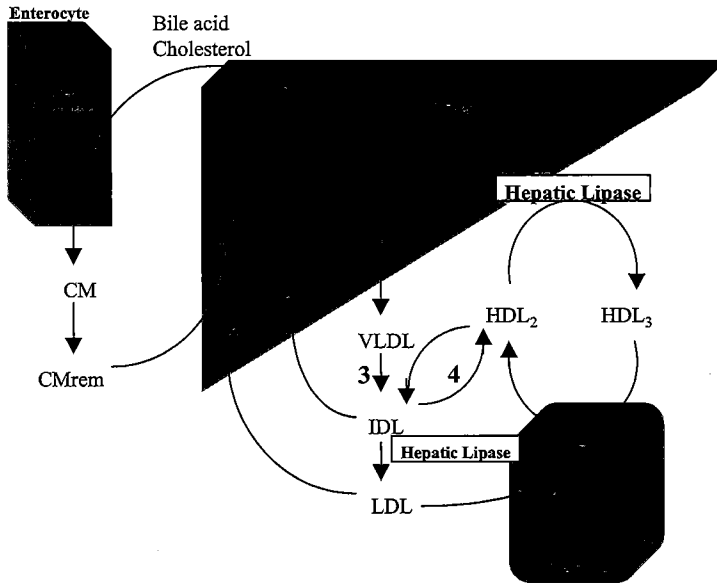

Figure 1. **Lipoprotein metabolism**

Hepatic lipase (HL) plays a role in the conversion of IDL to low density lipoprotein (LDL) and high density lipoprotein $(HDL)_2$ to HDL_3. Dietary cholesterol and cholesterol secreted by the liver into the intestine returns to the liver as Chylomicron remnant (CMrem). Cholesterol enters the endogenous pathway via very low density lipoprotein (VLDL) which after conversion to LDL delivers cholesterol to the peripheral tissues or returns to the liver. HDL_3 is able to take cholesterol from extrahepatic tissues to bring it to the liver in a process known as reverse cholesterol transport. 1. Remnant receptors, 2. Apo B/E receptors, 3. Lipoprotein lipase (LPL), 4. Cholesteryl ester transfer protein (CETP), 5. Scavenger receptor BI (SR-BI) and ATP binding cassete (ABC-A1) transporter.

Liver cells, besides having the capacity to synthesize cholesterol and triglycerides, are specialized in cholesterol clearance from circulating lipoproteins. The liver can also dispose excess of cholesterol into the intestine in the form of bile acids, which can be excreted or reabsorbed. Part of the hepatic cholesterol, together with triglycerides, is secreted into the circulation as VLDL. VLDL is the major transporter of triglycerides in plasma during fasting. The lipid core of VLDL undergoes hydrolysis catalyzed by LPL and is converted to IDL. In liver, some of the IDL is removed through interaction of its apoE with LDL receptors (apoB/E receptor) or it is further processed to LDL in plasma. In this case IDL exchanges its triglycerides with cholesterol esters from HDL by the action of CETP and after hydrolysis of triglycerides by hepatic lipase is subsequently converted to LDL [103]. LDL particles bring

cholesterol to the liver after binding to LDL receptors (apoB/E). Additionally, LDL can also deliver cholesterol to peripheral cells via the LDL-receptor pathway [104]. HDL_2 is susceptible to HL-mediated hydrolysis in the liver and is converted into a smaller en denser HDL_3 [66]. In the periphery HDL_3 binds to its putative receptor SR-BI or to ABC-A1 transporters.

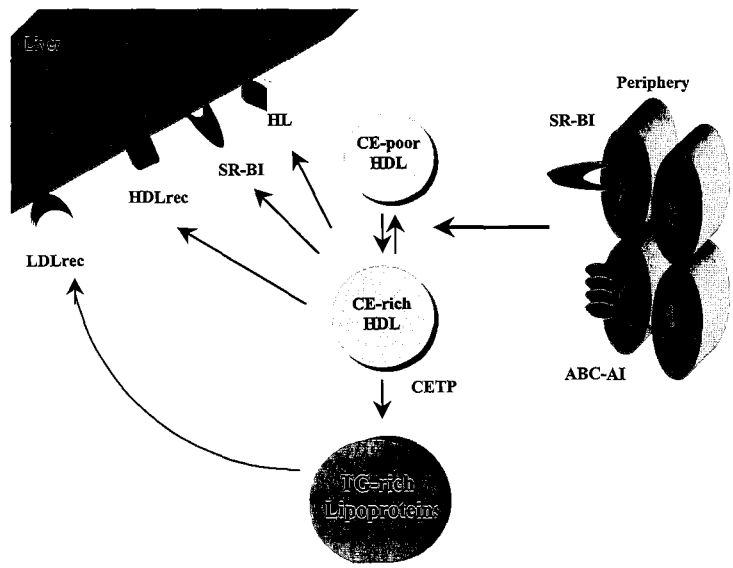

Figure 2. **Role of hepatic lipase in HDL metabolism**

HDL transports cholesterol from the periphery to the liver. HDL phospholipids are hydrolyzed by hepatic lipase promoting the cholesterol uptake by the liver. Alternatively, HDL binds to HDL receptors as HB_2 and HBP or to SR-BI. In this way HL and SR-BI may be both involved in the selective uptake of the HDL-cholesterol by the liver. In an indirect pathway, HDL-cholesterol can be transferred to other lipoproteins as LDL and subsequently taken up via LDL receptor.

Reverse cholesterol transport is a very important process by which excess cholesterol may be removed from peripheral cells to return to liver through the circulation [105, 106]. Once in the liver it can be recycled and incorporated into lipoproteins as VLDL to be secreted or further catabolized into bile acids as described above. However, in peripheral tissues, for example the arterial wall, endothelial cells and macrophages cholesterol accumulation may lead to atherosclerosis [107]. Therefore, an efficient reverse cholesterol transport is important in the prevention and/or treatment of atherosclerosis. Reverse cholesterol transport can be affected

by diverse factors. In this review only a couple of them will be discussed in detail with special emphasis on hepatic lipase. Hepatic lipase has been proposed to play an important role in reverse cholesterol transport by influencing HDL metabolism [7, 108] (Fig. 2).

In plasma the HDL forms a heterogenic pool of particles with varying composition [109]. Nascent apo A-I rich HDL is able to bind to receptors on peripheral cell surface such as SR-BI or ABC-AI and accept unesterified cholesterol and phospholipids becoming a mature spherical particle [110]. On the HDL particle unesterified cholesterol is esterified to cholesteryl esters by LCAT. The cholesteryl esters are then delivered to the liver via two pathways: indirectly via LDL or/and directly via HDL. In the indirect pathway, the HDL exchanges its cholesteryl esters for triglycerides from apoB-containing lipoproteins through the action of CETP. Subsequently, LDL is taken up via the LDL receptor [111]. HDL can directly deliver cholesterol via an endocytotic pathway or via selective uptake [112, 113]. In the endocytotic pathway, HDL receptors at the liver cell surface as HB_2 and HBP may be involved in the HDL uptake [114]. The selective cholesterol uptake does not require the endocytosis of the whole lipoprotein particle. Instead, the lipid moiety is delivered without apolipoprotein uptake after docking to SR-BI [115] and/or after undergoing hydrolysis of phospholipids and triglycerides by HL.

Many studies on the contribution of the hepatic lipase to HDL metabolism and to reverse cholesterol transport have been carried out. Hepatic lipase deficient mice show large phospholipid- and apoE- rich HDL particles [116, 117], while HL deficient mice, in whom HL overexpression was induced by adenoviral-mediated gene transfer in liver, showed a reduction of HDL-cholesterol and smaller denser HDL particles [118-121]. Transgenic rabbits overexpressing HL showed the same tendency [103, 122]. In humans, genetic HL deficiency is associated with elevated HDL-cholesterol levels and presence of larger HDL particles, however the effect is modest [123, 124] and humans displaying an elevated HL activity in post-heparin plasma have decreased levels of HDL-cholesterol [125]. These observations give strong evidence that HL activity is a modulator of HDL-cholesterol and particle composition in plasma and that the HL gene is an important determinant of HDL level.

Not only the catalytic activity of HL seems to be important in the HDL metabolism. Expression of a catalytic inactive form of the enzyme in mice led to a decrease in HDL-cholesterol by about 40% [10]. The authors explained this observation by assuming that HL may mediate HDL binding to the surface of hepatocytes by forming a bridge between the lipoprotein particle and the HSPGs. These observations are in line with our results described in Chapter 3.1. where hepatic lipase is shown to mediate the HDL binding to rat adrenal cells.

Lambert and collaborators [8] showed that HL in both active and inactive form could mediate HDL-cholesteryl ester uptake in a cell system by enhancing the capacity of HDL to bind to SR-BI. By studies using cell models it was shown that hepatic lipase can directly stimulate HDL-CE uptake [103, 119, 122]. Another study showed that administration of heparin and antibodies raised against HL activity inhibited the uptake of CE [126]. However, we showed that in vivo inhibition of HL activity may elicit changes in the expression of other genes e.g. SR-BI, which may affect HDL cholesterol uptake [94; chapter 3.2.]. The precise biochemical mechanism underlying the increase of HDL-cholesterol uptake under these circumstances is presently unknown. One possible explanation is that hepatic lipase plays a direct role in uptake of HDL unesterified cholesterol but only indirectly in cholesteryl ester uptake. In this way, hepatic lipase would stimulate the uptake of unesterified cholesterol by hydrolyzing phospholipids on the HDL surface. Consequently it may generate a deficiency in cholesteryl ester in the cell. In an attempt to restore this desequilibrium, SR-BI expression is increased and HDL-cholesteryl esters are finally taken up via this pathway.

Based on the studies described above it is evident that hepatic lipase plays a crucial role in the metabolism of HDL.

1.3.3. Association of HL with clinical disorders

In view of the apparent important, although not completely elucidated, role of HL in HDL metabolism a relation to heart and vascular disease is to be expected.

To understand the role of HL in atherosclerosis, several animal models have been developed. Overexpression of HL in cholesterol fed mice was reported to reduce aortic cholesterol content but also plasma HDL cholesterol [119]. In addition, overexpression of HL in transgenic rabbits [103] as well as overexpression of apoB in transgenic mice and apoE deficient mice [120] leads to reduction of circulating pro-atherogenic lipoproteins and HDL cholesterol. HL knockout mice do not have increased atherosclerosis [117]. HL knockout mice with apo E deficiency [127] have increased plasma cholesterol but decreased susceptibility to develop atherosclerosis. These results suggest that the development of atherosclerosis via HL is independent of plasma cholesterol levels and may take place via other pathways.

Also clinical studies pointed to the importance of hepatic lipase in lipoprotein metabolism and its anti- or pro-atherogenic character. In humans, increased HL activity has been associated with atherosclerosis due to its ability to form atherogenic lipoproteins. HL might be responsible for accumulation of small dense LDL particles and also for a low HDL-cholesterol level in plasma [128]. A low hepatic lipase activity is frequently associated with

coronary artery disease (CAD). HL deficiency is mostly due to functional mutations in exon 5, 6 or 8 leading to an inactivation of lipase activity or to an impaired enzyme secretion [124, 129, 130]. It is also associated with an increased risk to develop atherosclerosis, especially in the presence of other genetic factors that modulate lipid levels in plasma [131]. In fact, in a population with low HL activity the risk to develop CAD was increased exclusively when combined with CETP genetic deficiency [132]. The role of HL and CETP in an efficient reverse cholesterol transport can explain this. When one of the pathways fails, it can be compensate by another pathway (i.e. lipoprotein receptors). Polymorphism in the regulatory 5' region of the human hepatic lipase gene (LIPC) were also described [35, 37]. A G-216A and a C-480T substitution are simultaneously present and have an allele frequency of 0.15-0.20 in Caucasians [35, 37] and about 0.54 in African-Americans [133]. In Caucasians, these polymorphisms were associated with a decrease in hepatic lipase activity in postheparin plasma and hyperlipidemia [37, 133-135]. Furthermore, the C-480T base substitution was suggested to be functional. Since it leads to a decrease in HL expression it explains the association between the -480T allele and the low HL activity shown in man [62, 136]. The -480T allele was shown to be associated with insulin-resistance [137] and in combination with APOC3 -482T allele was associated with an increased glucose and insulin response during oral glucose tolerance tests [138]. Patients with diabetes mellitus type II (DM type II) have predisposition to develop CAD. Hepatic lipase activity is increased in these patients and since this increase is accomplished by a decrease in HDL-cholesterol levels it is suggested to be an atherognic factor. However, CAD has been positively correlated with both high [139] and low [140] HL activity. HL also has anti-atherogenic properties since it is involved in the conversion and removal of remnant particles that are highly atherogenic [141-143]. Moreover, the low HDL-cholesterol level that is seen in DM type II could reflect a rapid reverse cholesterol transport and subsequently a more efficient clearance of plasma/peripheral cholesterol. In this case it would be favorable against the development of atherosclerosis.

To determine whether HL has a pro- or anti-atherogenic character and whether its activity and presence of polymorphism are determinants to predict clinical disorders, more studies are needed. Furthermore, it is important not to underestimate the contribution of other genetic factors, including the ethnicity of the population that will certainly contribute to the clinical feature.

1.3.4. HL and SR-BI in steroidogenesis

Steroidogenic tissues (adrenal glands, gonads and placenta) undergo physiologic modifications during hormonal stimulation. They utilize intracellular and extracellular cholesterol to synthesize steroid hormones. The intracellular cholesterol is supplied by the de novo synthesis or after hydrolysis of CE stored in cytoplasmic lipid droplets. Alternatively, cholesterol can enter the cell as a component of circulating lipoproteins [144]. Lipoprotein particles (LDL and HDL) bind to specific receptors (LDL receptor and SR-BI) at the cell surface and deliver cholesterol (Figure 3 A). ACTH stimulates de synthesis and secretion of steroid hormones by adrenocortical cells [145]. Similar observations are described for granulosa cells of luteinized ovaries after PMS-hCG administration. When the production of steroids is increased the steroidogenic cell undergoes ultrastructural changes. In adrenal cortex, the cytoplasmic volume of the zona fasciculata (the most steroidogenic tissue of the adrenal cortex) is enlarged and lipid droplets become smaller as result of CE hydrolysis to supply cholesterol demanded for hormone synthesis. After an acute ACTH treatment, the cholesterol is mobilized from its esterified storage, de biosynthesis is increased and the intracellular transport of cholesterol to the mitochondria is stimulated [146]. During long/chronic stimulation with ACTH the hyperplasia and hypertrophy proceed in parallel to the cholesterol demand. The intracellular source of cholesterol is depleted and the cell relies on extracellular cholesterol sources such as lipoproteins [147-149]. HDL delivers cholesteryl esters via selective uptake [150] and LDL via receptor-mediated endocytotic pathway [151]. Under circumstances of enhanced steroid hormone output, the number of lipoprotein receptors increases and lipoproteins become the most important cholesterol source (Figure 3 B) [152, 153].

As described above (chapter 1.2.4), HL is present in adrenocortical cells and in ovarian corpora lutea. Its activity that parallels the output of steroid hormones, its localization at the extracellular surface together with its function in lipoprotein lipolysis, suggests a role for HL in the cholesterol supply to these organs. In fact, Jansen and collaborators [6] already suggested that also in non-hepatic tissues HL would modulate the unesterified cholesterol uptake by hydrolyzing phospholipids and triglycerides on the surface HDL particles. Moreover, in steroidogenic organs HL was suggested to mediate tissue uptake of cholesterol without requiring involvement of lipoprotein receptors [81]. In this model, HL serves itself as a binding site for HDL.

However, only recently the role of HL in steroidogenesis got a new perspective with the finding of the HDL receptor. In fact, scavenger receptor BI (SR-BI) was recently identified as a putative HDL-receptor [154]. It has two transmembrane domains and a large extracellular loop.

SR-BI is anchored to the plasma membrane and is a multiligand and multifunctional receptor active in liver, non-placental steroidogenic tissues and atherosclerotic lesions (see for review [155]). In steroidogenic cells, SR-BI expression follows the pattern of the hormone production [156]. Immunocytochemical studies have shown that SR-BI is expressed on the cell surface of the zona fasciculata and reticularis of the adrenal cortex, the corpora lutea of the ovaries and the Leydig cells of the testes [156-158]. In these tissues SR-BI was proposed to have a main function in the selective HDL-cholesterol uptake [159].

The tissue distribution of hepatic lipase and SR-BI largely match each other. Their extracellular localization at the same cell type, similar hormonal regulation and their suggested function in the lipoprotein metabolism suggest that SR-BI and hepatic lipase could cooperate in the HDL-cholesterol (ester) uptake (Figure 3) [95]. There are several indications that HL and SR-BI are involved in HDL-cholesterol uptake by the cell. They can act together or in parallel pathways. In a cooperative pathway, hepatic lipase modulates the HDL particle by hydrolyzing the phospholipids from the lipoprotein surface [160]. In this way, cholesterol ester could easier be taken up via SR-BI. Lambert and collaborators suggested that hepatic lipase would act as a bridge between the HDL and its receptor [8]. Also, in CETP transgenic mice it was found that the combined activity of CETP and HL may optimize the clearance of triglyceride-rich HDL particles via SR-BI in liver [161]. Furthermore, antibodies against SR-BI inhibit the stimulation of HDL-CE uptake due to HL, suggesting the importance of both proteins in selective uptake [126].

HL and SR-BI could also act in parallel pathways. Female HL knockout mice showed depletion of adrenal cholesterol stores and increase of SR-BI expression [95]. In line with this, incubation with cyclodextrin resulted in depletion of intracellular cholesterol and in an increase of SR-BI expression. Other studies pointed to a role of hepatic lipase and SR-BI on the clearance of phospholipids/unesterified cholesterol-rich lipoproteins in PLTP-deficient mice [162]. In PLTP deficient mice fed with a coconut oil-rich diet, the accumulation of lipoproteins rich in phospholipid/free cholesterol ratio dramatically increased when mice were also HL deficient. About 50% of the particles were taken up via SR-BI in hepatocytes. However, on a coconut oil-rich diet SR-BI could not mediate the uptake of phospholipids and free cholesterol. The authors observed that under these conditions, the fatty acid composition of the plasma membrane was altered suggesting that SR-BI is dysfunctional. In a situation that SR-BI is dysfunctional an active pathway for the clearance of the particles, e.g. a pathway involving HL, is essential. Taken together, HL and SR-BI can act synergistically or offer two parallel routes to deliver cholesterol to the cell. When one of the routes is not operative, the

cell can rely on an alternative pathway. In fact, we showed that when HL activity is inactivated with antibodies, the SR-BI expression is increased in rat adrenal glands [94].

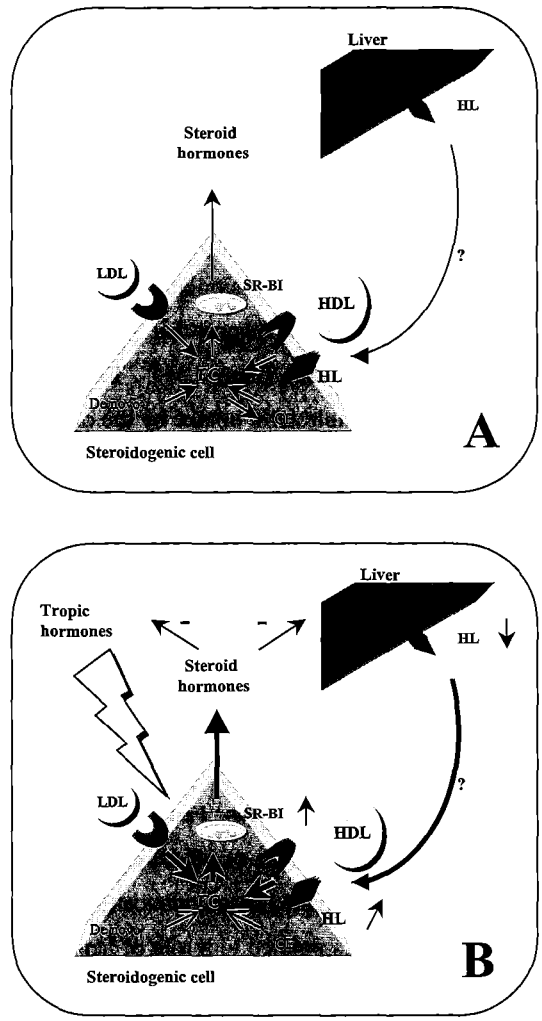

Figure 3. **Role of HL and SR-BI in steroidogenesis**

Steroidogenic cells, as adrenocortical and ovarian cells, rely on various cholesterol sources to produce steroid hormones. (A) The de novo cholesterol synthesis, hydrolysis of CE or circulating lipoproteins are the main sources. (B) Tropic hormones as ACTH or hCG, stimulate the hormone output and the cholesterol demand. Under these conditions the intracellular store is not enough and lipoproteins become the most important cholesterol donor. Lipoprotein receptors as LDL receptor, SR-BI and HL expression are induced by tropic hormones to compensate the reduction in intracellular cholesterol. HL and SR-BI are involved in an efficient selective HDL-cholesterol(ester) uptake by the cell. Steroid hormones down-regulate the HL expression in the liver.

1.4. Scope of this thesis

Hepatic lipase has triacylglycerol hydrolase and phospholipase A_1 activity towards a wide variety of substrates. It is extracellularly localized in liver and in steroid hormone producing organs. The enzyme plays an important role in both intracellular cholesterol homeostasis and lipoprotein metabolism. Furthermore, in adrenal glands and ovaries the lipase activity may play a role in the cholesterol uptake from circulating lipoproteins to be used in hormone synthesis. In this process SR-BI may also be involved.
In this study we intended to characterize the expression of the hepatic lipase in steroidogenic tissues under different conditions. Further, we studied its relation to the recently proposed putative HDL receptor, SR-BI, in the cholesterol homeostasis in those organs.

Expression and regulation of the LIPC gene in steroid hormone producing tissues
In rat adrenals the full-length HL mRNA is absent but a variant form missing the first two exons could be detected. In rat adrenals and ovaries hepatic lipase activity is increased after stimulation with tropic hormones. We first intended to study the HL gene expression in rat ovaries after hormonal induction of superovulation and to investigate how gonadotropic hormones regulate the variant HL mRNA (Chapter 2.1.).

Our results showed that trophic hormones modulate the variant HL mRNA and protein levels. Based on these observations we hypothesized that the variant HL mRNA would own an alternative promoter region involved in the enzyme expression in steroidogenic tissues. We proposed to localize this novel 5'-region, to identify a possible transcription start site and to study the regulation of this promoter (Chapter 2.2.).

From our results it is not clear whether the HL activity found in rat ovaries and adrenal glands is a product of the variant HL mRNA or synthesized by the liver and transported to those organs. In an attempt to better understand this matter we studied the functional molecular mass of hepatic lipase in those organs and compared it to that found in liver (Chapter 2.3.).

HL and cholesterol homeostasis
Adrenals and ovaries rely on cholesterol delivered by circulating lipoproteins as a substrate for steroid hormones. In these organs an efficient cholesterol uptake is crucial. Several studies had previously suggested that hepatic lipase is involved in the uptake of cholesterol in

steroidogenic tissues. More recently, the HDL receptor SR-BI was suggested to play a similar role in the process. We hypothesized that HL and SR-BI cooperate in the HDL binding. We first studied whether HL can modulate HDL binding to rat adrenal membranes in vitro in relation to SR-BI expression (Chapter 3.1.).

HL seems to be able to modulate HDL binding to SR-BI in vitro. Therefore, we investigated the effect of the inactivation of HL activity in vivo on uptake of HDL-cholesteryl ether by the adrenal gland of the rat (Chapter 3.2.).

Since our results indicate that there is an interaction between HL and SR-BI we questioned whether this may be a direct interaction by studying their localization in rat adrenal glands (Chapter 3.3.).

Finally, we studied to what extent HL and SR-BI are differently regulated in rat liver and adrenal glands (Chapter 3.4.).

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Chapter 2

Expression and regulation of the LIPC gene in steroid hormone producing tissues

Chapter 2.1

Hepatic lipase gene expression is transiently induced by gonadotropic hormones in rat ovaries

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Hepatic lipase gene expression is transiently induced by gonadotropic hormones in rat ovaries

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Abstract

Hepatic lipase (HL) gene expression was studied in rat ovaries. A transcript lacking exons 1 and 2 could be detected by reverse transcription-polymerase chain reaction (RT-PCR) in the ovaries of mature cyclic females and of immature rats treated with pregnant mare serum followed by human chorionic gonadotropin (hCG) to induce superovulation. By competitive RT-PCR the HL transcript was quantified. Low levels of HL mRNA were detected in ovaries of mature cyclic females and of immature rats. During superovulation HL mRNA was several fold higher than in mature cyclic rats and transiently increased to a maximum at 2 days after hCG treatment. Pulse-labelling of ovarian cells and ovarian slices with [^{35}S]methionine followed by immunoprecipitation with polyclonal anti-HL IgGs showed de novo synthesis of a 47 kDa HL-related protein. Expression of the protein was transiently induced by gonadotropins with a peak at 2 days after hCG treatment. Induction of liver-type lipase activity occurred only after HL mRNA and synthesis of the HL-related protein had returned to pre-stimulatory levels. We conclude that in rat ovaries the HL gene is expressed into a variant mRNA and a 47 kDa protein. The expression of the HL gene in ovaries is inducible and precedes the expression of the mature, enzymatically active liver-type lipase.

Keywords: Hepatic lipase; Liver-type lipase; Steroidogenesis; Ovary; Superovulation; Polymerase-chain reaction

1. Introduction

Ovaries and adrenals of rats and humans have been shown to contain a lipase activity (liver- or *l*-type lipase) similar to hepatic lipase (HL) normally present in liver sinusoids [1–5]. Whereas the liver enzyme plays a role in the uptake of circulating lipoproteins and its components, the *l*-type lipase in the steroidogenic organs is suggested to mediate the delivery of high-density lipoprotein cholesterol required for hormone synthesis [6]. In rat ovaries, the *l*-type lipase activity is mainly localized in the corpora lutea, which are most active in steroidogenesis [4,7] and is increased several-fold in immature rats upon induction of superovulation [8]. The *l*-type lipase activity in the ovaries varies during the oestrous cycle and lactation in parallel with progesterone and 20α-hydroxyprogesterone output [2,7,8].

The *l*-type lipase present in steroidogenic organs is thought to originate from the liver since full-length HL mRNA was not detected in adrenals and ovaries [9,10]. In this model, the lipase is secreted by the parenchymal liver cells, and then transported through the circulation

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to the steroidogenic organs. Rat ovaries are indeed shown to selectively sequester [^{125}I]HL from the circulation [9]. We recently found that the HL gene is transcribed in rat ovaries as well as in human and rat adrenals [11]. In rat adrenals, however, the mRNA product markedly differed from that in liver [12]. In the adrenal mRNA, the exons 1 and 2 of the HL gene are replaced by a novel sequence, so that it no longer encodes for the normal HL protein. Instead, a candidate translation product of about 45 kDa was identified [12]. This prompted us to study HL gene expression in rat ovaries. We show here that the HL gene products in the ovaries resemble that found in adrenal glands. Moreover, HL gene expression in ovaries is transiently stimulated several-fold upon induction of superovulation and was found to precede the acquisition of enzymatically active *l*-type lipase.

2. Materials and methods

2.1. Tissues

Locally bred Wistar rats were housed under controlled conditions of temperature, humidity and light (12 h light/12 h dark; light on at 07:00 a.m.), with free access to tap water and to a standard pellet diet. Animals were killed by decapitation under light diethyl ether narcosis. Ovaries were dissected from mature cyclic rats weighing 250–300 g. Alternatively, ovaries were isolated from 25–30 days old female rats in which superovulation was induced by a s.c. injection of 10 IU pregnant mare serum (PMS) (Gestyl, Organon, Oss, Netherlands) followed 2 days later with an injection of 10 IU human chorionic gonadotropin (hCG; Pregnyl, Organon, Oss, Netherlands). The day of hCG injection was designated as day 0. The dissected ovaries were placed in ice-cold phosphate-buffered saline (PBS) and the adherent fat tissue was rapidly trimmed off. Liver and whole adrenals were dissected from young adult males weighing 200–250 g. The tissues were either used immediately, or frozen in liquid nitrogen and stored at −80°C.

2.2. RNA analysis

Total RNA was isolated from whole organs by the method of Chomczynski and Sacchi [13]. RNA concentrations were determined by spectrophotometry at 260 nm [14]. The quality of the RNA preparations was judged from the pattern of ribosomal RNA after gel-electrophoresis [14]. HL mRNA was determined by a single tube reverse transcription-polymerase chain reaction (RT-PCR) starting from 1 μg of total RNA [12]. Random-primed cDNA was amplified for 30–35 cycles using different HL-specific primers. HL-1 (5′-GTG GGC ATC AAA CAG CCC-3′, nt 712–729; numbering according to the rat cDNA sequence [15] and HL-2 (5′-CAG ACA TTG GCC CAC ACT-3′, nt 1307–1289) were used in quantitative PCR. The sense oligonucleotides HL-12 (5′-TGG CTT GCT AGA AAC CTG G-3′, nt 297–315), HL-13 (5′-TGT CAT GAT CAT CCA CGG G-3′, nt 267–285) and INT (5′-GCA TTG TCC TTG AGC CTG AG-3, nt-112 to −93 according to the sequence upstream of exon 3 in adrenal HL mRNA [12]) were used in combination with the antisense oligonucleotide HL-9 (5′-GGC ATC ATC TGG AGA AAG GC-3′, nt 660–641) to determine the 5′-end of the HL mRNA. Quantitation of HL mRNA was done by competitive RT-PCR, in which 1 μg tissue RNA was mixed with 0.5 μg yeast RNA (Pharmacia, Uppsala, Sweden) containing increasing amounts of competitor RNA prior to reverse transcription and amplification [12]. The competitor RNA was obtained by in vitro transcription of an HL cDNA clone in pBluescript KS$^-$ which had been modified by deletion of an internal 80-bp Sau3A fragment (nt 1009–1088, numbering according to [15]). The amount of target RNA was determined from ethidium-bromide-stained gels by taking (or extrapolating to) the amount of internal competitor RNA that produced an equal signal intensity. All RT-PCR experiments included no-template and no-RT controls that remained negative.

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

Pulse-labelling studies were performed either with whole-ovary slices, or with ovarian cells isolated from female rats 2 days after treatment with hCG. Freshly dissected ovaries were roughly minced using a razor blade. The resulting slices were incubated in 1 ml methionine-free minimum essential medium, Eagle's modified (MEM) containing 25 U/ml heparin (Leo Pharmaceuticals, Weesp, Netherlands) and 20% bovine serum (dialysed and lipoprotein-free) at 37°C in a shaking water bath under an atmosphere of 5% CO_2/95% O_2. In some experiments, the tissue slices were first incubated for 45 min at 37°C with 3 mg/ml of collagenase (type I, Sigma, St. Louis, USA); thereafter, intact cells were collected by centrifugation (10 min, 100 × *g*, 20°C) through a cushion of 0.5% BSA in PBS [16]. The cell pellet was resuspended in methionine-free MEM and incubated in a final concentration of $1–2 \times 10^6$ cells/ml as described above for the tissue slices. After 30 min incubation, 80 μCi of Tran^{35}S-label (ICN, Cosa Mesa, USA) was added and the pulse-labelling was allowed to proceed for 2 h. The incubation was stopped on ice, and the cells/tissue and cell-free media were separated by centrifugation (10 000 × *g*, 20 s, 4°C). The cell pellet was washed twice with ice-cold PBS and then lysed for 45 min at 4°C in PBS containing 1% Triton X-100, 1% sodium deoxycholate, 10 mM Hepes (pH

7.4), 25 U/ml heparin, 1 mM L-methionine, 1 mM EDTA, 10 μg/ml benzamidine, 10 U/ml Trasylol, and 1 μg/ml each of leupeptin, antipain, chymostatin and pepstatin. After centrifugation (10 min, 10 000 × g, 4°C), the lysates were used for immunoprecipation.

2.4. Immunoprecipitation

Tissue and cell lysates were pre-cleared by incubation for 2 h at 4°C with non-immune goat IgG immobilized onto Sepharose-4B (Pharmacia, Uppsala, Sweden). After removal of the beads, the lysates were incubated overnight with polyclonal goat anti-rat HL IgGs coupled to Sepharose-4B. Some immunoprecipitations were carried out in the presence of an excess unlabelled, partly purified rat hepatic lipase [17]. The beads were collected by centrifugation and then washed with 1 ml of, successively, PBS, 1 M NaCl in PBS, 0.2% Tween-20 in PBS and PBS (all at 4°C). The final pellet was resuspended in Laemmli sample buffer [18], heated for 5 min at 95°C and the beads were removed by centrifugation. The released proteins were analyzed by SDS-PAGE on a 10% polyacrylamide gel, followed by fluorography using Amplify (Amersham, UK). The autoradiograms were scanned with the HP ScanJet II CX densitometer, and the integrated optical density was expressed in arbitrary units.

2.5. l-Type lipase assay

The ovaries were homogenized in 10 Vol. of ice-cold PBS (pH 7.4) containing 5 U/ml of heparin and 1 mM benzamidine. After centrifugation (10 000 × g, 2 min, 4°C), the post-nuclear supernatant was assayed for triacylglycerol hydrolase activity at pH 8.5 in 0.6 M NaCl using a gum-acacia stabilized glycerol [^{3}H]trioleate emulsion as substrate [3]. In immunoinhibition assays, the supernatant was pre-incubated for 1 h at 4°C with excess goat anti-rat HL IgGs immediately before the lipase assay. HL activity is defined here as the triglyceridase activity that was sensitive to immunoinhibition [17]. Enzyme activities were expressed as mU (nmol of free fatty acids released per min).

3. Results

3.1. Effect of gonadotropins on HL mRNA in rat ovaries

RT-PCR on total ovary RNA isolated from mature cyclic rats and from immature superovulating rats using the primers HL-1 and HL-2 yielded the expected 596-bp product, whose identity with part of HL cDNA has been confirmed by restriction mapping and internal re-amplification [11]. In order to obtain UV-detectable DNA bands, 5–10 extra cycles of amplification were required with RNA from mature rats than from that of superovulating animals, suggesting that the amount of HL mRNA was higher in the gonadotropin-stimulated ovaries. Quantitation of HL mRNA was done by competitive RT-PCR using HL RNA that lacked an internal 80-nt fragment as competitor (Fig. 1). In five independent ovaries from cyclic female rats, the amount of HL mRNA ranged from 0.002 to 0.005 attomoles per μg of total RNA. In the ovaries from superovulating rats, the amount of HL mRNA was at least ten-fold higher at 0.05–0.4 attomoles per μg total RNA, depending on the treatment with gonadotropins (see below). Using the same assay, rat liver was shown to contain 5–10 attomoles HL mRNA per μg total RNA. Hence, the HL gene is expressed in rat ovaries to a level two to three orders lower than in liver.

Although part of the HL gene transcript could be detected by RT-PCR in ovaries, we were unable to amplify the entire HL cDNA from ovary RNA, either isolated from mature cyclic rats or from young superovulating rats. For rat adrenals, we recently reported that the HL gene is transcribed into a variant mRNA in which exons 1 and 2 are replaced by a unique sequence [12] representing part of intron 2 immediately upstream of exon 3 (manuscript in preparation). To test whether this also holds for the ovaries, RT- PCR was performed using HL-9, a down-stream primer recognizing exon-5, in combination with different upstream primers (Fig. 2). With HL-12, an exon-3 specific oligonucleotide, the expected 481-bp product was obtained with RNA from

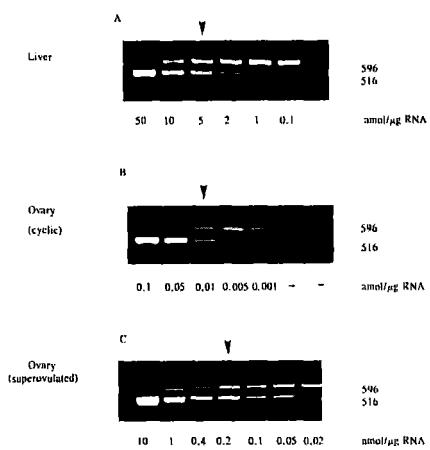

Fig. 1. Quantitation of HL mRNA by competitive RT-PCR. Competitive RT-PCR was performed in total tissue RNA from rat liver (A), ovaries from mature cyclic (B) and from immature superovulated rats 2 days treated with hCG (C). To 1 μg RNA the indicated amount of competitor was added. Amplification was performed in 20 (liver), 30 (superovulated rats) or 35 cycles (cyclic rats). The arrowheads indicate the lanes where intensities of both bands are identical.

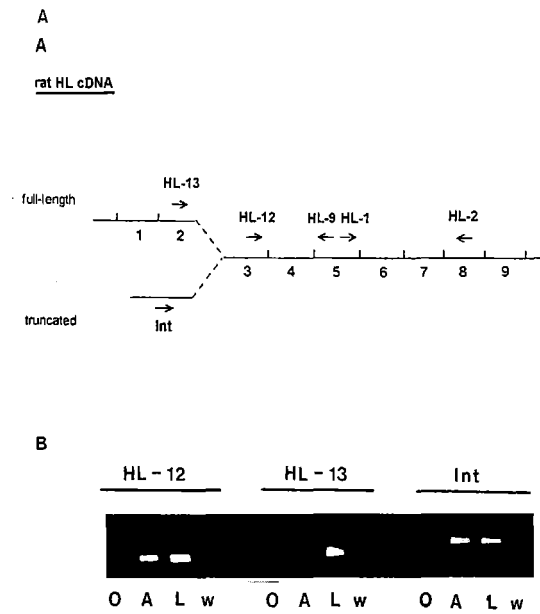

Fig. 2. Detection of the HL mRNA by RT-PCR in liver and steroidogenic tissues. The exon organization of the rat full-length HL cDNA and the alternative 5'-end found in steroidogenic tissues is represented in (A). The arrows show the position and orientation of the oligonucleotides used in RT-PCR. HL mRNA in ovary (O), adrenal (A) and liver (L) was detected by RT-PCR (B). A water control (w) was used. RNA was reverse-transcribed and amplified as described in Materials and methods using the given upstream primer and HL-9 as a common downstream primer. The resulting cDNA was amplified for 30 (liver and adrenal) or 40 (ovary) cycles.

stimulated ovaries as well as with adrenals and liver. With HL-13, an exon-2 specific primer, the expected 511-bp band was observed with liver, but not with ovaries or adrenals. Using INT, which anneals to the unique 5'-end of the variant HL mRNA transcript found in adrenals and liver [12], a 598-bp product was obtained with ovaries, similar to the expected band seen with adrenals and liver. The 598-bp amplimers from ovaries and adrenals were identical, as determined by direct sequencing (data not shown). We conclude therefore, that in rat ovaries the HL gene is transcribed virtually only into the variant HL message also found in adrenals.

3.2. De novo synthesis of HL-related proteins

The de novo synthesis and secretion of HL-related proteins was studied in freshly isolated ovarian cells and in ovarian slices, by pulse-labelling with [^{35}S]methionine followed by immunoprecipitation with polyclonal anti-HL IgGs. In ovarian cells no radiolabelled protein was detected in the 55–60 kDa range corresponding to mature HL synthesized and secreted by rat liver (Fig. 3). Instead, four major ^{35}S-labelled protein bands with apparent M_w of 200, 67, 50 and 47 kDa were obtained. Even after 2 h pulse-labelling, these bands were not detectable in the cell-free medium, indicating that these proteins remained intracellularly. These bands co-migrated with the proteins immunoprecipitated from pulse-labelled rat adrenocortical cells (data not shown). In contrast to the 67 and 50 kDa band, immunoprecipitation of the 200 kDa and 47 kDa bands was markedly suppressed by the inclusion of excess cold HL partially purified from rat liver perfusates. Qualitatively similar results were obtained using a mixture of five different monoclonal anti-HL antibodies. In all conditions tested thus far, the ^{35}S-radioactivity of the 200 and 47 kDa bands varied in parallel.

3.3. Effect of gonadotropins on HL gene expression

The expression of the variant HL mRNA in rat ovaries was compared with that of the 47 kDa HL-related protein at different times after hCG treatment. As shown in Fig. 4, the amount of the variant HL mRNA was increased approximately four-fold 2 days after treatment with hCG. Six days after treatment with hCG the HL mRNA had decreased again to pre-stimulatory levels. The de novo synthesis of the 200 and 47 kDa proteins was also transiently increased during superovulation (Fig. 4). After 2 days of treatment with hCG

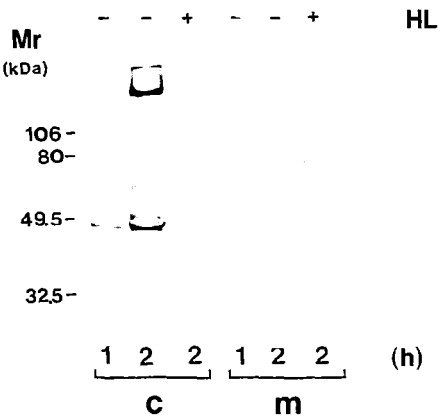

Fig. 3. Pulse-labelling of ovarian cells with [^{35}S]methionine. Freshly isolated ovarian cells from 2 days treated females with hCG were pulse-labelled for 1 or 2 h. Cell lysates (c) and cell-free medium (m) were immunoprecipitated with polyclonal anti-HL IgGs coupled to Sepharose-4B beads in presence (+) and in absence (−) of an excess of unlabelled purified HL. The adsorbed proteins were analyzed by SDS-PAGE on a 10% gel followed by fluorography. The position of the molecular weight standards is given in kDa.

the ^{35}S-incorporation into both HL-related proteins increased several-fold. Thereafter, the ^{35}S-incorporation decreased again and returned to pre-stimulatory levels 6 days after hCG treatment. Thus, the expression pattern of the variant HL mRNA and ^{35}S incorporation into the 47 kDa protein was similar. The effect of hCG on the *l*-type lipase activity in the stimulated ovaries showed a strikingly different pattern (Fig. 4). The enzyme activity began to increase after 2 days of treatment with hCG and continued to increase thereafter. On a mg protein basis, the *l*-type lipase activity present in the ovaries was maximal after 4 days of hCG treatment and remained high at least until 6 days after hCG injection.

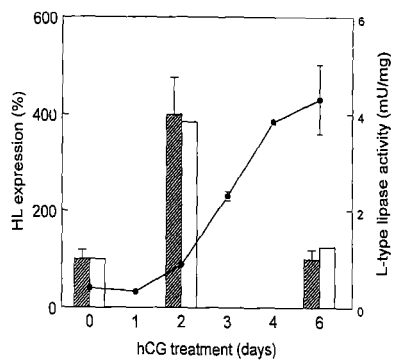

Fig. 4. Transient HL gene expression in immature rat ovaries induced by hCG. Female rats (25–30 days old) were treated with 10 IU PMS followed 2 days later by 10 IU hCG (day 0). Of each animal, one ovary was used for RNA measurement, whereas the other ovary was used for protein measurements. Quantitation of HL mRNA (hatched bars) was carried out by RT-PCR using primers HL-1 and HL-2; data are given as % of results of day 0; 0.075 ± 0.01 amoles/μg; (mean ± SE, $n = 3$). Ovarian tissue slices were pulse-labelled with [^{35}S]methionine for 2 h followed by immunoprecipitation and SDS-PAGE. The gel was then exposed to a film and the 47 kDa protein band was scanned. Data (open bars) are expressed in arbitrary units of relative blackness given as % of day 0, and are representative for 2 independent experiments. *l*-Type lipase activity (●) was measured in tissue homogenates and given as the lipase activity sensitive to polyclonal anti-HL antibodies (mean ± SE, $n = 3$).

4. Discussion

We show here that the rat HL gene is transcribed into a variant mRNA, not only in adrenals, but also in ovaries. This transcript appears to be the major, if not only, HL gene product in these steroidogenic organs. In the liver, it is also present but as a minor component compared to the full-length message encoding HL [12]. As discussed before, this second HL gene product may represent an alternatively spliced form, or may be the product of an alternative transcription start site in the intron preceding exon-3. Since this variant mRNA lacks the first two coding exons of the full-length form found exclusively in liver, its possible translation product would be considerably shorter than the 58 kDa HL protein. Moreover, the protein product would probably remain intracellularly because of the absence of a signal peptide. Whether such a protein product would be catalytically active is presently unknown. It is unlikely, however, that such a protein is responsible for the *l*-type lipase activity found in the steroidogenic organs, since (i) the abundance of HL gene transcripts in adrenals and ovaries is 10- to 100-fold less than in liver, despite the presence of a similar lipase activity per mg protein [8] and (ii) the pattern of expression of the *l*-type lipase activity in ovaries during superovulation differs markedly from that of HL mRNA. This study therefore strengthens the hypothesis that the *l*-type lipase activity found in rat ovaries originates from liver [9,10]. Hepatic lipase injected into the circulation is selectively sequestered by the ovaries [9]. During PMS/hCG-induced neovascularization and differentiation of the ovary the hepatic lipase appeared specifically in the blood vessels of the theca interna of follicles, corpora lutea and interstitial tissue [19]. How the lipase is transported from the liver to the steroidogenic organs and how the protein is specifically sequestered into these organs remains to be determined.

Two immunoreactive proteins of 47 and 200 kDa were found in ovaries as well as in adrenals (data not shown) after pulse-labelling and immunoprecipitation. The 47 kDa protein would be an obvious candidate as a translation product of the variant HL mRNA. This is

strongly supported by the resemblance in the transient expression pattern of both the HL-related protein and the HL mRNA upon induction of superovulation. Since the unique 5'-end of the variant HL mRNA contains multiple translation stop codons and lacks potential start codons, translation must have started at the first in-frame AUG, which occurs in exon 4 upstream of the catalytic triad [12]. Hence, this protein may possess esterase activity towards some ester substrates. The 200 kDa band always varied in parallel with the 47 kDa in the immunoprecipitates, suggesting that their expression is somehow related. Whether the high M_w protein represents aggregates of the 47 kDa form, or a 47 kDa protein covalently linked to another protein, is presently under investigation in our laboratory.

The finding that the HL gene is transcribed into a variant mRNA, and possibly is translated into a 47 kDa HL-related protein, exclusively in the steroid-producing organs, suggests a role for this gene product in cholesterol metabolism. Induction of follicle development and corpora lutea formation by treatment with PMS and hCG, results in a marked increase in steroid hormone production by the ovaries and hence in a demand for cholesterol [7]. Cholesterol needed for steroid production is either mobilized from endogenous cholesterol ester stores, synthesized de novo, or delivered to the ovaries by circulating high- and low-density lipoprotein (HDL and LDL), processes mediated by the *l*-type lipase and the LDL receptor, respectively [20]. The transient expression of the HL gene in ovaries of superovulating rats observed here (Fig. 4) coincides with the fall in intracellular cholesterol esters and parallels the transient increase in cholesterol de novo synthesis [21]. Taken together, these data suggest a role, either structural or catalytical, for the variant HL gene product in the de novo synthesis and/or mobilization of endogenous cholesterol in these organs. At the time that the endogenous cholesterol becomes limited, the extracelluarly localized *l*-type lipase is increased. This enzyme facilitates the influx of HDL-cholesterol in a process possibly involving the recently identified SR-BI receptor [22].

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Chapter 2.2

Identification of an alternative promoter in intron-2 of the rat hepatic lipase active in liver, adrenal glands and ovaries

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ABSTRACT

In rat steroidogenic organs the hepatic lipase (HL) gene is exclusively transcribed into a variant mRNA, in which the first two exons are substituted by an intron-like sequence; in parallel, these organs express a 47 kDa protein that is immunologically related to HL [1]. Here we examined the origin of this novel sequence. Southern blot analysis of rat genomic DNA using 13 different restriction enzymes indicated the presence of only a single HL gene copy in the rat genome. By screening a rat genomic library for the novel sequence, and by PCR analysis, we located the 5'-end of the variant HL mRNA in intron 2 immediately upstream of exon 3. With primer extension analysis the transcription start site was mapped at 465 bp upstream of exon 3. The 5'flanking region contains a number of potential regulatory sites, but lacks a clear TATA box. The activity of the novel promoter region was tested in HepG2 cells by transient transfection of CAT-reporter constructs. Compared to the normal rat HL promoter region (-437/+9), the activity of the alternative rat HL promoter (-253/+350) was very low. By Northern blot analysis, the variant HL mRNA in liver was 2.6 kb. The longest open reading frame in the variant transcript would translate into the C-terminal part of the HL protein of about 47 kDa, but a translational startsignal is not present at an appropriate location. Moreover, this open reading frame is preceded by other potential startsites. When rats were treated for 2 days with ACTH, the variant HL mRNA increased from 0.4 to 3.5 attomoles per μg RNA. Thereafter, HL mRNA decreased again to control values at day 6. This transient induction was paralleled by the de novo synthesis of a 47 kDa HL-related protein. In contrast, HL activity in the adrenals started to increase after 2 days of treatment, and remained elevated thereafter. We conclude that the rat HL gene has an alternative promoter within intron 2, which is active in liver, adrenals and ovaries. In rat adrenals, this promoter is transiently induced by corticotropic hormones, and is regulated independently of the normal HL gene promoter.

INTRODUCTION

Hepatic lipase (HL; E.C. 3.1.1.34) is extracellularly located in the liver of most vertebrates, where it is plays an important role in lipoprotein metabolism. The lipase is involved in the conversion of IDL into LDL, and in the uptake of VLDL- and chylomicron (CM)- remnants by the liver (see for review [2]). The enzyme also plays a major role in the metabolism of HDL. Notably HL is known to facilitate the uptake of HDL-cholesterolesters by liver cells [3,4].

Adrenal cortex and ovaries of a number of species including rat and human contain an *l*-type lipase activity that is indistinguishable from HL. This *l*-type lipase activity is thought to originate from liver, and to be transported via the bloodstream to the steroidogenic organs [5]. The *l*-type lipase activity in these organs varies in parallel with steroid hormone output. At least in the rat, these organs depend largely on HDL cholesterol as a source for steroid hormone production. The putative HDL receptor, scavenger receptor B1 (SR-B1), mediates delivery of HDL cholesterol to the sites of steroidogenesis . The tissue distribution of *l*-type lipases is similar to that of SR-B1. The *l*-type lipases may therefore facilitate the uptake of HDL cholesterol via SR-B1. In line with this, SR-B1 expression in adrenal glands is upregulated when HL and *l*-type lipase activity is inhibited by injection with anti HL antibodies [6].

In contrast to mouse adrenals [7], synthesis and secretion of HL in rat adrenals and ovaries has not been detected. In rat adrenals and ovaries, the HL gene is not expressed into a full-length HL mRNA. Instead, an alternative form of HL mRNA is found in these tissues [1,8], in which the first two exons of the HL gene are replaced by an intron-like sequence of unknown origin. We designate this novel sequence as exon 1A. This variant HL mRNA is predicted to be translated into a protein that lacks the N-terminal part of liver HL, including the signal sequence and the lid that covers the catalytic pocket. This variant HL mRNA is also expressed in rat liver but at a much lower level than full-length HL mRNA.

In rat ovaries the amount of variant HL mRNA varied in parallel with cholesterol demand for steroidogenesis [8]. In rat adrenals and ovaries, the expression of the variant HL mRNA coincides with the de novo synthesis of a 45-47 kDa protein that is immunologically related to HL [1,8]. For rat ovaries, we have shown that the immuno-related protein is transiently induced by gonadotropic hormones in parallel with the induction of the truncated HL mRNA[8]. This HL-related protein remained mainly intracellular. Taken together, these observations suggest a possible role for this HL-related protein in intracellular cholesterol handling in the steroidogenic organs.

Since the first two exons are not used in the expression of the variant HL mRNA, we assumed that transcriptional regulation occurs from another, hitherto unidentified, promoter region. In order to get a better understanding of the regulation and promoter function of the variant transcript and related translation product, we decided to identify the alternative promoter and its 5'flanking region in the rat genome. In addition, we determined whether in rat adrenals expression of the alternative HL gene products is regulated by corticotropic hormones.

MATERIAL AND METHODS

Genomic library screening for exon 1A and the 5'regulatory region of the rat HL gene

To isolate rat genomic clones containing the exon 1A sequence, we screened a rat genomic library in λ DASH II (Stratagene, La Jolla, CA, USA) with the oligonucleotide INT (5'-GCA TTG TCC TTG AGC CTG AG-3', position – 112 to – 93 upstream of exon 3; Verhoeven 1994). Briefly, 10^6 plaque-forming units were plated and blotted to Hybond filters (Amersham Bio Sciences, the Netherlands). Duplicate filters were screened with oligonucleotide INT. The oligonucleotides were end-labelled using [γ-^{32}P]ATP (Amersham Bio Sciences) and polynucleotide kinase (Roche Diagnostics, Almere, the Netherlands), followed by gel filtration on a 1 ml Sephadex G50 column (Amersham Bio Sciences). Filters were hybridised overnight at 42°C with 50 ng of labelled oligonucleotide INT [9]. After hybridisation the filters were extensively washed at room temperature for 5 min with, successively, 5 x NaCl/Na-citrate, 2 x NaCl/Na-citrate and 1 x NaCl/Na-citrate in 0.1% SDS (1 x NaCl/Na-citrate buffer contains 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0). Finally, the damp filters were exposed overnight to Fuji X-ray films with intensifying screens at -80°C. Two out of 10^6 plaques were positive on both filters; these clones were plaque-purified three times [9].

The same genomic library was also used for isolation of the upstream regulatory region of the rat HL gene, using an HL cDNA probe corresponding to exons 1 and 2. The probe was generated by RT-PCR on 1 μg liver RNA using the oligonucleotides (5'-GGT AAG ACG AGA GAC ATG G-3', nt 1-19 numbering to [10]) and (5'-CCC GTG GAT GAT CAT GAC AA-3', nt 266-285) as forward and reverse primers, respectively. The RT-PCR product was isolated by agarose gel electrophoresis, and ^{32}P labelled using [α^{32}-P]dCTP and the Megaprime kit from Amersham Bio Sciences. Filters containing 10^6 plaques were hybridised overnight at 42°C with 50 ng of the labelled cDNA probe in 50 % formamide, 0.5 % SDS. 0.1 mg/ml denaturated herring sperm DNA and 2 x PIPES buffer [9]. After washing in 0.2 x NaCl/Na-citrate/0.5% SDS at 65°C for 5 min, the filters were exposed to autoradiography film. Two positive clones were identified, which were plaque purified three times. One of these clones was selected for further analysis. Phage DNA was isolated and digested with EcoRI. A 7 kb fragment [11] was cloned into pBluescript KS$^-$ and its identity with the 5'regulatory region of the rat HL gene was verified by sequence analysis. Sequencing of both strands was done with the Thermo-sequenase dye terminator kit (Amersham Bio Sciences) and the ABI 377 sequencer.

Restriction mapping and sub-cloning

The two λ clones that were positive for the INT oligonucleotide were analysed by restriction mapping using the endonucleases BamH*I*, Bgl*II*, Hind*III*, Kpn*I*, Pvu*II*, Sac*I*, Sma*I* and Xho*I* (Roche Diagnostics), either alone or in combination with EcoR*I*. Of the λ DNA, 10 µg was subjected to overnight digestion with 10 U of the restriction enzymes at the temperature recommended by the manufacturer. The digestion products were separated on a 0.7% agarose gel, followed by denaturation with 0.5 M NaOH, 1.5M NaCl and overnight blotting to Hybond membranes. The membranes were hybridised with different ^{32}P-end labelled HL specific oligonucleotides: HL-11 (5'-CTG TGG ACA AGG CGT GGG-3', nt 78-95), HL-13 (5'-TTG TCA TGA TCA TCC ACG GG-3', nt 266-285), HL-14 (5'-CAC CCA CTA TCT TCC AGA TCC-3', nt 314-334), HL- 8 (5'-TTA ATT GGG TAC AGC CTG GG-3', nt 508-527) and INT, which recognise exons 1, 2, 3, 4 and exon 1A, respectively. After stripping, the filters were re-hybridised with a different probe. A 5.6 kb EcoR*I*-EcoR*I* fragment that was positive for INT was isolated from clone 1, and subcloned into pBluescript KS$^-$. From this construct a 577 bp Hind*III*-Bgl*II* fragment was isolated and sub-cloned in pBluescript KS$^-$ for further analysis.

Southern blot analysis of genomic DNA

Rat genomic DNA was isolated from the liver of a healthy 3-month-old male Wistar rat [9]. In parallel incubations, 10 µg of this genomic DNA was digested overnight with 10 U of the indicated restriction enzymes. After size separation by agarose gel-electrophoresis, the DNA fragments were transferred to a Hybond membrane and denaturated in 1.5 M NaCl, 0.5 M NaOH. The filter was hybridized overnight at 42°C in hybridisation buffer (50 % formamide, 0.5 % SDS. 0.1 mg/ml denaturated herring sperm DNA and 2 x PIPES buffer) with 20 µg of the 577 bp Hind*III*/Bgl*II* DNA fragment, that had been ^{32}P-labelled as described above. After washing the blot at 60°C in 0.2xNaCl/Na-citrate/0.5% SDS, the blot was exposed overnight to autoradiography film at –80 °C.

Northern blotting

A rat multiple tissue Northern blot (Clontech, Palo Alto, CA, USA), which contained 2 µg poly(A) enriched RNA from several tissues, was probed with oligonucleotide AIB (antisense to INT). After stripping, the blot was reprobed successively with oligonucleotide HL-14 (specific for exon 3) and with a 1.1-kb human GAPDH cDNA probe, according to the manufacturers instructions. The oligonucleotides were end-labelled with [γ-^{32}P]ATP and

polynucleotide kinase, and the cDNA probe was labelled by random-priming using [α^{32}-P]dCTP, as described above. After hybridisation with the oligonucleotides, the blots were washed for 10 min at 60°C in 2xNaCl/Na-citrate, and then exposed for the indicated time to autoradiography film. After hybridisation with the cDNA probe, the blot was washed for 1 h at room temperature with 2x NaCl/Na-citrate/0.5 % SDS, followed by two 20-min washes with 0.1xNaCl/Na-citrate/0.5 % SDS at 65°C.

Primer extension analysis

Primer extension analysis was performed according to Sambrook [9] using rat liver RNA. Total RNA was isolated from the liver of a healthy 3-month-old Wistar rat by the method of Chomczynski & Sacchi [12]. Three different primers, corresponding to sequences located in intron 2 upstream of exon 3, were used: AIB (anti-sense to INT), ext-1 (5-GAT TTC TCA ATC TCG TGC AG-3', nt -169 to -150) and ext-2 (5'- GTC ATT GTC TGA ATC TTT CCC-3', nt -342 to -322). Primers were ^{32}P-labelled with [γ^{32}P]ATP and polynucleotide kinase (Roche Diagnostics), and isolated by gel purification on Sephadex G50. Of these primers, $2x10^5$ dpm was hybridised to 10-50 µg total RNA in the presence of hybridisation buffer (0.15 M KCl, 1 mM EDTA, 10 mM Tris-HCl, pH 8.3). The primer-RNA mixture was treated for 2 min at 95°C, incubated for 5 min at 70°C followed by slow cooling to room temperature. After ethanol precipitation, the pellet was resuspended in 25 µl of reverse transcriptase cocktail (0.56 mM of each dNTP, 50 µM Tris pH 8.3, 50 µM KCl, 5 mM DTT, 5 mM $MgCl_2$, 20 U RNAsin (Promega, Madison, WI, USA), 100 U MMLV RT (Promega)), and primer extension was performed by incubation at 42°C for 90 min. After phenol-chloroform extraction and ethanol precipitation, the extended primer was resuspended in 5 µl 10 mM Tris-HCl, 1 mM EDTA buffer (pH 8.0). After addition of 3 µl formamide loading buffer (United States Biochemicals, Cleveland, Ohio, USA) and denaturation at 70°C, the mixture was run on a 6% polyacrylamide; 7 M urea sequencing gel alongside a radioactive sequencing ladder. The latter was prepared from the 577 bp Hind*III*-Bgl*II* fragment using the same primer as in the primer extension reaction. DNA radioactive sequencing was performed using the Sequenase 2 kit from United States Biochemicals. After gel-electroforesis, the sequencing gel was exposed to autoradiography –film.

Promoter activity

For in vitro studies, fragments of the 5'upstream regulatory region of the rat HL gene and of the alternative rat HL promoter region located in intron 2 were cloned into the pCAT-Basic

reporter plasmid (Promega). From the 7 kb EcoR*I*-EcoR*I* fragment containing the 5'-regulatory region of the HL gene, a Pst*I*-Xba*I* (-437 to +9) fragment was isolated and subcloned into pCAT-Basic in front of the CAT reporter gene. For the alternative promoter region in intron 2, the 577 bp Hind*III*-Bgl*II* fragment was cloned into pCAT-Basic.

HepG2 cells were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (ICN, Costa Mesa, CA, USA) supplemented with 10% fetal calf serum (Gibco BRL, Breda, NL) and penicillin/streptomycin. At 24 h before transfection, the cells were plated in 60 mm culture dishes at 20-30 % confluence. At 3 h before transfection, the medium was refreshed. Transfections were performed by the calcium-phosphate co-precipitation method using 10 μg of the CAT reporter test plasmid and 0.4 μg of the RSV β-galactosidase expression plasmid (Promega). Parallel transfections with SV40-CAT-Control and CAT-Basic plasmids were used as controls. Seventy-two h post-transfection, cell lysates were prepared and the amounts of CAT and β-galactosidase antigen were measured by ELISA using kits from Roche Diagnostics. Promoter activity was expressed as pg CAT/ng β-galactosidase to correct for differences in cell number and transfection efficiency.

Rat adrenal glands

Male Wistar rats (200-250 g body weight) were fed *ad libitum* with a standard chow diet (Hope Farm, Wilnis, The Netherlands). Animals were killed by decapitation under light diethyl ether narcosis. Adrenal glands were quickly excised and the surrounding adipose tissue was trimmed off. Hypertrophic adrenals were obtained by treating the animals for the indicated time by daily s.c. injections with 50 μg of a synthetic corticotrophin analogue (Synacthen, Ciba-Geigy, Basel, Switzerland). For each animal one adrenal was rapidly frozen in liquid nitrogen and used for RNA isolation or measurement of L-type lipase activity; the second adrenal was placed in medium and immediately used in pulse-labelling experiments (see below).

RNA analysis

Total RNA was isolated from 2-4 adrenals by the method of Chomczynski & Sacchi[12]. RNA concentrations were determined by spectrophotometry at 260 nm [9]. The quality of the isolated RNA was judged from the ratio of 28S over 18S ribosomal RNA and electrophoresis in a 1 % agarose/TBE gel [9]. HL mRNA and GAPDH mRNA were determined by RT-PCR starting from 1 μg total tissue RNA. After synthesis of random-primed cDNA, the mixture was divided into two PCR incubations. Amplification was performed for 35 cycles with HL-

specific primer pairs, or 20 cycles with GAPDH-specific primer pairs. HL-specific forward primers were either RHL-3 (5'-CGG GGG CTC CTT CCA GCC TGG-3'; nt 756-776; numbering according to the rat cDNA sequence [10] or INT -, whereas RHL-2 (5'-CAG ACA TTG GCC CAC ACT-3'; nt 1307-1289) and RHL-9 (5'-GGC ATC ATC TGG AGA AAG GC-3', nt 660-641) were used as reverse primers. The GAPDH-specific primers 5'-TCT TCT TGT GCA GTG CCA GC-3' (nt 35-54) and 5'-CTC TCT TGC TCT CAG TAT CC-3' (nt 1120-1101) span the entire coding sequence [13]. All RT-PCR experiments included no-template and no-RT controls.

HL mRNA was semi-quantified by competitive RT-PCR [14]. Of total tissue RNA, 0.5 µg was mixed with 0.5 µg yeast RNA (Pharmacia, Uppsala, Sweden) containing known amounts of competitor RNA. The competitor RNA was obtained by in vitro transcription of a HL cDNA clone in pBluescript KS⁻that had been modified by deletion of an internal 80-bp Sau3A fragment (nt 1009-1088 [14]). Parallel RT-PCR reactions were performed with increasing amounts of competitor RNA added to 0.5 µg of tissue RNA. After synthesis of random-primed cDNA, amplification was performed for 35 cycles using RHL-2 and RHL-3 as reverse and forward primers, respectively. With this primer combination, target and competitor RNA yielded PCR-products of 552 bp and 472 bp, respectively. The amount of target RNA was determined from ethidium-bromide stained gels by taking (or extrapolating to) the amount of internal competitor RNA that produced an equal signal intensity.

Pulse-labelling experiments

Three freshly dissected adrenal glands from 3 animals were pooled and roughly minced using a razor blade. The slices were incubated in 1 ml of methionine-free Minimal Essential Medium containing 25 U/ml of heparin (Leo Pharmaceuticals, Weesp, The Netherlands) and 20 % bovine serum (dialysed against phosphate buffered saline (PBS)) at 37°C in a shaking water bath under an atmosphere of 5 % CO_2/95 % O_2. After 30 min, 80 µCi of Tran^{35}S-label (ICN, Cosa Mesa, CA, USA) was added and the incubation was continued for an additional 2 h. The incubation was stopped on ice, and the tissue and medium were separated by centrifugation (10000 g, 20 s, 4°C). The tissue was lysed for 45 min at 4°C in PBS containing 1 % Triton-X100, 1 % sodium deoxycholate, 10 mM Hepes (pH 7.4), 25 U/ml heparin, 1 mM methionine, 1 mM EDTA, 10 µg/ml benzamidine, 10 U/ml Trasylol, and 1 µg/ml each of leupeptin, antipain, chymostatin and pepstatin. The lysate was cleared by centrifugation (10 min, 10000 g, 4°C) and then incubated overnight with goat anti-rat HL IgG's immobilized onto Sepharose 4B beads [1]. Some immunoprecipitations were carried out in the presence of

100 mU unlabelled HL that had been partly purified from rat liver heparin perfusates [15]. After collection by centrifugation, the beads were washed at 4°C with 1 ml of, successively, PBS, 1 M NaCl in PBS, 0.2 % Tween-20 in PBS, and PBS. The immunoprecipitated proteins were released from the beads by heating for 5 min at 95°C in Laemmli's sample buffer [16], and analysed by SDS-PAGE in 10 % polyacrylamide gels followed by fluorography using Amplify filters (Amersham Bio Sciences). The autoradiograms were scanned with the HP ScanJet II CX densitometer, and the integrated optical density was expressed in arbitrary units.

L-type lipase activity

Three adrenals from 3 different animals were pooled and homogenized in 10 volumes of PBS (pH 7.4) containing 5 U/ml of heparin and 1 mM benzamidine (4°C) using a Polytron homogenizer. After centrifugation (2 min, 10000 g, 4°C), the post-nuclear supernatant was assayed for triacylglycerol hydrolase activity at pH 8.5 in 0.6 M NaCl using a gum-acacia stabilized emulsion of glycerol tri[9,10(n)-^{3}H]oleate (Amersham Bio Sciences) as substrate[17]. In immunoinhibition assays, the supernatant was pre-incubated for 1 h at 4°C with excess goat anti-rat HL IgGs immediately before the lipase assay. L-type lipase activity is defined here as the triglyceridase activity that was sensitive to immunoinhibition with anti-HL IgGs. Enzyme activities were expressed as mU (nmol of free fatty acids released per min).

RESULTS

Mapping of exon-1A to intron 2 of the rat HL gene

In rat liver, the HL gene is expressed not only into the full-length HL mRNA but also into a variant form [1]. Interestingly, in adrenals and ovaries only this variant form could be detected. In this mRNA, exons 1 and 2 are replaced by a novel sequence, from which to date only 126 bp has been described. We designate this novel sequence here as part of exon-1A. In order to determine the transcription start site present in exon-1A a rat genomic library was screened using the oligonucleotide INT, which recognizes the known part of exon-1A. From the library, two positive clones (I and II) were identified and isolated. A restriction map of both clones was obtained after digestion with a number of restriction enzymes, blotting and hybridisation with different oligonucleotide probes of exons 1, 2, 3,4 and 5 and 1A (Fig.1).

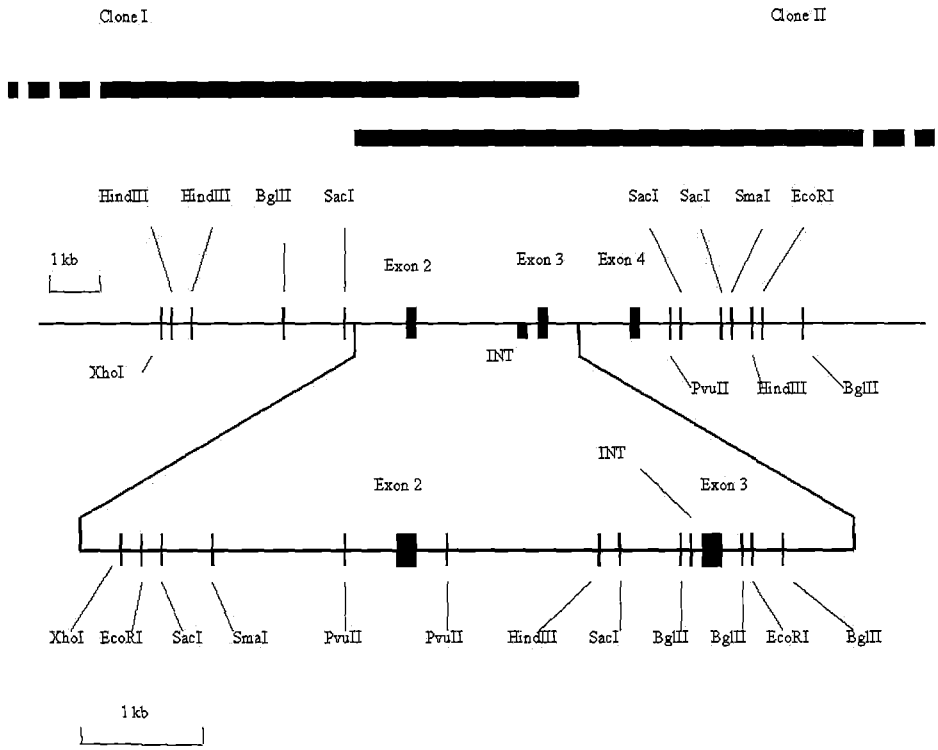

Figure 1. **Restriction map of two positive clones from the rat genomic library.** The restriction maps of the two overlapping λ recombinant clones (clone I and clone II) are shown. The positions of exons 2 to 4 are indicated, as well as the position where the oligonucleotide INT hybridises.

The map reveals that the clones contained overlapping sequences of the rat HL gene. Clone I contains exons 1 to 3 and clone II contains exons 2 to 4. Neither clone hybridised with an oligonucleotide specific for exon 5. From this analysis, we can deduce that intron-2 spans ± 2.5 kb, whereas intron 3 spans ± 3 Kb. Since exon 5 is not included in clone I, the length of intron 4 exceeds 5 kb. Exon 1 was mapped at approximately 9 kb upstream of exon 2. INT hybridised with DNA fragments that contained intron 2 sequence (Fig. 1). A 5.7 kb EcoR*I* fragment containing the entire intron 2 was isolated from clone I and subcloned into pBluescript KS$^-$. Further digestion and sequencing analysis of this clone localised the exon-1A sequence in intron 2 immediately upstream and contiguous with exon 3 (Fig. 2).

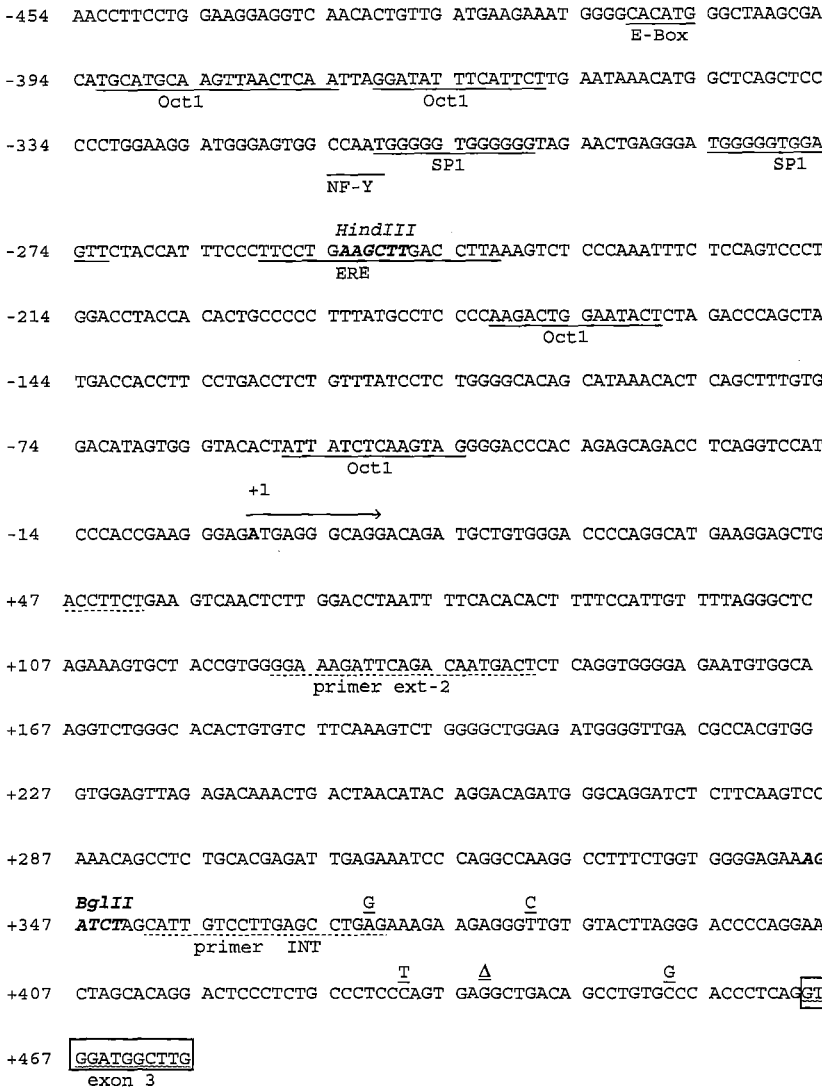

Figure 2. **Sequence of the exon 1A and part of the 5'-regulatory region (prom 1A) fragment of rat HL gene.** Numbering is according to the transcription start site (indicated by an arrow); the first nucleotide of the transcript is denoted as +1. Exon 3 is boxed. The primer sequences used for primer extension analysis and genomic library screening are indicated by arrows (5' → 3') underneath. Restriction sites used to generate promoter fragments for cloning and transfection are indicated in bold. An analysis of the sequence revealed several putative transcription factor binding sites (underlined). Nucleotides that differ from the previously described novel sequence upstream of exon 3 are given above the sequence; the Δ indicates the insertion of an extra G.

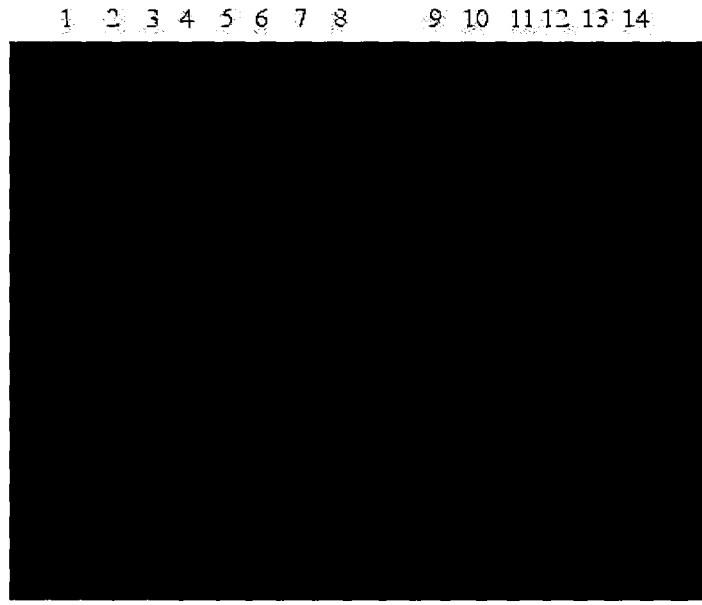

Figure 3. **Southern blot analysis.** Rat genomic DNA was digested with different endonucleases as indicated in the figure, separated in an agarose gel and blotted as described in Material and Methods. Membranes were hybridized with ^{32}P-labelled 577 bp Hind*III*-Bgl*II* fragment and exposed to autoradiography film. Used restriction enzymes were respectively: 1) Aoc*I*, 2) Apa*I*, 3) BamH*I*, 4) bcl*I*, 5) Bgl*II*, 6) EcoR*I*, 7) EcoRV, 8) Hind*III*, 9) Nco*I*, 10) Pst*I*, 11) Sac*I*, 12) Sau3A, 13) Sca*I* and 14) Xba*I*.

From this EcoR*I* fragment, a 577 bp Hind*III*-Bgl*II* fragment containing the boundary of exon-1A and exon 3 (Fig. 2) was isolated and subcloned also into pBluescript KS$^-$ using the Hind*III* and BamH*I* site of the poly-linker.

The location of exon-1A within intron 2 was confirmed by a PCR on genomic rat DNA using primer pairs that flank intron 2. The used upstream and downstream primers were (5'- TTG TCA TGA TCA TCC ACG GG-3' and 5'-CAC CCA CTA TCT TCC AGA TCC-3') respectively. The resulting PCR product was approximately 2.5 kb, and hybridised with the exon-1A specific oligonucleotide INT (data not shown). Furthermore, sequence analysis of this PCR product confirmed that the novel sequence previously described [1] was localised immediately upstream of exon 3.

To test the possibility that multiple HL-like genes exist in the rat genome, we performed a Southern blot analysis of rat genomic DNA. With 13 out of 14 restriction enzymes used, the 577 bp Hind*III*-Bgl*II* fragment, hybridised to a single band (Fig. 3). No hybridising bands were obtained after Sau*3A* digestion. Since the digestions produced only one hybridizing band, we conclude that the rat genome contains a single copy of the HL gene. It is unlikely therefore that the alternative transcript observed in rat liver and steroidogenic organs is product of a gene distinct from the HL gene.

Expression of the variant HL mRNA in rat liver

Northern blot analysis of poly(A)-rich RNA isolated from different rat tissues is shown in Figure 4. The exon-1A specific oligonucleotide AIB hybridised with a single RNA band of approximately 2.6 kb. This signal was obtained with RNA from liver, but not with RNA from kidney or skeletal muscle. As expected [10] the exon 3-specific oligonucleotide HL-14 hybridised with a RNA band of approximately 1.9 kb in liver, but not in any of the other tissues tested. An additional band at about 2.6 kb was not clearly detectable in this blot. These data indicate that the variant HL mRNA that contains the exon-1A sequence is 2.6 kb long and is expressed in rat liver at a much lower level compared to full-length HL mRNA.

Primer extension

The transcription start site that leads to the expression of exon 1A was localized by primer extension using rat liver RNA. The primers AIB and ext-1, which recognize a sequence immediately upstream of exon 3 and the sequence at –169 nt – 150 nt upstream of exon 3, respectively, gave distinct products larger than 350 and 250 nucleotides (data not shown). With primer ext-2 (–342 –322 nt) a product of ± 140 nucleotides was obtained (Fig. 5). Alignment of this product with the sequencing ladder of the 577 bp Hind*III*-Bgl*II* fragment pinpointed the transcription start site at an A residue, 465 nucleotides upstream of exon 3 .

Sequence analysis

The DNA sequence of exon 1-A as well as the sequence of the 5'- flanking region of the alternative rat HL promoter is shown in Fig. 2. Sequence analysis of the variant HL transcription product revealed the presence of a number of relatively short open reading frames (ORF) with an AUG start codon. The first potential startcodon from the 5'end is present at nt 16-18, and would predictably generate a 66 aminoacid protein with a molecular weight of 7 kDa. Screening a protein database did not reveal significant homology with any of

the known proteins. Further downstream AUG codons would open reading frames with even shorter translation products, except for an AUG within exon 4 (nt 634-636 in the variant HL mRNA). None of these AUG codons were in a favorable Kozak context [18]. The longest ORF in the variant HL mRNA runs form nt 391 tot 1650, and would correspond to a protein of maximally 47 kDa consisting almost entirely of the C-terminal part of the HL protein. The 5'-end of this ORF lies just upstream of exon-3, but the first AUG codon in this frame occurs in exon 4, where it would result in a 38 kDa HL-related protein (aminoacids 134 to 472 [10]. Despite careful inspection of the sequencing reactions, no potential in-frame AUG codon was found in this region of the sequence.

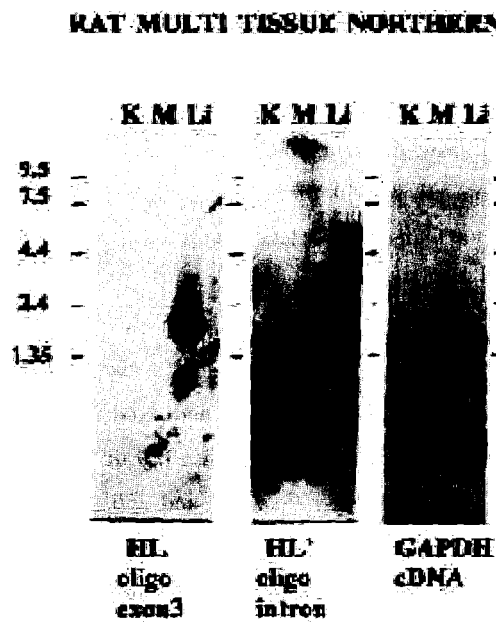

Figure 4. **Northern blot analysis.** Approximately 2 µg poly(A) rich RNA isolated from rat kidney (K), skeletal muscle (M) and liver (L) were separated on a 1.2 % agarose gel under denaturing conditions, transferred to a charge-modified nylon membrane, and probed successively with ^{32}P-labelled oligonucleotide AIB (specific for intron-2; specific activity 0.5x10^{9}dpm/µg; 5x10^6 dpm/ml), ^{32}P-labelled oligonucleotide HL-14 (specific for exon-3; specific activity 0.4x10^{9}dpm/µg; 0.5x10^6 dpm/ml), and finally with a ^{32}P-labelled human GAPDH cDNA probe. The migration of RNA markers is indicated.

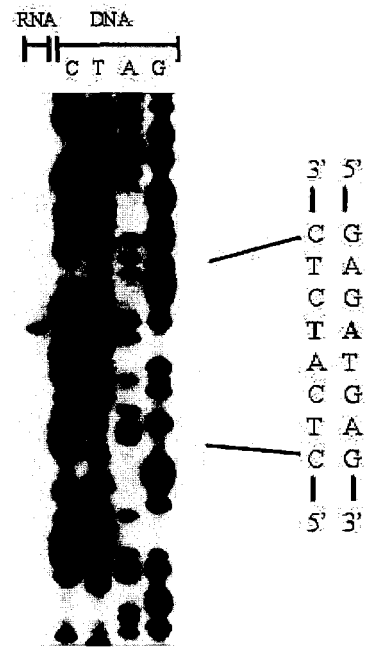

Figure 5. **Primer extension analysis.** The ^{32}P-labelled oligonucleotide ext-2, complementary to part of exon-1A sequence was hybridised to rat liver RNA, and the oligonucleotide was extended by incubation with reverse-transcriptase. The extended primer was run in a denaturing polyacrylamide gel (left lane) alongside the C, A, T and G sequencing reactions on the Hind*III*/Bgl*II* 577 bp fragment using the primer ext-2.

The 5'-flanking region of the alternative HL promoter was analysed for the presence of binding sites for transcription factors with MatInspector V2.2 software [19] using the transcription factor database Transfac [20]. Several putative gene regulatory elements were identified, such as an oct-1, SP1 and NF-Y binding sites, as well as an estrogen responsive element and an E-box. A clear TATA box could not be identified.

Promoter activity

To test the transcriptional activity of the alternative promoter and its 5' flanking region, reporter assays were performed by transient transfections of HepG2 cells. The intron 2 Hind*III*-Bgl*II* 577 bp fragment was cloned into a promoter-less CAT-vector (pCAT-Prom1A). As a reference we used a similar pCAT construct with the –437/+9 region of the rat HL gene as insert (pCAT-437). The pCAT-437 construct showed a promoter activity of 53 ± 9 % of the

SV40 promoter driven control plasmid (117 ± 17 %). Compared to pCAT-437, the pCAT-Prom1A construct displayed only a weak promoter activity (Fig. 6), which was not significantly different from that of the promoter-less pCAT. This is in line with the much lower expression of the variant compared to the full-length HL mRNA in rat liver [1]. Qualitatively similar results were obtained with transient transfections of NCI-H295R human adrenocortical cells (data not shown).

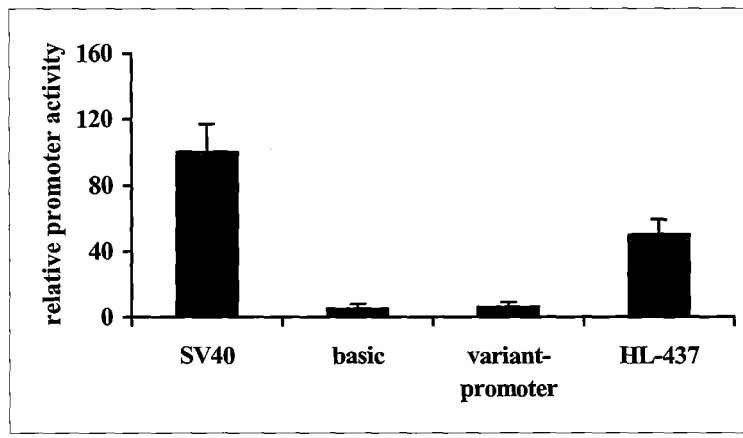

Figure 6. **Activity of the alternative HL promoter in HepG2 cells. The promoter region of the alternative promoter (-253/+350), and of the regular promoter (-437/+9)** were inserted in a pCAT reporter plasmid. The resulting plasmids were co-transfected with a control reporter plasmid (β -Galactosidase) in the HepG2 cells. At 48 h post-transfection, CAT and β-galactosidase expression was determined. As a reference, parallel transfection assays with pCAT basic and pCAT-SV40 were performed. The results are expressed as the ratio between the CAT and β-galactosidase mass. Data are means ± SD (n=4). The results are representative of 4 similar experiments.

Expression of HL mRNA in control and hypertrophic adrenals

RT-PCR on total RNA isolated from control adrenals using the primers RHL-2 and RHL-3 yielded the expected 552-bp product, of which the identity with part of HL cDNA has been confirmed by restriction mapping [14]. Upon treatment of the rats with ACTH, the amount of PCR product transiently increased, with highest levels observed at day 1 and day 2 of treatment (Figure 7, inset). In contrast, the amount of PCR product generated with GAPDH

specific primers was hardly affected by ACTH treatment. Quantification of HL mRNA by competitive RT-PCR using a HL RNA with an internal 80 nt deletion as competitor showed that control adrenals contained approximately 0.4 attomoles of HL mRNA per µg total RNA (Fig. 7), in agreement with our previous report [1]. Upon ACTH treatment, the amount of HL mRNA gradually increased to 3.5 attomoles per µg total RNA at day 2. Thereafter, the amount of HL mRNA decreased again to near-control levels after 6-9 days.

Although part of the HL gene transcript was detected in rat adrenals, and shown to be transiently up-regulated by stimulation with ACTH, we were unable to amplify the entire HL cDNA either from control adrenals or from adrenals of rats that had been treated for 2-9 days with ACTH. Using the oligonucleotide INT and RHL-9, which are specific for exon 1A and exon 5, respectively, the expected 481-bp PCR product was obtained with all adrenal RNA preparations. The amount of PCR product generated by this primer pair was also transiently increased after one and two days of ACTH treatment, in parallel with PCR product generated with the primers RHL-2 and RHL-3 (data not shown). In contrast, we were unable to amplify exon 1 and exon 2 sequences from adrenal mRNA, either from control or from two days ACTH treated rats. Hence, we conclude that the transient increase in the amount of HL gene transcript in rat adrenals is entirely due to up-regulation of the variant form in which exons 1 and 2 are replaced by exon 1A.

De novo synthesis of HL-related proteins

The *de novo* synthesis of HL-related proteins was studied by pulse-labelling with [^{35}S] methionine in whole-adrenal slices followed by immunoprecipitation with polyclonal anti-HL IgGs. With the adrenals from control rats, no immuno-reactive proteins were found in the 55-60 kDa range corresponding to full-length HL. Instead, two major ^{35}S-labelled protein bands with apparent molecular weight of 200 and 47 kDa were detected (not shown). Immunoprecipitation of these bands was almost completely suppressed by the inclusion of excess non-labelled HL partially purified from rat liver perfusates. Upon *in vivo* stimulation of the adrenals with ACTH, the ^{35}S-incorporation into the 47 kDa protein band increased several-fold (Fig. 8). The incorporation of radioactivity in the 47 kDa band was highest at day 2, and decreased again thereafter to near-control levels after 6-9 days of ACTH treatment. The radioactivity in the 200 kDa band varied in parallel to that of the 47 kDa band (data not shown). Hence, the ACTH-induced up-regulation of *de novo* synthesis of the 47 and 200 kDa proteins occurred concomitantly with the up-regulation of the variant HL gene transcript in the adrenals.

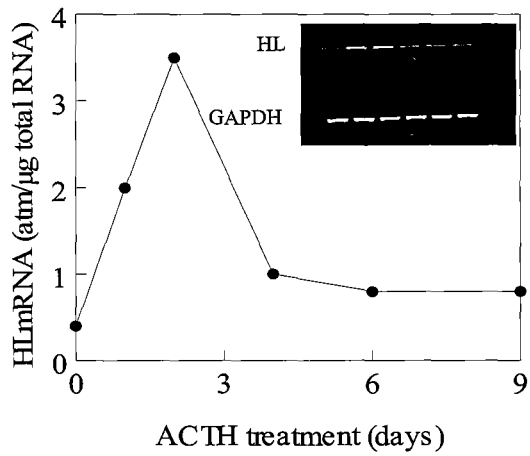

Figure 7. **HL mRNA contents in rat adrenal glands.** Rats were treated with ACTH for 0 to 9 days, and HL mRNA expression in rat adrenal glands was measured by competitive RT-PCR. In the inset, HL mRNA was qualitatively detected by RT-PCR using the primer RHL-2 and RHL-3. GAPDH mRNA was used as external standard. The figure is representative for 3 experiments.

The *l*-type lipase activity in the adrenals increased from 10.16 ± 0.83 to 15.4 ± 0.6 mU/2 adrenals (n=3, $p<0.05$) at day 2 of treatment, and continued to increase to 64.4 ± 1.1 mU/2 adrenals at day 6 of treatment (n=3; $p<0.05$). On a mg protein basis, the *l*-type lipase activity was maximal after 6 days, and remained high at least until 9 days of ACTH treatment (data not shown). Hence, the effect of *in vivo* ACTH treatment on *l*-type lipase activity in rat adrenals showed a strikingly different pattern compared to adrenal HL mRNA and *de novo* 47 kDa protein synthesis.

DISCUSSION

Rat, mouse, human, hamster and bovine steroidogenic organs contain an *l*-type lipase activity that is indistinguishable from hepatic lipase present in liver of these species. It is generally accepted that most if not all of the *l*-type lipase activities originate from the liver and that no HL synthesis occurs in adrenals and ovaries [5].

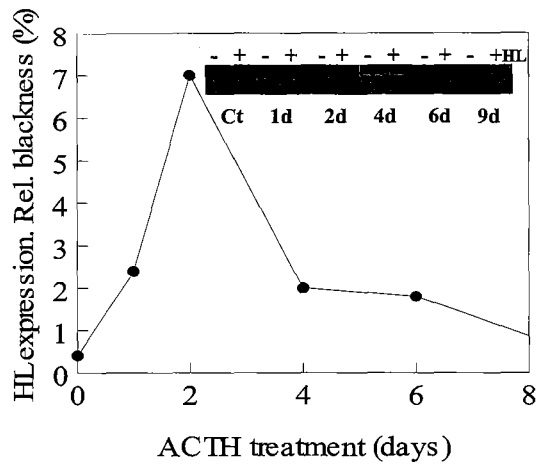

Figure 8. **Expression of HL-related proteins on rat adrenals.** Rat adrenal slices from control and ACTH treated animals were pulse-labelled with [^{35}S]methionine for 2 h. After cell lysis, lysates were incubated with polyclonal goat anti-rat HL IgG's coupled to Sepharose 4B. Incubations were carried out in presence (+) or absence (–) of 100 mU unlabelled rat HL. The immunoprecipitated proteins were separated by SDS-PAGE, and visualised by fluorography (inset). The 47 kDa band was scanned and optical density was expressed in arbitrary units of relative blackness. Each point represents the HL-related protein, given as the difference between the band intensity in the absence and presence of unlabelled hepatic lipase during immunoprecipitation.

A recent report by the group of Scow [7] suggests that adrenals of newborn mice are able to synthesise and secrete proteins that are recognised by anti HL antibodies. In rat adrenals and ovaries, no evidence for *de novo* synthesis and secretion of HL has been obtained. Despite this, our group reported the presence of a HL-like mRNA in rat adrenals and ovaries. This mRNA is identical to that of full length HL mRNA except that exons 1 and 2 are replaced by a novel exon 1A sequence [1]. In rat liver both forms of HL mRNA are present. Here, we showed that exon 1A lies within intron 2 of the rat HL gene immediately upstream of, and contiguous with, exon 3. By primer extension analysis, we pinpointed the transcription start site at 465 bp upstream of exon 3 (Fig. 5). In adrenals and ovaries only the transcript starting with exon 1A which is synthesized from an alternative promoter is used. This promoter is also

active in rat liver but to a much lower extent than the normal promoter. From the data presented by Verhoeven [1] one can deduce that rat liver expresses about 0.4 attomoles of the variant HL mRNA compared to 16 attomoles of the full length HL mRNA in 1 μg total RNA. In line with this, activity of the alternative promoter-CAT construct in HepG2 cells was much lower than that of the normal HL (–437/+9) promoter CAT construct (Fig. 6). The variant mRNA in adrenals and ovaries appears to be unrelated to the *l*-type lipase activity found in these organs, because: a) induction of HL mRNA synthesis and synthesis of the 47 kDa, HL-related protein by corticotropic or gonadotropic hormones, respectively, is only transient, and precedes the long lasting expression of *l*-type lipase activity, and b) the expression of the HL gene in these organs is restricted to the variant form of HL mRNA and its putative 47 kDa protein product. In contrast to the *l*-type lipase activity , the 47 kDa protein that cross-reacts with anti-HL IgGs remains mainly intracellular [1]. Since the N-terminal part of HL protein encoded by exons 1 and 2 is missing, it is unlikely that the catalytic activity is similar to that of HL and the l-type lipases.

Sequence analysis of the variant HL transcript revealed the presence of a number of possible open reading frames (ORF) that start with an AUG. The longest ORF starts from an AUG within exon 4 (codon 134 [10]) and would translate into the C-terminal 38.5 kDa part of the HL protein. Because it lacks a signal sequence, its localization would be predominantly intracellular. The observed expression and induction of a 47 kDa HL immunorelated protein parallel with the variant HL mRNA in both adrenals and ovaries would fit with this predicted translation product, except for its relatively large molecular mass on SDS-PAGE. The discrepancy in molecular size may be explained by extensive post-translational modification, the nature of which is unclear. Alternatively, we may have missed an additional in frame AUG upstream of exon 3 in exon-1A, despite repeated double-strand sequencing analysis of this area in the rat genomic clone. In order to be translated from the variant HL mRNA, the translation machinery must ignore the several preceding AUGs in the sequence. Incorporation of [^{35}S]methionine into the 200 kDa HL-related protein always varied in parallel with the 47 kDa protein. Its identity is unknown at present, but it may represent covalent aggregates containing the 47 kDa protein.

The 5'flanking region of the alternative promoter in intron 2 contained a number of potential binding sites for regulatory transcription factors, but lacks an unequivocal TATA box. The presence of an E-box, a CAAT-box, an Sp1, NF-Y and an ER binding site opens the possibility for hormonal regulation of transcription from this promoter. The activity of the alternative promoter was tested in HepG2 cells using a CAT-reporter construct. This construct

also contained 350 bp of exon-1A sequence. Compared with the normal HL promoter region, the alternative HL promoter displayed a low activity. This low promoter activity may be due to the absence of a clear TATA box, or to the long 5'-untranslated region with several AUG translational start codons. Anyway, the low promoter activity of the alternative promoter in HepG2 cells is in line with the relatively low expression level of the variant HL mRNA species in rat liver.

The expression of HL mRNA in adrenal glands was studied in control and in ACTH treated rats. Similar to what we described previously for ovaries under PMS-hCG stimulation [8], the variant HL mRNA was transiently increased in hypertrophic adrenals in parallel with *de novo* synthesis of the 47 kDa HL related protein. Since both mRNA and HL related protein are transiently increased upon ACTH treatment in adrenals and PMS-hCG treatment in ovaries [8], it suggests that this response may be related to changes in cholesterol homeostasis of these steroid producing cells in the early days of stimulation. This time frame overlaps with the reduction of the intracellular cholesterol ester content, and parallels the increase in *de novo* cholesterol synthesis. Therefore, the endogenous cholesterol levels may have become limited. As a consequence, extra-cellular *l*-type lipase activity may have increased, which may facilitate the influx of HDL cholesterol in cooperation with SR-BI. The role of the intracellular, HL-related protein in cholesterol homeostasis of steroid producing cells remains unclear.

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Chapter 2.3

Functional molecular mass of rat hepatic lipase in liver, adrenal gland and ovary is different

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Functional molecular mass of rat hepatic lipase in liver, adrenal gland and ovary is different

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Lipoprotein lipase (LPL) is functionally active only as a dimer. It is also generally assumed that the highly homologous hepatic lipase functions as a dimer, but no clear evidence has been presented. A hepatic lipase-like activity, also indicated as L-type lipase, is present in adrenal and ovary tissues. This enzyme is thought to originate from the liver and to be identical to hepatic lipase. We determined the functional molecular mass of hepatic lipase in rat liver, adrenal gland and ovary by radiation inactivation, a method for determining the functional size of a protein without the need of prior purification. Samples were exposed to ionizing radiation at −135 °C. Hepatic lipase activity in liver homogenate showed a single exponential decay. The functional molecular mass was calculated to be 63±10 kDa. Hepatic lipase activity in adrenal homogenate was found to have a functional molecular mass of 117±16 kDa. The functional molecular masses of the lipases partially purified from rat liver perfusate, adrenal homogenate or ovarian homogenate showed the same pattern, a target mass for the liver enzyme of 56±6 kDa and a target mass of 117±14 kDa for the enzyme from adrenal gland or ovary. In Western blot analysis the mass of the structural units of hepatic lipase in liver was 57 kDa and in adrenal and ovary tissue 51 kDa. We conclude that the functional unit of hepatic lipase in the liver is a monomer. The enzyme in adrenal gland and ovary is different from the liver and the functional unit may be a dimer.

INTRODUCTION

Two extracellular lipases, lipoprotein lipase (LPL) and hepatic lipase (HL), serve important roles in lipoprotein metabolism [1–4]. Both enzymes are highly homologous.

LPL is found in extrahepatic tissues, i.e. heart, skeletal muscle and adipose tissue. Its main function is the hydrolysis of triacylglycerols in very-low-density lipoprotein and chylomicrons [5,6]. HL is present in the liver and is believed to play a role in the metabolism of high-density lipoprotein, intermediate-density lipoprotein and chylomicron remnants [7–11]. In addition to the liver, an immunologically related enzyme, sharing characteristics with HL, has been found in adrenal gland and ovary [12,13]. This enzyme is also indicated as L-type lipase.

After synthesis in the parenchymal cells LPL and HL are secreted and extracellularly bound to specific binding sites [14–19]. For adrenal gland and ovary the situation seems to be more complex. In these tissues the HL gene is transcribed into a truncated mRNA [20]. Function or catalytic properties of these HL gene products are not known. The full-length message of HL is missing in adrenal gland and ovary. It has been hypothesized that these tissues acquire catalytically active HL from the liver. In this model it is assumed that the enzyme, after being secreted by the liver, is transported to the adrenal gland and ovary, where it binds to specific binding sites [21,22]. Therefore the HL in adrenal gland and ovary is supposed to be identical to the liver enzyme.

It has been shown in studies of sedimentation equilibration, gel filtration and radiation inactivation studies that the homologous enzyme LPL is only catalytically active as a dimer [23–25]. The functional unit of HL has not been established yet. Because of its close homology with LPL it is generally assumed that HL is also a dimer. Gel-filtration experiments yield conflicting results. Depending on the salt concentration molecular masses of the native enzyme of 65–200 kDa have been found [26–29]. HL easily aggregates when free in solution. Therefore we employed radiation inactivation to estimate the functional molecular mass of HL in liver, adrenal gland and ovary.

MATERIALS AND METHODS

Animals

Normally fed male Wistar rats, 200–250 g, were used for most experiments. Female Wistar rats were used for the experiments with ovaries. They were housed under controlled conditions: temperature 20–22 °C, light on 07:00–19:00 h. They had free access to Purina rat chow and water.

Tissue preparations

Rats were killed by decapitation and 20% (w/v) liver homogenate and 10% (w/v) adrenal homogenate were prepared in PBS containing 10% (v/v) glycerol, pH 7.4 (15 s, Polytron, setting 4). Aliquots (400 μl) of homogenate were sealed in 1 ml glass ampoules, frozen in liquid nitrogen and stored at −80 °C until use.

Isolation of HL

HL was isolated from heparin-containing rat liver perfusate or heparin-containing tissue extracts from rat adrenals and ovaries

Abbreviations used: LPL, lipoprotein lipase; HL, hepatic lipase.

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as described previously [13] with minor modifications. The perfusate and tissue extracts were applied to a Sepharose–heparin column. After washing the column with 3–6 column volumes of 10 mM sodium phosphate buffer, pH 7.4, containing 0.2 M NaCl and 10% (v/v) glycerol, HL activity was eluted with the same buffer containing 1 M NaCl and 1% BSA in addition. The peak fractions were combined and the buffer was changed to PBS containing 1% BSA and 10% glycerol by gel filtration using PD10 columns. Aliquots (400 μl) of partially purified HL from liver perfusate or tissue extracts of adrenals or ovaries were sealed in 1 ml glass ampoules and frozen in liquid nitrogen. Samples were stored at −80 °C until use. SDS/gel electrophoresis and Western blotting of the samples were performed as described previously [30]. To quantify the amount of protein, gels or blots were scanned with the Hewlett Packard Scan Jet IICX and analysed.

Radiation inactivation and dosimetry

Samples were irradiated in a cryostat at −135 °C, employing a 3 MeV Van de Graaff electron accelerator. The duration of the electron pulse was 250 ns and the pulse had a 2 A peak current as described previously [31]. To improve dose homogeneity a 0.3-mm-thick aluminium scatter plate was used on top of the beam window of the cryostat. Dosimetry was performed before each irradiation experiment using FWT-60 radiochromic nylon film dosimeters (1 cm × 1 cm), between 3-mm-thick nylon plates. The dose received was determined from the change in absorbance at 510 nm and calculated from the formula:

$$\text{Dose (kGy)} = 151.6 \times (\Delta A)^{1.186}$$

obtained by dose calibration of the FWT-60 films using a ^{60}Co source.

This resulted in a dose of 0.082 Gy/nC with an accuracy of ±5%. Electron pulses were given at a rate of 5 Hz. The charge per pulse was determined before and after each irradiation and was found to be stable within 1%. The relationship between the molecular mass (M) and the dose is given by the equation $M = c \cdot K \cdot St$ where $c = 6400$ kGy/kDa, $St = 2.8$ (correction factor for the irradiation temperature of −135 °C) and K = slope of the ln (fractional activity A/Ao) versus dose plot [32]. A least-square fit was calculated for the ln (remaining activity versus radiation dose). The validity of our method was verified by determination of the target mass of yeast glucose-6-phosphate dehydrogenase [33]. We observed a monoexponential inactivation for this protein corresponding to a target mass of 99±6 kDa, which is in line with the molecular mass of the enzyme of 104 kDa.

Radiation inactivation of HL protein was followed by SDS/PAGE according to the method of Laemmli on a 3% stacking gel and a 10% resolving gel and subsequent Western blotting on nitrocellulose paper. HL was identified on the blot using polyclonal goat anti-HL IgG and an alkaline phosphatase-conjugated anti-(goat IgG) [30].

HL assays

HL triacylglycerol hydrolase activity was measured using an artificial glycerol-[9,10(n-^{3}H)]trioleate emulsion in gum arabic, pH 8.5 [12]. To avoid any possible contamination with other neutral lipase activities in the adrenal and liver homogenates, HL activity was measured in samples preincubated with or without an anti-HL-specific polyclonal antibody [34].

Materials

All chemicals used were of analytical grade. Heparin was purchased from Leo Pharmaceutical Products, Weesp, The Netherlands and BSA from Sigma (St. Louis, MO, U.S.A.). Glycerol [9,10(n-^{3}H)]trioleate was obtained from Amersham International, Amersham, Bucks., U.K. Alkaline phosphatase-conjugated anti-(goat IgG) was purchased from Tago, Burlingame, CA, U.S.A.

RESULTS

HL in rat liver

HL activity in homogenate from rat liver, prepared in PBS containing 10% (v/v) glycerol and 1% BSA, is stable and can be frozen and thawed without loss of activity.

If frozen rat liver homogenate fractions were exposed to increasing doses of radiation triacylglycerol hydrolase activity showed a simple exponential decay. The functional molecular mass was calculated to be 63±10 kDa (Figure 1). A similar decay curve was observed on exposure of HL partially purified from rat liver heparin perfusate. The calculated target mass was 56±6 kDa.

The target mass was also evaluated by SDS/gel electrophoresis and immunoblotting. On Western blots HL had an apparent molecular mass of 57 kDa. Upon radiation, immunoreactivity at this position in the gel was lost. In parallel immunoreactive material increased at the front of the gel, suggesting that irradiation resulted in fragmentation of the protein (Figure 2). The loss in HL-immunoreactive material at 57 kDa on the Western blot upon irradiation yielded a target mass of 53±5 kDa (Figure 3). We also followed the decrease in the amount of albumin during irradiation at 66 kDa on the gel. For albumin a target mass of 70±6 kDa was found.

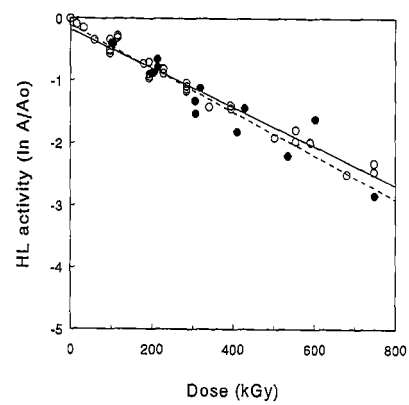


Figure 1 Radiation inactivation of HL in liver

HL triacylglycerol hydrolase activity was measured in irradiated liver homogenate (●, broken line) or in irradiated hepatic lipase (HL) purified from liver heparin perfusate (○, solid line) as described in the Materials and methods section. Data are expressed as ln [activity measured in the samples after irradiation (A) divided by the HL activity of non-irradiated samples (Ao)]. The fitted line is from least-square analysis. Values plotted are the means of three determinations from five separate experiments.

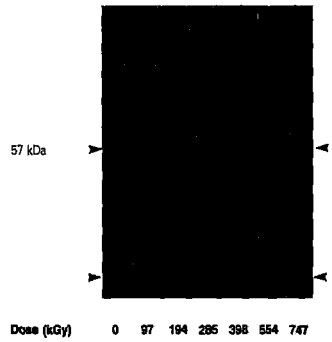


Figure 2 Western blot of irradiated partly purified HL

Partly purified HL was irradiated as described in the Materials and methods section. After radiation the samples were mixed with SDS/PAGE sample buffer, electrophoresed on SDS/PAGE and subjected to Western blot analysis.

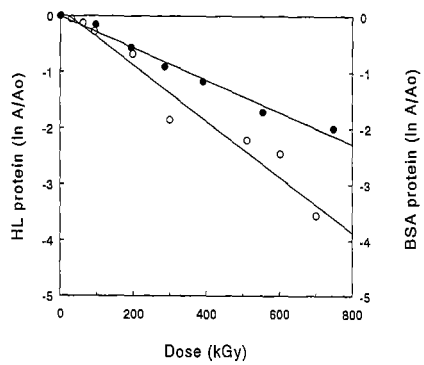
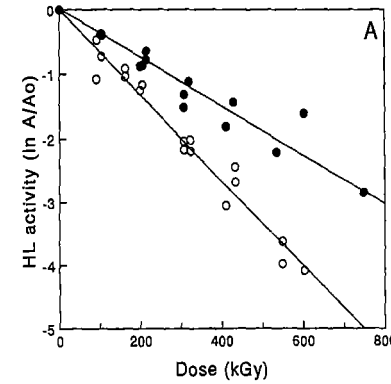


Figure 3 Irradiation fragmentation of HL protein

The fraction of surviving immunoreactive protein of hepatic lipase (HL, ●) or BSA (○) [ln (A/Ao)] is plotted as a function of radiation dose. HL-immunoreactive protein was measured at 57 kDa on Western blots. The amount of BSA was measured as the amount of protein at 66 kDa on SDS-gels. To quantify the amount of protein, gels or blots were scanned with the Hewlett Packard Scan Jet IICX and analysed.



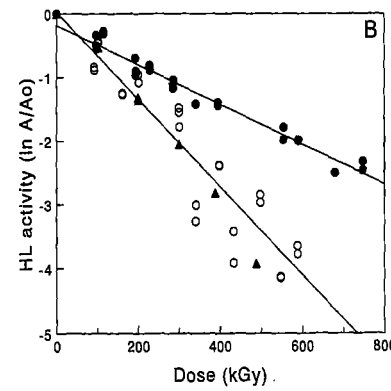


Figure 4 Radiation inactivation of HL in liver, adrenal gland and ovary

HL triacylglycerol hydrolase activity was measured in (**A**) irradiated liver homogenate (●) or adrenal homogenate (○); or (**B**) irradiated HL partly purified from liver heparin perfusate (●), or heparin-containing homogenates of adrenals (○) or ovaries (▲) as described in the Materials and methods section. Data are expressed as ln [activity measured in the samples after irradiation (A) divided by the activity in non-irradiated samples (Ao)]. The fitted line is from least-square analysis. The values plotted are the means from 3–5 different experiments.

HL in rat adrenal gland and ovary

Tissue homogenate of rat adrenals was exposed to increasing doses of radiation. A target mass of 117 ± 16 kDa was found, which is much larger than in liver (Figure 4A). Because the HL activity in ovary was not sufficient to employ radiation inactivation in the homogenate, we purified the enzyme from ovaries and adrenals. The same target mass as for the lipase activity in the adrenal homogenate was obtained for the purified HL from rat adrenal gland and ovary, i.e. 117 ± 14 kDa (Figure 4B).

Structural unit of HL

Besides the size of the functional unit, we also compared the size of the structural unit of the liver enzyme and the enzyme in adrenal gland and ovary with SDS/gel electrophoresis and immunoblotting. In liver a structural unit of 57 kDa was found compared with a structural unit of 51 kDa in adrenal gland and ovary (Figure 5).

DISCUSSION

Employing radiation inactivation, we show that the functional target mass of HL in rat liver is 63 kDa, in the adrenal gland and in the ovary it is 117 kDa. The structural unit in liver is 57 kDa and in the adrenal and ovary 51 kDa, as measured by Western blotting. Using radiation target mass analysis the molecular mass of the enzyme can be determined in its native state, bound to its binding site, without prior purification. In this way purification artefacts are avoided. Moreover, radiation inactivation reveals functional interactions among enzyme complexes with receptors or in oligomeric enzyme systems. After purification the sizes of the functional units of the liver and the adrenal or ovarian enzyme were respectively 56 and 117 kDa, similar to the values observed in crude homogenate. Therefore it seems that interaction of the enzyme with its binding site does not influence

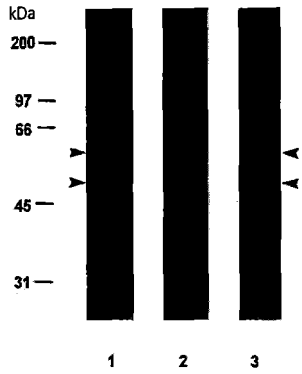


Figure 5 Determination of the size of the structural unit of HL in adrenal gland and ovary

Partly purified HL from heparin-containing liver perfusate (lane 1) or from heparin-containing homogenate of adrenals (lane 2) or ovaries (lane 3) were subjected to SDS/PAGE and Western blotting to determine the size of the structural unit.

the activity of the enzyme. The reported molecular mass of HL in liver, as estimated by SDS/gel electrophoresis, is in the range 55–66 kDa [28,29,35,36]. The value obtained with gel filtration varies between 65 and 200 kDa [26–29]. The variation in the observed values of the enzyme in its native state may be due to association of the enzyme with specific or non-specific HL-binding proteins or to aggregation of the enzyme. The monomeric value of the molecular mass of HL after gel filtration as observed by Ikeda et al. [29] gives no information about the size of the functional unit; formation of dimeric HL in the lipase assay cannot be excluded, because for LPL also, which is clearly a dimer, Ikeda observed a monomeric value of the molecular mass after gel filtration. The target size of both the native liver enzyme in the homogenate and the purified liver enzyme as measured by radiation inactivation indicates that the functional unit of HL in liver is a monomer. This is in contrast to LPL. Using a number of techniques including radiation inactivation the functional unit of LPL has been shown to be a dimer [23–25]. The dimeric functional unit of LPL refers to its triacylglycerol hydrolase activity, for its esterase activity there is no need for a dimeric structure [37]. For LPL it is assumed that the dimeric structure is a prerequisite for the binding of the water-insoluble triacylglycerol. The proposed model for the LPL structure is a model consisting of two monomeric LPL units arranged in such a manner that the C-terminal domain, the lipid-binding domain of one subunit, is close to the N-terminal domain, the catalytic domain of the other subunit, in a so called head-to-tail arrangement [38,39]. Based on the close relationship between HL and LPL it is assumed that the functional unit of HL is also dimeric. However, it appears that HL in liver is a monomer rather than a dimer. Therefore, it may well be that the interaction of HL, or at least the liver enzyme, with its substrate triacylglycerol is different from the proposed interaction of LPL with its substrate.

For the size of the functional unit of the enzyme from adrenal gland or ovary a much higher target mass was found than for the liver enzyme, i.e. 117 kDa. Despite this higher target size, we found a smaller size of structural unit of the HL from adrenal gland and ovary, 51 kDa. This is lower than for the liver enzyme, but similar to the size that was reported for the adrenal gland by Doolittle et al. [21]. Since we found a functional unit of 117 kDa for the adrenal and ovarian enzyme, both in the crude homogenate and after purification, we conclude that the enzyme in adrenal gland and ovary is in a dimeric form. The difference between the liver enzyme and the enzyme in the adrenal gland or ovary is unexpected as it was assumed that the enzyme in these tissues is derived from the liver [21,22]. This suggests that HL is present in the liver as a monomer and is dimerized after or during its transport to the adrenals and ovaries. Radiation inactivation of the liver enzyme would lead to inactivation of only one monomeric subunit, radiation inactivation of the adrenal or ovarian enzyme on the other hand, would lead to inactivation of both subunits because of energy transfer between the two monomeric subunits. The size of the structural unit in the adrenal gland and ovary is also different from that in the liver, indicating that post-translational modification of the enzyme occurs in the liver or during its transport from the liver to the adrenal. Liver and adrenal enzymes differs also in binding characteristics. The release of HL from the liver and from the adrenal gland by protamine is different [40]. No significant release of HL was found in the adrenal gland after injection of protamine, while protamine was as effective as heparin in the release of HL from liver. It was concluded that this may be due to a difference in the binding site for HL in liver and adrenal gland. However, the observed difference in the release of HL could also indicate that the adrenal enzyme is more tightly bound because of its dimeric functional unit. The release of the homologous dimeric enzyme LPL by protamine injection was also very low and only 10 % of the amount of LPL released by heparin.

In this paper we have shown that HL in liver is in the monomeric form and different from HL in the adrenal gland and ovary, which is in the dimeric form. Whether the size of the functional and structural unit as well as the substrate specificities or physiological function of HL in the adrenal gland and ovary are different is currently under investigation.

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Chapter 3

Role of hepatic lipase and SR-BI in cholesterol homeostasis

Chapter 3.1

Role of hepatic lipase in HDL binding to rat adrenal membranes

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ABSTRACT

High density lipoproteins (HDL) bind and deliver cholesterol (esters) to rat steroidogenic tissues. We investigated characteristics of HDL binding to rat adrenal plasma membranes, and whether this binding is modulated by hepatic lipase. ^{125}I-HDL specific binding was saturated at 20-30 μg/ml with a K_d=10 μg/ml and a B_{max}=1 μg/mg. HDL binding was partly heparin-sensitive. Pretreatment of plasma membranes with heparin decreased HDL binding by about 60%. Furthermore, two classes of binding sites were identified, one with K_d= 8 μg/ml and B_{max}=0.30 μg/mg which is heparin-insensitive, and a second heparin-sensitive site, which accounts for the major part of specific binding (K_d=11 μg/ml and B_{max}=0.75 μg/mg). The decrease in HDL specific binding to membranes pretreated with heparin could be completely restored by addition of purified rat hepatic lipase. We further studied the effect of hepatic lipase on HDL binding after treatment of the rats with ACTH during 9 days. ACTH treatment did not lead to an increase in HDL binding. In ACTH treated rats hepatic lipase greatly enhanced the HDL binding to heparin pretreated membranes. The effect of hepatic lipase was more pronounced in ACTH treated rats than in controls. ACTH treatment also led to an increase (20-fold) in HDL receptor, SR-BI. Our findings suggest that hepatic lipase modulates the binding of HDL to rat adrenal glands and that its role is more relevant during induction of steroidogenesis. This modulation probably occurs simultaneously with or may precede the binding of HDL to its receptor SR-BI.

INTRODUCTION

The rat adrenal gland preferentially utilizes cholesterol derived from circulating high-density lipoprotein (HDL) as a substrate for steroid hormone synthesis [1, 2]. HDL cholesterol uptake in steroid hormone producing tissues may not require the internalization of the whole lipoprotein particle [3]. Instead, HDL-cholesterol(esters) are taken up by the cells through a selective pathway [4] while further internalization involves a network of vesicles [5]. The HDL particle is supposed first to bind to the transmembrane Scavenger Receptor class B type I (SR-BI), the putative HDL receptor prior to deliver its cholesterol [6]. SR-BI is expressed in liver and non-placental steroidogenic tissues [7] and it is upregulated under hormonal stimulation of steroidogenesis [8].

Hepatic lipase (HL) may mediate the uptake of HDL-cholesterol (esters) by its enzymatic,

phospholipase A-1 activity [9-11] and by binding lipoproteins [12-14]. The binding of HDL via HL in vitro was reported to be dependent on heparan sulfate proteoglycans [15]. In steroidogenic tissues HL also may play a modulating role in the uptake of HDL-cholesterol (esters) [9, 16, 17]. In line with this, hepatic lipase in adrenals is enhanced upon stimulation with ACTH [18]. More recent in vitro studies suggested that hepatic lipase is able to promote the HDL-cholesterol esters uptake via SR-BI [19, 20]. The mechanism by which HL promotes HDL-binding / cholesterol uptake remains unclear.

In the present study we investigated HDL binding to rat adrenal plasma membranes and the potential role of hepatic lipase in HDL binding. We show that HL modulates the HDL binding to membranes of steroidogenic tissues in vitro. In the binding SR-BI may be involved.

MATERIALS AND METHODS

Animals and membrane preparation

Young adult male Wistar rats (6-9 weeks old, weighting 200-300 g), controls and corticotrophin treated (Synacthen - synthetic ACTH analogue, Ciba, 0.2 mg /Kg body weight, daily subcutaneously administrated during 9 days) were used. Control animals were treated with saline simultaneously. Adrenal membranes were isolated at 4°C as described elsewhere [21] with minor modifications and plasma membranes were obtained after centrifugation on a discontinuous sucrose gradient.

Binding assays

Human HDL (density 1.063-1.21) was isolated by sequential centrifugation [22] and subsequently subfractionated on sepharose-heparin column to remove apo E-rich fractions [23]. Radio-iodinated HDL was prepared as previously described [24]. The radioactivity ranged 130-190 cpm/ng protein. Hepatic lipase was purified from rat liver heparin-perfusate [25]. When indicated, the plasma membranes were incubated with heparin (10 IU/ml, Leo Pharmaceutical Products) for 15 minutes at 4°C prior to assay and/or to incubation with purified HL (100 mU/ml incubation, specific activity ~ 20 mU/µg enzyme protein). In both cases, excess of unbound ligand (heparin or HL) was removed by centrifugation, 100,000 x g for 30 min at 4°C, twice washed with ice-cold PBS and again centrifuged. HDL binding studies were carried out for 1 h utilizing 30 µg of plasma membrane protein and varying concentrations of

radio-iodinated HDL in a final volume of 200 µl ice-cold phosphate buffered saline (PBS) containing bovine serum albumin (BSA, 10 mg/ml). Unbound HDL was removed by centrifugation at 100,000 x g for 30 min. The recovered membrane pellet was washed twice with PBS-BSA buffer. Iodine label in the pellet was counted in a Gamma counter. Specific HDL binding is given as the difference between total binding and the binding in presence of a 20-fold excess unlabelled lipoprotein.

SR-BI gene expression

Total tissue RNA was isolated using the Chomczynski and Sacchi method [26] from freshly dissected adrenals from control and ACTH treated rats. RNA concentration was determined by spectrophotometry at 260 nm and quality of the preparations judged by agarose gel electrophoresis. RT-PCR was performed starting from 1 µg of total RNA. Random-primed cDNA was amplified for 25 cycles using SR-BI specific primers, SR1-For (5'-CGG AAT TCA GGG GTG TTT GAA GGC-3') and SR1-Rev (5'-CGG GAT CCT GAA TGG CCT CCT TAT CC-3') according to the human cDNA sequence. All RT-PCR experiments included no-template and no-RT controls that remained negative. The expected 598 bp band obtained shared >90% sequence homology with human SR-BI sequence. This PCR product was extracted and used as probe in Northern Blotting analysis. Five µg of total tissue RNA was fractionated on agarose gel transferred to a nylon membrane and then probed with a ^{32}P-labelled rat SR-BI cDNA. Blots were analyzed using a Molecular Imager from Bio-Rad.

RESULTS

Effect of heparin on HDL binding

We investigated the role of hepatic lipase in HDL binding to rat adrenal plasma membranes. Fig. 1 shows the binding of ^{125}I-HDL in presence and absence of excess unlabelled lipoprotein. Specific binding reached saturation at an HDL concentration of 20-30 µg protein/ml with a equilibrium constant, K_d, of about 10 µg protein/ml and a maximal binding capacity, B_{max}, of about 1 µg protein/mg membrane protein. The specific ^{125}I-HDL binding was significantly reduced by heparin. In the presence of heparin, the residual (heparin-insensitive) binding (K_d = 8 µg protein/ml and B_{max} = 0.3 µg protein/mg) was less than 40% of the total capacity indicating that the HDL binding is partially heparin-sensitive (Fig. 2). The heparin-sensitive binding of HDL was saturated at 20-30 µg protein/ml with an equilibrium dissociation constant, K_d, of 11 µg/ml and a B_{max}, of 0.75 µg/mg.

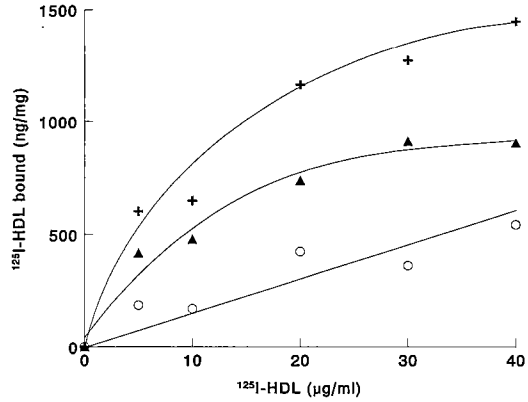

Figure 1. **Binding of ^{125}I-HDL to rat adrenal plasma membranes as a function of labelled lipoprotein concentration.** Aliquots of control adrenal plasma membranes (30μg protein) were incubated with the indicated concentrations of ^{125}I- HDL in 0.2 ml of incubation medium containing 1% BSA (w/v) for 1 h at 4°C. Specific binding (▲) was obtained by subtracting the binding found in presence of 20-fold excess of unlabelled lipoprotein (o) from the total binding (+). One out of several similar experiments is shown.

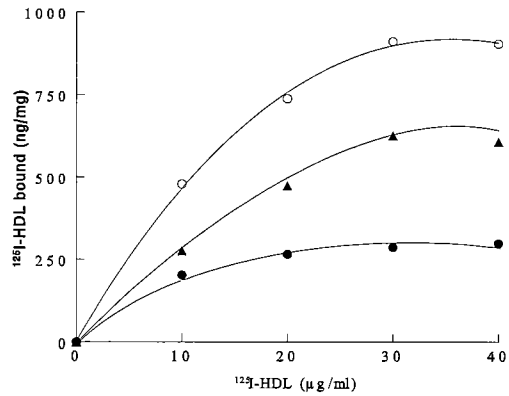

Figure 2. **Specific binding of ^{125}I-HDL to rat adrenal plasma membranes. Effect of heparin treatment.** Membranes (30 μg protein) were incubated with or without heparin (10 U/ml) during 15 minutes at 4°C. Membranes were then washed to remove unbound heparin and incubated with the indicated ^{125}I-HDL concentrations. Heparin-sensitive binding (▲) is given as the difference between the HDL specific binding in absence of heparin (o) and the heparin-insensitive binding (specific binding to heparin pre-washed membranes) (●). One typical experiment is shown.

The decrease in binding after heparin was not due to a loss of membrane protein in the preparations used since neither a difference in protein content (1.03 ± 0.1 mg/ml vs 0.93±0.01 mg/ml before and after heparin, respectively), nor in the pattern of the major protein bands migrating in a acrylamide gel electrophoresis could be detected (data not shown).

Effect of hepatic lipase on HDL specific binding

Our next objective was to determine whether the loss of heparin-sensitive binding of the HDL could be due to release of hepatic lipase by heparin and if so, whether it could be restored by addition of purified hepatic lipase. Rat adrenal plasma membranes contain hepatic lipase (l(iver)-type lipase) activity (1.3-2.99 mU/mg protein) which can be displaced by heparin (data not shown).

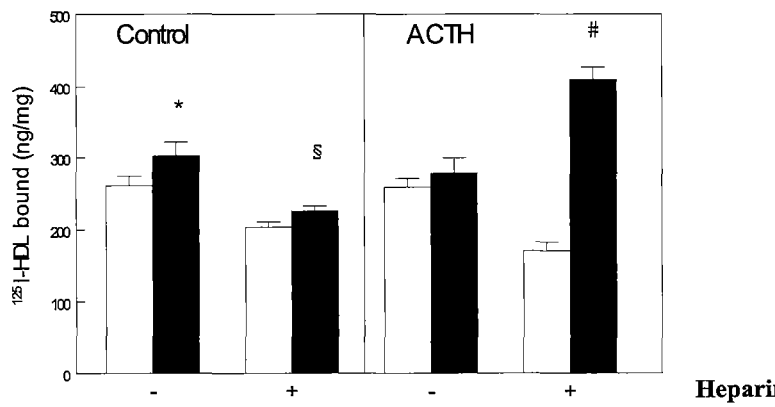

Figure 3. **Hepatic lipase modulation of the specific binding of ^{125}I-HDL to adrenal plasma membranes. Effect of ACTH stimulation (9 days).** Membranes were pretreated with heparin followed by incubation without (open bars) or with purified hepatic lipase (100 mU/ml) (filled bars). Binding assays were performed in presence of a constant ^{125}I-HDL concentration (15 μg/ml). Each point represents the mean ± S.D. of triplicates. *p<0.05, §p<0.02 and #p<0.001 compared with results in absence of hepatic lipase.

Figure 3 illustrates the effect of HL on ^{125}I-HDL binding to adrenal membranes of control and ACTH stimulated rats. In control animals, HDL binding to adrenal membranes untreated with heparin was increased after addition of hepatic lipase ($p<0.05$). Hepatic lipase added to heparin-washed membranes lead to a significant increase in HDL binding especially after ACTH treatment. In control rats (0 d ACTH) the decrease in HDL binding caused by heparin could not completely be restored by addition of purified hepatic lipase. ACTH treatment did not lead to an increase in HDL binding to membranes. Membranes previously treated with heparin showed a lower HDL binding especially after 9 days of ACTH treatment. Addition of hepatic lipase however, restored completely the HDL binding in stimulated rats leading to 2.4-fold ($p<0.001$) increase in HDL binding. We also investigated the expression of the putative HDL receptor, SR-BI, before and after ACTH treatment. ACTH greatly increased (20-fold) SR-BI mRNA after 9 days of treatment (Fig. 4).

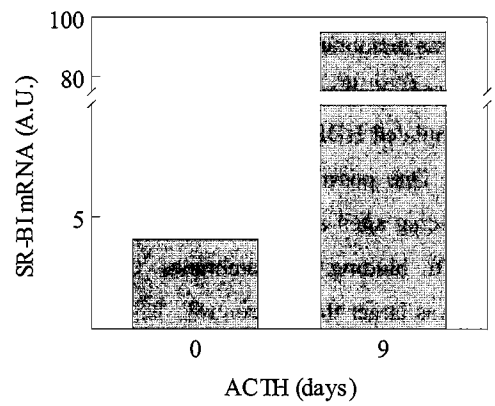

Figure 4. **Effect of ACTH treatment (9 days) on adrenal SR-BI expression.** Northern-blot was performed as described in Materials and Methods. Five µg of adrenal total RNA was fractionated on agarose gel, transferred to a nylon membrane and incubated with a SR-BI cDNA probe. SR-BI mRNA was quantified by scanning of the blots, and results are given in arbitrary units.

DISCUSSION

HDL particles bind to adrenal cells from rodents and humans. Its kinetics differs from that of LDL binding because the classical binding pattern to a well characterized receptor in not found [27]. The mechanism by which binding, internalization or selective uptake of HDL-cholesterol esters occurs is not completely understood. We studied the ^{125}I-HDL binding to rat adrenal glands, especially whether hepatic lipase modulates the binding. Our results indicate that rat adrenal plasma membranes contain two classes of HDL binding sites, one sensitive and another insensitive to heparin (Fig. 2). The decrease in HDL binding by heparin possibly implicates the release of a specific heparin-sensitive protein or factor associated with the plasma membrane and involved in the HDL binding. Previous work by Jansen and coworkers [18, 28, 29] showed the existence of a salt-resistant, hepatic lipase immuno-related enzyme in steroidogenic tissues. This enzyme is heparin-releasable and was suggested to play a role in the reverse-cholesterol transport by promoting the transfer of cholesterol (esters) to the liver [11, 30, 31], it may also be involved in the cholesterol uptake by steroidogenic tissues [9, 32, 17]. Therefore, we studied whether the heparin-sensitive HDL binding was modulated by hepatic lipase. In freshly isolated plasma membranes of control and of ACTH stimulated rats addition of HL did not greatly influence HDL binding. However, if the plasma membranes were first washed with heparin, HL stimulated HDL binding so that loss of HDL binding capacity by heparin was restored by HL. One possible explanation for this observation is that under control conditions all HL binding sites are occupied. Addition of extra enzyme cannot be matched by much higher HDL binding. In membranes of ACTH pretreated rats, the HL stimulation of HDL binding was larger than in controls. These data suggest that the heparin-sensitive HDL binding is HL mediated. ACTH treatment greatly induced the expression of SR-BI, a putative HDL receptor [7]. As can be seen in Fig. 3, HDL binding per mg heparin non-treated plasma membrane did not differ between control and ACTH- treatment of plasma membranes. Since ACTH treatment induces hyperplasia of the cortical cells HDL binding per organ did increase upon ACTH treatment. Fig.3 shows that this is partly mediated by HL. Our results are compatible with binding of HDL mediated by an interaction between HL and SR-BI. These observations are in line with the work of Ji [15]. The authors found that part of the HDL binding and cholesterol ester uptake by cells overexpressing HL was mediated by HL and that it could be disrupted by heparinase.

Our hypothesis is that, under basal conditions, both HL and SR-BI are involved in the HDL binding to rat adrenal membranes. They can act independently or together. Furthermore, this

hypothesis is supported by the work of Lambert and collaborators [14]. In their work the authors found that in cells overexpressing HL the HDL binding was two-fold enhanced and co-overexpression of HL with SR-BI also enhanced the uptake of HDL-cholesteryl esters. Furthermore, the effect of HL on HDL association and HDL-CE uptake by the cells was displaced by heparin, suggesting that HL is the major modulator of HDL binding and SR-BI mediated HDL-CE uptake.

The data here described give evidence that hepatic lipase modulates the HDL binding to rat adrenal plasma membranes and that the role of enzyme is more important after ACTH stimulation. SR-BI may be involved in the HL mediated binding.

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Chapter 3.2

Induction of adrenal SR-BI and increase HDL-cholesteryl ether uptake by in vivo inhibition of hepatic lipase

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Induction of Adrenal Scavenger Receptor BI and Increased High Density Lipoprotein-Cholesteryl Ether Uptake by *in Vivo* Inhibition of Hepatic Lipase*

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Hepatic lipase (HL) and scavenger receptor type B class I (SR-BI) have both been implicated in high density lipoprotein (HDL)-cholesteryl ester uptake in cholesterol-utilizing tissues. Inactivation of HL by gene-directed targeting in mice results in up-regulation of SR-BI expression in adrenal gland (Wang, N., Weng, W., Breslow, J. L., and Tall, A. R. (1996) *J. Biol. Chem.* 271, 21001–21004). The net effect on HDL-cholesteryl ester uptake is not known.

We determined the impact of acute *in vivo* inhibition of rat adrenal HL activity by antibodies on SR-BI expression and on human and rat HDL-[^{3}H]cholesteryl ether (CEth) uptake in the adrenal gland. Rat HDL was isolated from rats in which HL activity had been inhibited for 1 h. The rats were studied under basal conditions (not ACTH-treated) and after previous treatment with ACTH for 6 days (ACTH-treated). Intravenous injection of anti-HL resulted in 70% lowering of adrenal HL activity in both conditions which were maintained for at least 8 h. In not ACTH-treated rats, inhibition of adrenal HL increased adrenal SR-BI mRNA (5.2-fold) and mass (1.6-fold) within 4 h. HL inhibition resulted in 41% and 14% more adrenal accumulation of human HDL-[^{3}H]CEth during 4 and 24 h, respectively. The adrenal uptake of rat HDL-[^{3}H]CEth increased by 68%, 4 h after the antibody injection. ACTH treatment increased total adrenal HL activity from 3.7 ± 0.5 milliunits to 34.0 ± 17.2 milliunits, as well as adrenal SR-BI mRNA from 2.9 ± 0.7 arbitrary units (A.U.) to 86.8 ± 41.1 A.U. and SR-BI mass from 7.7 ± 1.8 A.U. to 63.16 ± 46.7 A.U. The human HDL-[^{3}H]CEth uptake by adrenals was also significantly increased from 0.58 ± 0.11% of injected dose to 7.24 ± 1.58% of injected dose. Inhibition of adrenal HL activity did not result in further induction of SR-BI expression and did not affect human HDL-[^{3}H]CEth uptake.

These findings indicate that SR-BI expression may be influenced by changes in HL activity. HL activity is not needed for the SR-BI-mediated HDL-cholesteryl ester uptake by rat adrenal glands.

In the rat, hepatic lipase (HL[1]; E.C. 3.1.1.34) is extracellularly localized at the parenchymal cell microvilli of the liver (1–3). A related enzyme, also indicated as liver (L)-type lipase, is present in the zona fasciculata of the adrenal gland and in the corpora lutea of the ovary (2, 4–6). We proposed a role of HL in the uptake of HDL-unesterified cholesterol and cholesteryl esters in the lipase-containing tissues (7, 8). *In vitro* studies, with either isolated cell systems or perfused rat liver, showed that HL activity may stimulate the uptake of HDL-cholesteryl esters as well as unesterified cholesterol (9–11). However, *in vivo* only indirect support for a role of HL in HDL-cholesterol and cholesteryl ester uptake has been obtained. Jansen *et al.* (12) showed that plasma HDL-cholesterol increased by *in vivo* inhibition of HL. At the same time *de novo* cholesterol synthesis in liver (13) and in superovulated rat ovaries (14) is induced. These findings are compatible with the involvement of HL in the uptake of extracellular cholesterol. HDL-cholesteryl ester uptake has been studied in a wide range of tissues under different metabolic conditions and may be taken up via several mechanisms. Besides the classical endocytotic pathway (see Ref. 15 as review) a selective uptake mechanism, in which HDL-cholesteryl esters are taken up without concomitant internalization of the protein part, has been proposed (8, 16, 17). The scavenger receptor class B type I (SR-BI), exclusively present in liver and non-placental steroidogenic tissues, is involved in this process (18–20). In endocrine tissues the SR-BI expression is regulated by trophic hormones (21). Additionally, cellular cholesterol levels may modulate SR-BI expression (22, 23). Investigations in HL-deficient (knock-out) mice suggest a link between HL and SR-BI expression (22). In female HL knock-out mice, SR-BI expression in adrenal gland was strongly enhanced. The induction of SR-BI was suggested to result from a lowering of intracellular cholesterol stores because of HL deficiency. An alternative mechanism may be that SR-BI expression is stimulated compensatory to changes in plasma lipoprotein metabolism because of the long-term HL deficiency. Remarkably, despite the greatly enhanced SR-BI levels (3.5-fold), adrenal cholesterol (ester) stores were largely depleted suggesting that the increase in SR-BI did not result in adequate cholesterol supply to support steroidogenesis. This may indicate that HL activity is required for the optimal activation of selective HDL-cholesteryl ester uptake.

In the present investigation we studied the impact of acute *in vivo* inhibition of HL activity on adrenal SR-BI expression. In addition, we measured the consequences for HDL-cholesteryl ester uptake under these conditions.

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[1] The abbreviations used are: HL, hepatic lipase; HDL, high density lipoprotein; CEth, cholesteryl ether; SR-BI, scavenger receptor class B type I; IgG, immunoglobulin G; PCR, polymerase chain reaction; ACTH, adrenocorticotrophic hormone; A.U., arbitrary unit(s).

EXPERIMENTAL PROCEDURES

Animals—Male Wistar rats (200–300 g) were kept under controlled conditions of humidity, light, and temperature with free access to tap water and chow diet. The animals were fasted overnight before use. ACTH treatment consisted of daily subcutaneous administration of 0.2 mg of Synacthen (a synthetic ACTH analogue, Ciba) per kg body weight for 6 days. Control rats were injected daily with saline for the same period.

In Vivo Inhibition of Hepatic Lipase Activity—The IgG fraction of goat anti-rat HL and non-immune goat serum was isolated by protein G affinity chromatography. The IgGs were dialyzed against 5 mM $(NH_4)HCO_3$ and lyophilized. The obtained pellets were resuspended in 0.15 M NaCl to a concentration of 30 mg of protein/ml. The antibody preparation was tested for its ability to inhibit HL activity of heparin-containing rat liver perfusate. To inhibit adrenal lipase activity, the rats were treated with an amount of antibodies that inhibited the enzyme activity equivalent to all heparin-releasable HL activity in rats of the same weight. Control animals were injected with the same amount of control IgG. Four, eight, or twenty-four hours after antibody injection, adrenals were homogenized in 10 volumes of ice-cold phosphate-buffered saline (pH 7.4) containing 10 IU/ml heparin and 1 mM benzamidine. After centrifugation (10,000 × *g*, 2 min, 4 °C), the post-nuclear fraction was assayed for hepatic lipase activity as described elsewhere (24). Enzyme activity was determined as triacylglycerol hydrolase and expressed as milliunits (nmol of free fatty acids released per min).

Northern Blot and Immunoblot Analysis of SR-BI—Total tissue RNA was isolated from adrenal glands as described (25). Adrenal RNA (7.5 μg/lane) was electrophoresed on a formaldehyde-agarose gel and transferred to a nylon membrane (Highbond-N+, Amersham). The SR-BI cDNA probe for *in situ* hybridization was prepared by reverse transcriptase-PCR using 1 μg of adrenal RNA. The reverse transcriptase-PCR was performed as described previously (26), using the primers SR-BI(1)-(5′-CGG AAT TCA GGG GTG TTT GAA GGC-3′) and SR-BI(2)-(5′-CGG GAT CCT GAA TGG CCT CCT TAT CC-3′) according to the human cDNA sequence (27). This primer combination yields a PCR product of 550 base pairs, which is ~98% homologous with that of the rat (data not shown). The RT-PCR product (550 base pairs) was extracted from agarose gel and resuspended in sterile water. A glyceraldehyde-3-phosphate dehydrogenase probe (570 base pairs) was also prepared by reverse transcriptase-PCR from rat heart RNA and used as reference. Both probes were labeled using 1 mCi of [^{32}P]ATP. Membranes were hybridized following standard methods (28). Radioactive bands were analyzed using a GS363 Molecular Imager System from Bio-Rad. Values of the SR-BI mRNA were normalized for glyceraldehyde-3-phosphate dehydrogenase contents in the same samples. Crude membranes of adrenal glands were isolated as described elsewhere (29). Twenty μg of membrane protein were separated by 10% SDS-polyacrylamide gel electrophoresis under reducing conditions (30) and transferred to a nitrocellulose membrane (Schleicher & Schuell). The membranes were incubated for 2 h at room temperature with a rabbit polyclonal anti-rat SR-BI antibody followed by incubation for 1 h with alkaline phosphatase-conjugated goat anti-rabbit IgGs as secondary antibody. SR-BI protein bands were detected and scanned using a Hewlett Packard ScanJet 4C and quantified.

HDL Isolation and Labeling—Human HDL was isolated from blood of healthy volunteers at a density between 1.063 and 1.21 g/ml by sequential ultracentrifugation using standard techniques (31). HDL was passed over a Sepharose-heparin column to remove apoE-containing lipoproteins (32). After dialysis against 0.15 M NaCl, containing 1 mM EDTA, pH 7.4, the lipoprotein was labeled in its lipid moiety with [1α,2α-^{3}H]cholesteryl oleyl ether (Amersham Pharmacia Biotech) as described previously for LDL (33). The labeled HDL was reisolated by gradient ultracentrifugation (33) and dialyzed against 0.15 M NaCl. Before use the preparation was filtered through a 0.45-μm Millipore filter. Rat HDL was isolated from blood of control animals injected with polyclonal anti-HL antibody and sacrificed 1 h later. During this period HL activity in the liver is inhibited 90–98%. The HDL fraction was isolated and handled as described above for human HDL, except that the lipoprotein fraction was collected at density between 1.050 and 1.21 g/ml. The labeling of rat HDL occurred in the presence of human lipoprotein-deficient serum as a source of cholesteryl ester transfer protein (33).

HDL-[^{3}H]CEth Uptake in Vivo—To study adrenal HDL[^{3}H]CEth uptake *in vivo*, two different procedures were used. In the first procedure rats were intravenously injected with 0.1 ml of concentrated anti-HL or non-immune IgG preparation. Four hours later 0.2 ml of human HDL-[^{3}H]cholesteryl ether solution, corresponding to 124 nmol of total cholesterol and 1 × 10^6 dpm, was injected intravenously. Animals were sacrificed 4 or 24 h after the injection of labeled HDL. In the second procedure, animals were intravenously injected with 0.1 ml of concentrated anti-HL or non-immune IgG preparation. Two hours later 0.2 ml of rat HDL[^{3}H]CEth corresponding to 100 nmol of total cholesterol and 1 × 10^6 dpm was intravenously injected. Two hours after the injection of the labeled HDL, 0.1 ml of concentrated anti-HL or control IgGs were administrated again. Animals were sacrificed 4 h after the labeled lipoprotein injection. The adrenals were excised, cleaned from adherent fat tissue, and weighed. Tissue samples were dissolved in Soluene-350 (Packard Instrument) for 4 h at 55 °C and analyzed for radioactivity. The radioactivity in the adrenals was corrected for contamination of plasma radioactivity and used 9.9% (v/w) plasma per organ (34). In the experiments with rat HDL the radioactivity in the adrenals was also corrected for the increase in HDL-cholesteryl esters between 2 and 4 h after the antibody injection (12.3%) (12).

TABLE I
In vivo effect of anti-HL antibody administration on adrenal HL activity and SR-BI expression in control rats

Animals were injected with goat non-immune IgG (control IgG) or with a goat polyclonal anti-hepatic lipase antibody (anti-HL) and sacrificed after 4 h or 24 h. Adrenal glands were removed, homogenized, and assayed for HL activity. HL activity was assayed as triacylglycerol hydrolase and expressed as milliunits (nanomoles of free fatty acids released per min) per 2 adrenals. SR-BI mRNA and mass were determined as described under "Experimental Procedures." Values are mean ± S.D. (n = 7) except for HL activity at 24 h (n = 3) and for SR-BI expression (n = 3). All values are statistically significant if compared with control IgG, except when NS is indicated using one way analysis of variance with the Student-Newman-Keuls test.

	HL activity	SR-BI mRNA	SR-BI mass
	(total milliunits/organ)	*(A.U./organ)*	*(A.U./organ)*
Control IgG (4 h)	3.68 ± 0.46	2.9 ± 0.7	7.7 ± 1.8
Anti-HL (4 h)	1.18 ± 0.48[a]	15.1 ± 4.2[a]	12.8 ± 0.4[a]
Anti-HL (24 h)	8.38 ± 1.08[a]	2.4 ± 0.6[NS]	13.7 ± 3.5[a]

[a] $p < 0.0001$.

RESULTS

HL activity was lowered *in vivo* by administration of HL activity inhibiting antibodies. Four hours after anti-HL administration to control (not ACTH-treated) rats, the adrenal lipase activity was inhibited by 68% (Table I). The adrenal HL activity remained inhibited for at least 4 h. After 8 h the adrenal HL activity was still 40% lower than in controls (non-immune) (2.21 ± 0.36 *versus* 3.68 ± 0.46 milliunits/organ). After 24 h, the adrenal lipase activity had increased to 2- to 3-fold over the basal activity. Four hours after the injection of anti-HL antibody into control (not ACTH-treated) animals the adrenal SR-BI mRNA content was greatly increased (5.2-fold) (Table I). SR-BI mass increased during the same period by 66%. Twenty-four h after the injection of anti-HL, when HL activity had increased above the basal activity, SR-BI mRNA was about 18% below control values (n.s.). SR-BI mass, however, remained increased at the level already reached 4 h after inhibition of HL activity.

In the following experiments we studied the consequences of the changes in HL activity and SR-BI expression for the adrenal uptake of HDL-cholesteryl ester. To this end, the rats were injected with HDL labeled with a non-degradable cholesteryl ester analogue, [^{3}H]cholesteryl ether (HDL-[^{3}H]CEth). The accumulation of radioactivity represents the uptake of HDL-[^{3}H]cholesteryl ether (HDL-[^{3}H]CEth) as an estimation of HDL-cholesteryl ester. In the first 4 h after administration of labeled human HDL, 72.0 ± 2.4% of the injected HDL-[^{3}H]CEth was cleared from the plasma compartment in control rats, compared with 66.0 ± 2.7% in antibody-treated rats (n.s). Most of this label is removed by the liver (not shown). The adrenals took up 0.58 ± 0.11% of the total injected dose during this time period (Fig. 1). In rats treated with anti-HL, the

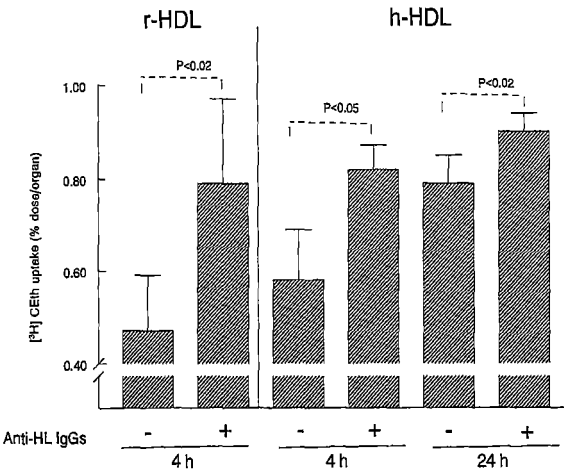

FIG. 1. **Effect of HL activity inhibition on HDL-[^{3}H]CEth uptake by adrenal glands of control rats.** The rats were injected with control IgGs or with a polyclonal anti-rat HL antibody preparation. Labeled rat (*r*) or human (*h*) HDL was intravenously injected 2 or 4 h later, respectively, as described under "Experimental Procedures." The animals were sacrificed 4 h or 24 h later. HDL-[^{3}H]CEth uptake by adrenal glands was expressed as percentage of the injected dose per organ. Values are mean ± S.D. (n = 4). The effect of anti-HL was tested using one-way analysis of variance with the Student-Newman-Keuls test.

adrenal uptake of HDL-[^{3}H]CEth was 41% higher than in the controls. This effect on adrenal HDL-[^{3}H]CEth uptake was also present when the rats were studied 24 h after HDL-[^{3}H]CEth injection, although it tended to be smaller (Fig. 1). Additional experiments were carried out using HDL isolated from rats in which HL activity had been functionally inactivated by anti-HL antibody for 1 h. In the first 4 h after the administration of rat HDL-[^{3}H]CEth about 50% (controls, 53.0 ± 5.2%; anti-HL-treated rats, 47.6 ± 3.0%, n.s.) of the label was cleared from the plasma compartment. During this period control adrenals took up 0.47 ± 0.12% of the injected dose per organ. In rats treated with anti-HL, the adrenal uptake was increased by 68% (Fig. 1) (0.47 ± 0.12, n = 4 *versus* 0.79 ± 0.18, n = 5, $p < 0.02$). From these experiments, we concluded that SR-BI rather than HL activity corresponds with the uptake of HDL-cholesteryl ester in the adrenal gland. On the other hand, SR-BI expression may be modulated by changes in HL activity.

Next we studied whether HL activity may affect HDL-cholesteryl ester uptake when the adrenal gland is stimulated by ACTH treatment and HL activity is greatly enhanced. Rats were treated with ACTH for 6 days, leading to about a 2-fold increase in HL activity (107 ± 13 *versus* 213 ± 50 milliunits/g wet weight). Because the adrenal weight increased during ACTH treatment (35 ± 5 *versus* 174 ± 61 mg/2 adrenals), the total lipase activity in the adrenals increased even more (9.2-fold) (Fig. 2). Under these conditions SR-BI expression is also greatly enhanced (Fig. 2). Total SR-BI mRNA in stimulated adrenals was 30-fold higher than in the control (2.9 ± 0.7 *versus* 86.8 ± 41.1 A.U./2 adrenals). SR-BI mass was less increased (4.9 ± 0.6 *versus* 19.5 ± 14.8 A.U./mg of protein) (Fig. 2), but total SR-BI mass in the adrenals was 8.1-fold higher than in the controls (7.7 ± 1.8 *versus* 63.2 ± 46.7 A.U./2 adrenals). Under these conditions, the stimulated adrenals took up 7.2% of the injected dose of (HDL-[^{3}H]CEth) in 4 h, which is about 12 times more than in the unstimulated adrenals (Fig. 2). Inhibition of HL activity under these conditions had no effect on SR-BI expression either in total mRNA (86.8 ± 41.1 *versus* 67.1 ± 9.9 A.U./2 adrenals) or in total SR-BI mass (63.2 ± 46.7 *versus* 60.9 ± 33.6 A.U./2 adrenals). In addition, inhibition of HL activity did not influence [^{3}H]CEth uptake (7.24 ± 1.58 *versus* 6.67 ± 1.40% of injected dose/2 adrenals) in ACTH-treated rats for 4 h.

	Control	ACTH
HL activity (mU/organ)	3.68±0.46	34.00±17.19
HDL-[^{3}H]CEth uptake (4 h) (% injected dose/ organ)	0.58±0.11	7.24±1.58

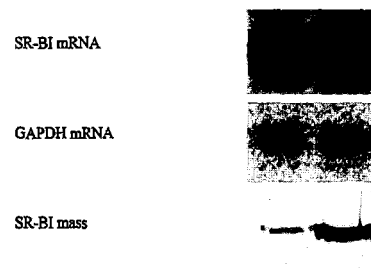

FIG. 2. **Effect of ACTH treatment on rat adrenal HDL-[^{3}H]CEth uptake and SR-BI expression.** Control and ACTH animals were treated with control IgG and sacrificed 4 h later. Both adrenals were removed subsequently and frozen. HL activity and SR-BI mRNA and mass were analyzed by enzymatic assay, Northern blot, and immunoblot, respectively, as described under "Experimental Procedures." The figure shows a representative experiment of Northern blot and immunoblot. HDL-[^{3}H]CEth was injected 4 h after the IgG and the animals were sacrificed 4 h later in order to measure the uptake of radioactivity in the adrenal glands. Values are mean ± S.D. (n = 4) except for HL activity (n = 7).

DISCUSSION

Several mechanisms have been proposed for the (selective) uptake of HDL-cholesteryl ester in the adrenal gland. Both L-type lipase, the adrenal form of HL, and SR-BI may play a role in adrenal cholesterol homeostasis. *In vitro*, several studies on the effect of HL on HDL-cholesterol (ester) uptake in cultured cells have been reported, but no *in vivo* data are available. HL and SR-BI expression may be coordinately regulated. Gene-targeted inactivation of HL in mice was found to be associated with increased expression of SR-BI. Despite the increase in SR-BI expression, adrenal cholesteryl ester stores were partly depleted (22). This suggested that the induction of SR-BI could not fully compensate for the loss of HL activity in cholesterol homeostasis. The effect of the increased SR-BI on HDL-cholesteryl ester uptake was not evaluated. In the pres-

ent study, we determined the effect of an acute inhibition of adrenal HL activity on SR-BI expression and on HDL-cholesteryl ether uptake *in vivo*. Administration of anti-HL to rats leads to a rapid inactivation of HL activity in adrenals and liver. The adrenal HL turnover is relatively slow. Once adrenal lipase activity is inhibited it remains lowered during at least 8 h, while the HL activity in the liver is restored to control values in 4 h. Twenty-four hours after the injection of antibody the adrenal lipase activity is increased above the control values. Acute inhibition of HL in the adrenal gland led to a greatly increased expression of SR-BI within 4 h, which was accompanied by a significant increase in HDL-[^{3}H]CEth uptake. Twenty-four hours after antibody administration, SR-BI expression had returned to control values. The actual rate of adrenal uptake of HDL-cholesteryl esters at this time point cannot be determined as the major part of HDL-[^{3}H]CEth uptake is by the liver (35) and takes place within the first 4 h after injection. Between 4 and 24 h after HDL-[^{3}H]CEth administration the increase in uptake of label in the adrenal gland was much smaller in the antibody-treated animals than in the controls. In this time period SR-BI mRNA decreased to control values in the antibody-treated animals. This may partly explain the lower rate of uptake of HDL-[^{3}H]CEth in the adrenals. We also used rat HDL isolated from animals in which HL activity had been functionally inactivated for 1 h. Therefore, this HDL had hardly been processed by HL *in vivo* prior to intravenous injection and is enriched in phospholipids and cholesterol (12). Uptake of [^{3}H]CEth from these "unprocessed" homologous rat HDL was similar to that from human HDL. Our data are compatible with a model in which adrenal HL activity is a determinant of SR-BI expression and SR-BI is the most important determinant of HDL-[^{3}H]CEth uptake. The latter is further supported by findings in ACTH pretreated rats. ACTH pretreatment led to a considerable increase in SR-BI expression, adrenal HL activity, and HDL-[^{3}H]CEth uptake. The increase in SR-BI mass was in line with the increased HDL-[^{3}H]CEth uptake. In stimulated rats the inhibition of HL did not affect either SR-BI expression or HDL-[^{3}H]CEth uptake. This clearly rules out adrenal HL activity as a major determinant of HDL-cholesteryl ester uptake under these conditions. The mechanism of the interaction between adrenal HL activity and SR-BI expression in the control rats can only be speculated about. HL is an enzyme with high phospholipase activity. Its preferred substrates are HDL-phospholipids. HL has been shown to be able to modulate HDL-unesterified cholesterol fluxes between HDL and cells and specifically to diminish the efflux of cholesterol from cells to HDL (36, 37). SR-BI expression is likely to be regulated by the cellular cholesterol content (22). Therefore, it could be that *in vivo* inhibition of HL leads to an increased efflux (or diminished influx) of non-esterified cholesterol in the adrenal gland which in turn gives rise to induction of SR-BI expression. Subsequently, SR-BI may stimulate HDL-cholesteryl ester uptake. In this model the primary role of HL would be in the modulation of fluxes of unesterified HDL-cholesterol and that of SR-BI in the mediation of HDL-cholesteryl ester uptake. Taken together, HL and SR-BI may be part of mechanisms ensuring an optimal cholesterol supply for steroid hormone synthesis under a variety of conditions.

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Chapter 3.3

Hepatic lipase colocalizes with SR-BI in rat adrenal glands

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ABSTRACT

Hepatic lipase (HL) and scavenger receptor class B type I (SR-BI) are both involved in high density lipoprotein (HDL)-cholesterol metabolism in liver and steroidogenic tissues. Both proteins participate in the reverse cholesterol transport and in cholesterol supply for steroid hormone synthesis. In several cell lines the lipoprotein-cholesterol flux across the cell membrane and the selective uptake of HDL-cholesteryl esters take place at the caveolae. In this study we investigated biochemically and cytochemically if hepatic lipase and SR-BI in rat adrenal glands are colocalized and whether this occurs at the membrane microdomain, caveolae. For this purpose we first isolated caveolae-enriched adrenal cell membranes in a sucrose gradient followed by immunoblotting analysis to detect HL, SR-BI and caveolin-1. HL protein and activity, SR-BI and caveolin-1 partly co-purified in rat adrenal cell membranes. By double immunofluorescence in confocal microscopy HL and SR-BI were found extracellularly localized mainly at the sinusoids of zona fasciculata of adrenal glands. By cytofluorometric analysis of the superimposed images, it appears that about 2/3 of HL and SR-BI colocalize. Based on the results described here we conclude that in rat adrenal glands the partial colocalization of HL and SR-BI is entirely found in the zona fasciculata. Moreover, only a small part of the localization does occur at the caveolae. Our results strongly indicate that a direct interaction between HL and SR-BI is possible. Probably this interaction is important for an efficient HDL-cholesterol uptake in adrenal glands to be used for hormone synthesis.

INTRODUCTION

Plasma high density lipoproteins (HDL) play a central role in cholesterol homeostasis. HDL takes up excess cholesterol from the peripheral tissues and transports it to cholesterol utilizing tissues, either for excretion from the body (liver) or as substrate for steroid hormone synthesis (adrenals and ovaries). The tissue uptake of HDL cholesterol esters may take place without concomitant internalization and degradation of the HDL particle [1, 2]. This process of selective HDL cholesterol uptake is highly active in adrenals and ovaries of rats and mice [3]. Recently, the scavenger receptor BI (SR-BI) was found to mediate the selective HDL cholesterol uptake in mice, rat and murine tissues [4, 5]. Rat and mouse adrenals and ovaries highly express SR-BI [6, 7], which is further enhanced if the demand for cholesterol is increased due to stimulation of

hormone synthesis [8-11]. In this way the tissues are supplied with sufficient cholesterol and can maintain a high rate of steroid hormone synthesis. Earlier, it has been suggested that hepatic lipase (HL) induces cholesterol delivery to steroidogenic tissues [12].

Like SR-BI, hepatic lipase is found in human [13] and in rat steroidogenic tissues, adrenal gland and ovaries. Here it was immuno-detected in zona fasciculata and corpora lutea, respectively [14-16]. HL activity in adrenal cortex and corpora lutea increases strongly after stimulation of hormone synthesis [17]. In female HL knockout mice the expression of SR-BI in adrenal glands was strongly enhanced probably due to a lowering of the cell cholesterol stores [18]. We showed that acute lowering of HL activity in rats also leads to a 5-fold increase in SR-BI expression. This increase was accompanied by a similar increase in HDL-cholesteryl ether uptake [19]. Recently, Lambert and collaborators [20] found that HL (not necessarily catalytic active) was required to obtain an optimal HDL-cholesterol uptake in kidney 293 cells overexpressing HL and/or SR-BI. It thus seems that both SR-BI and HL may mediate HDL-cholesterol uptake in these tissues.

In tissues containing caveolae, SR-BI was found to co-express with caveolin-1 [21-23]. Caveolin-1 binds cholesterol and is the most prominent protein of the caveolae. Caveolae are plasma membrane microdomains enriched in cholesterol and sphingomyelin and are specialized in the maintenance of the protein and lipids composition of the eukaryotic membranes and intracellular organelles [24]. It is also involved in the flux of the lipoprotein cholesterol through the cell membrane [25]. The co-expression of SR-BI with caveolin was associated with an increase in HDL-cholesteryl ether uptake [22].

Whether, how and to what extent SR-BI, HL and caveolin interact in HDL cholesterol uptake in vivo is not clear. Is there a direct interaction between SR-BI and HL, or do they serve as separate factors backing up for each other to insure an adequate supply of cholesterol for the highly important steroid hormone synthesis under all conditions? A direct interaction would require colocalization of SR-BI and HL; separate pathways may involve distinct localized processes. Therefore, we investigated the localization of SR-BI and HL in rat adrenal glands. We addressed two questions 1) to what extent are SR-BI and HL colocalized in rat adrenals and 2) is the colocalization at the caveolae? Our findings indicated that SR-BI and HL only partly colocalize in the zona fasciculata of rat adrenal glands. Both HL and SR-BI were co-expressed with caveolin-1. However this is restricted to a very small part of the total SR-BI and HL expression.

MATERIAL AND METHODS

Materials

Goat anti-rat HL antibodies were developed at our laboratory. Rabbit anti caveolin-1 IgGs were from Transduction Laboratories and rabbit anti-SR-BI/II from Abcam, UK. Donkey anti-sheep and swine anti-rabbit IgGs were from Dako and Chemicon International Inc., respectively. All antibodies used were polyclonal. For the immunoblotting, swine anti-goat- and goat anti-rabbit- alkaline phosphatase-conjugated IgGs were used, both from BioSource International.

Animals and tissue preparation

Male Wistar rats (200-300 g) were kept under standard conditions with free access to water and chow diet. For the detection of hepatic lipase by immunofluorescence, anaesthetized animals were injected (i.v.) with 0.1 ml of polyclonal goat anti-rat HL immunoglobulins (30 mg protein/ml). This amount of antibody was found to inhibit the total HL activity in the rat [19]. A control group was treated in the same way with non-immune immunoglobulins. Eight minutes after antibody injection, rats were perfused through the left ventricle with saline at room temperature (10 ml/min, 2 min.) to remove all blood components followed by fixation with paraformaldehyde 4% (w/v) in 0.1 M phosphate buffer pH 7.4 during 30 minutes. After the fixation the liver and adrenal glands were removed, cut in small pieces and stored in paraformaldehyde at 4°C until use.

Double immunofluorescence microscopy

Tissues stored in paraformaldehyde were washed with 0.1 M phosphate buffer pH 7.4 at 4°C followed by a overnight incubation with the same buffer containing 30% sucrose (w/v) at 4°C. Tissues were then frozen in tissue freezing medium (OCT) and 10 μm cryosections were obtained. When necessary, autofluorescence was quenched by incubating the coverslips with $NaBH_4$ prior to antibody incubation. Non-specific binding was reduced with 0.1 M phosphate buffer pH 7.4, 20 mM glycine containing 1% (w/v) bovine serum albumin (blocking solution) for 10 minutes at room temperature. To detect SR-BI a rabbit anti SR-BI/II polyclonal antibody was used and incubated during 2 hours at room temperature. As secondary antibodies, donkey anti-sheep tetramethyl-rhodamine conjugated (TRITC) IgG and swine anti-rabbit fluorescein-isothiocyanate conjugated (FITC) IgG were used to detect HL and SR-BI, respectively. Incubations were carried out for 1 h at room temperature. All antibodies

were diluted in blocking solution. Controls without primary antibody were processed in parallel. After washing twice (seven minutes each time) in phosphate buffer/glycine the coverslips were mounted with Mowiol. Colocalization studies were performed in presence of antibodies against the target proteins. Results were analyzed by confocal scanning laser microscopy (Leica TCS 4D, Leica Lasertechnik GmbH, Heidelberg, Germany). Acquisition of digital images at two fluorescence emission wavelengths, the confocal microscope was adapted to an inverted Leitz DMIRBE microscope and a 63X objective. Three dimensional projection images were calculated from six serial optical sections.

Isolation of caveolae-enriched membranes

Isolation of membranes was carried out as described by Sargiacomo [26] with a few modifications. Briefly, adrenal glands from six animals were pooled and homogenized in 0.025 M MES (morpholino sulfonic acid) pH 6.5 containing 1% Triton X-100, 0.15 M NaCl and 1 mM PMSF (30% w/v) using a teflon/glass homogenizer followed by passing several times through a 22 gauge needle at 4°C. The homogenate was adjusted to 40% sucrose by adding a sucrose solution of 80% (w/v) and loaded under 5% - 30% discontinuous sucrose gradient in 6 ml final volume and centrifuged at 300 000 x g for 19 h at 4°C. One-milliliter fractions were collected for further analysis.

SDS-PAGE and Immunoblotting analysis

Aliquots (15µg protein) of each sucrose gradient fraction were subjected to 4% / 12% SDS polyacrylamide gel electrophoresis under reducing conditions [27] and transferred to a nitrocellulose membrane (Schleicher and Schuell). Membranes were blocked with 3% gelatin in Tris buffered saline pH 7.5 overnight at 4°C. Blots were incubated for 2 h at room temperature with different antibodies, rabbit anti-SR-BI/II IgGs (1:1000), goat anti-HL IgGS (1:500) or rabbit anti-caveolin IgGs (1:1000), followed by incubation for 1 h with alkaline phosphatase-conjugated goat anti-rabbit (1:2000) or swine anti-goat (1:2000) as secondary antibodies. All incubations with antibodies were carried out in Tris-buffered saline containing gelatin (1% w/v) and Tween-20 (0.05% v/v). Between incubations the blots were washed with the same buffer but in absence of gelatin.

Enzymatic assay

Tissue homogenates and membrane fractions were assayed for hepatic lipase activity as described elsewhere [13]. Enzyme activity was expressed as milliunits (nmol of free fatty acids

released per min).

RESULTS

Immunobloting studies

We fractionated rat adrenal glands homogenates into six density fractions and analyzed them for the presence of SR-BI, hepatic lipase (activity) and caveolin (Fig.1). Immunoreactive hepatic lipase, SR-BI and caveolin-1 were detectable in several fractions. None of the proteins was confined to a single density fraction. HL protein (about 75% of total) appeared as a band of 58 kDa in the densest fractions between 30-40% sucrose (fractions 4-6). In these fractions hepatic lipase activity was also found (Fig.1).

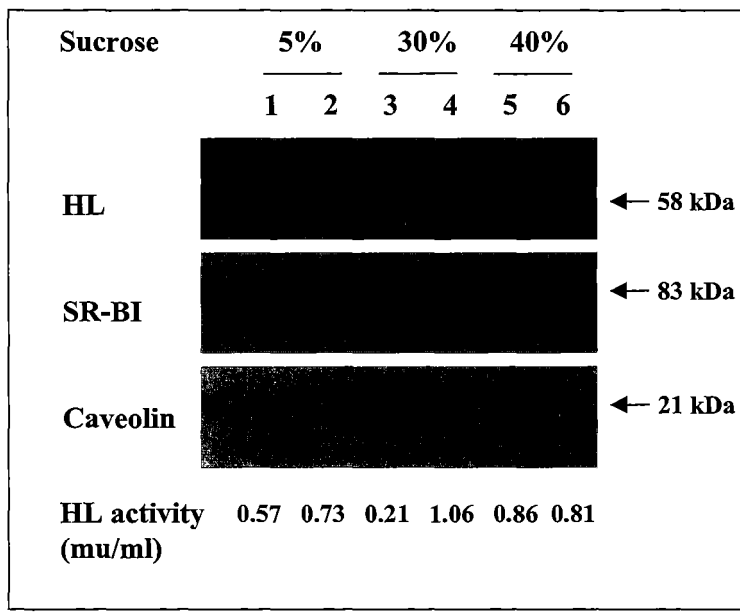

Figure 1. **Analysis of hepatic lipase, SR-BI and caveolin-1 expression in rat adrenal membranes by immunoblotting**

Tissue homogenate was subfractionated on a sucrose gradient (5% - 40%). Aliquots of each gradient fraction containing equal amounts of protein (15 µg) were separated in SDS-PAGE and transferred to nitrocellulose membrane. Blots were incubated with a variety of specific antibodies against the proteins of interest. Bound antibodies were detected by alkaline phosphatase conjugated secondary antibodies. Hepatic lipase activity was determined as described in Material and Methods and is given as mU/mg protein.

SR-BI (83 kDa) appeared to be present in most fractions, but predominantly in fractions 3

(30% of total) and 4 (40% of total). Caveolin was mainly found in fraction 3 (about 60% of total). In the gradient this fraction showed a white opaque band representing caveolin-rich membranes. From these patterns, it appeared that some fractions, mainly fraction 5, contain SR-BI as well as HL. Part of SR-BI was found in the caveolin-rich fraction 3. In this fraction virtually no hepatic lipase immunoreactivity and only low enzymatic activity was present. An appreciable amount of SR-BI was localized in a compartment (fraction 4) in which the amount of caveolin was very low. These data indicated that SR-BI partly coincided with HL and partly with caveolin-1. Little co-localization of HL protein with caveolin-1 existed.

Immunofluorescency studies

Localization of HL and SR-BI was further studied by immunofluorescence using confocal microscopy. Different structures of the adrenal cortex could be distinguished. Lipase was abundantly present in the sinusoids of the zona fasciculata (zf), while in the capsule (c) and zona glomerulosa (zg) no hepatic lipase was detectable (Fig 2 panel A). The enzyme was extracellularly detectable. SR-BI was also highly expressed in the zona fasciculata (Fig.2 panel B). The capsule and the zona glumerulosa were negative for SR-BI.

Double labelling was used to establish the extent of co-localization of HL and SR-BI in liver and adrenal glands (Fig. 3). The amount of SR-BI present in the liver was much lower than that of HL (Fig. 3 A-C) but the signal found was extracellular at the sinusoids of the liver. Since the amount of SR-BI in liver is low it is difficult to demonstrate colocalization of HL and SR-BI. Fig. 3 (D-I) shows in a detail of the zona fasciculata HL in red and SR-BI in green. Sites at which both signals are present appear in white. The degree of co-localization was further estimated by cytofluorometry. The insets show the cytofluorograms. Throughout the zona fasciculata HL and SR-BI colocalize. From the cytofluorograms it appeared that about 1/3 of SR-BI and HL did not co-localize. Parallel control experiments were performed in presence of non-immune IgGs and anti-SR-BI but in presence of both secondary antibodies. Under these conditions no signal was obtained (data not shown). Immunofluorescence studies showed little to none co-localization of HL with caveolin (not shown). Colocalization of SR-BI with caveolin was not studied. Colocalization of HL and SR-BI after ACTH treatment was also studied. Immunofluorescency showed similar results to that for control rats (not shown).

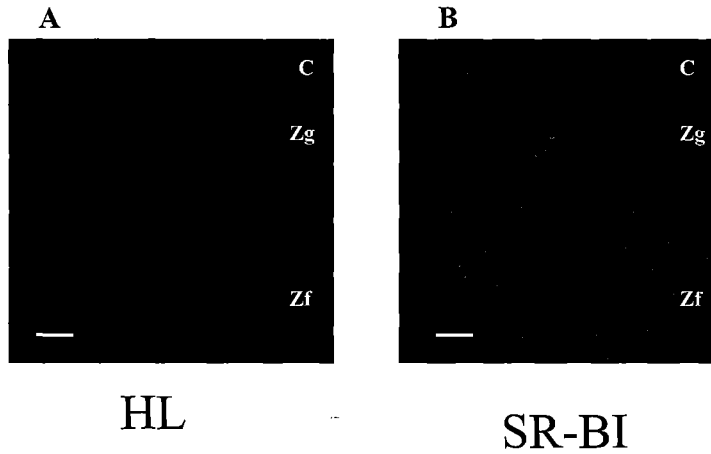

Figure 2. **Immunolocalization of hepatic lipase and SR-BI in rat adrenal glands by confocal microscopy**
Samples were prepared as described in Experimental Procedures. Cryosections of organs from animals injected with goat anti-rat HL IgGs were incubated with donkey TRITC- labelled anti-sheep IgGs (red fluorescence - panel A). For the detection of SR-BI, cryosections were incubated with rabbit anti-SR-BI antibodies followed by incubation with swine FITC-labelled anti-rabbit IgGs (green fluorescence - panel B). *c*, capsule; *zg*, zona glomerulosa; *zf*, zona fasciculata. Bar: 20 μm.

DISCUSSION

Hepatic lipase is suggested to participate in steroidogenesis by facilitating cholesterol uptake from HDL as substrate for hormone synthesis [28]. The lipase shares these properties with the scavenger receptor BI [8]. To what extent HL and SR-BI cooperate or act as additional systems for cholesterol supply is not clear. Neither is it clear by what mechanism SR-BI and HL may cooperate. To better understand this question we studied the colocalization of HL and SR-BI in rat adrenal glands. Lambert and coworkers [20] showed in 293 kidney cells transfected with SR-BI that interaction between HL and SR-BI leads to increased HDL cholesterol uptake. In line with this, we observed HDL binding to SR-BI in rat adrenal membranes to be enhanced by HL (see Chapter 3.1. of this thesis). On the other hand, we found that acute inhibition of HL activity did not impair HDL cholesterol ether uptake via SR-BI in intact rats [19]. However, is not known if the in vitro data can be extrapolated to the in situ situation. Tissue distribution of hepatic lipase and SR-BI largely match with each other. In adrenals both are extracellularly

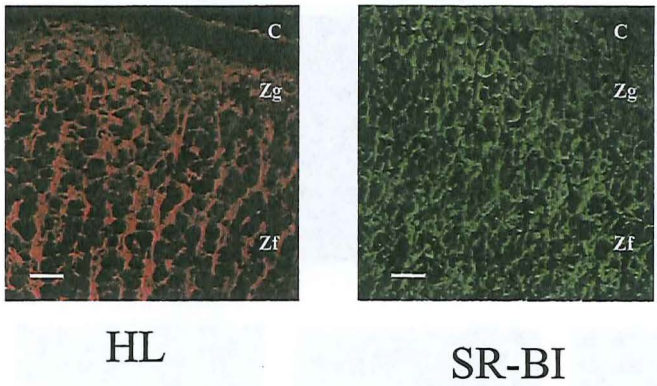

Figure 2. **Immunolocalization of hepatic lipase and SR-BI in rat adrenal glands by confocal microscopy** Samples were prepared as described in Experimental Procedures. Cryosections of organs from animals injected with goat anti-rat HL IgGs were incubated with donkey TRITC- labelled anti-sheep IgGs (red fluorescence - panel A). For the detection of SR-BI, cryosections were incubated with rabbit anti-SR-BI antibodies followed by incubation with swine FITC-labelled anti-rabbit IgGs (green fluorescence - panel B). *c*, capsule; *zg*, zona glomerulosa; *zf*, zona fasciculata. Bar: 20 μm.

localized at zona fasciculata (Fig.2). It is however not known whether direct interaction between HL and SR-BI is possible. Different membrane fractions of rat adrenals separated by density gradient centrifugation were found to contain HL as well SR-BI although the relative amounts varied greatly (Fig.1). About 75% of HL protein and 60% of SR-BI protein were present at the same membrane fractions. For the first time, immunofluorescence studies indicate that in the zona fasciculata about two thirds of HL co-localized with SR-BI while part of HL and SR-BI exist separable from each other (Fig. 3). These observations indicate that direct interaction between SR-BI and HL may exist. On the other hand, they do not exclude that SR-BI and HL also act independently from each other. A third component that may be involved in SR-BI mediated cholesterol uptake is caveolin. SR-BI has been suggested to localize in caveolae. In endothelial cells and macrophages [21] SR-BI was found to co-purify (although not entirely) with caveolin, the most prominent protein of the caveolae and a cholesterol chaperone. In those cell lines SR-BI together with caveolin might be involved in the first step of redistribution of cholesterol in the caveolar domains of the plasma membrane. However, the interaction of SR-BI with caveolin does not seem a prerequisite for its functionality. In monocyte-derived macrophages, SR-BI is upregulated but not caveolin [29]. In these cells SR-BI is still functionally active. Also experiments carried out in rat [30] and mouse[7] luteinizing granulosa cells suggested that SR-BI is not present at the caveolae even when expression of SR-BI and HDL-cholesterol uptake are increased. Recently, Matveev and collaborators showed that dependent on the cholesterol content/need of the cell, caveolin-1 can be a regulator of SR-BI-dependent selective uptake via SR-BI [31]. The fact that only part of SR-BI is present at caveolae suggests that besides a cooperation between the two proteins they also have individual functions. In a caveolin-independent process but with colocalization of HL and SR-BI as described here, both proteins would act to supply cholesterol for steroidogenic tissues, in a mechanism in which HL would modulate the binding/bridging of lipoprotein to SR-BI. Silver and Tall [32] suggested a model in which SR-BI together with HDL is internalized in liver cells and recycled, transporting cholesterol across the cell probably to be again excreted. It is possible that hepatic lipase is involved in these processes. Furthermore, such a mechanism would not require the simultaneous presence of HL, SR-BI and caveolin-1 in the same plasma membrane fraction. HL and SR-BI could function synergistically or in parallel. How precisely this occurs remains unclear. SR-BI was suggested to alter the plasma membrane cholesterol distribution with consequences for the intracellular lipid homeostasis [32]. On the other hand, HL may be of great importance for intracellular lipid homeostasis [12, 33] by facilitating the uptake of phospholipids and unesterified cholesterol.

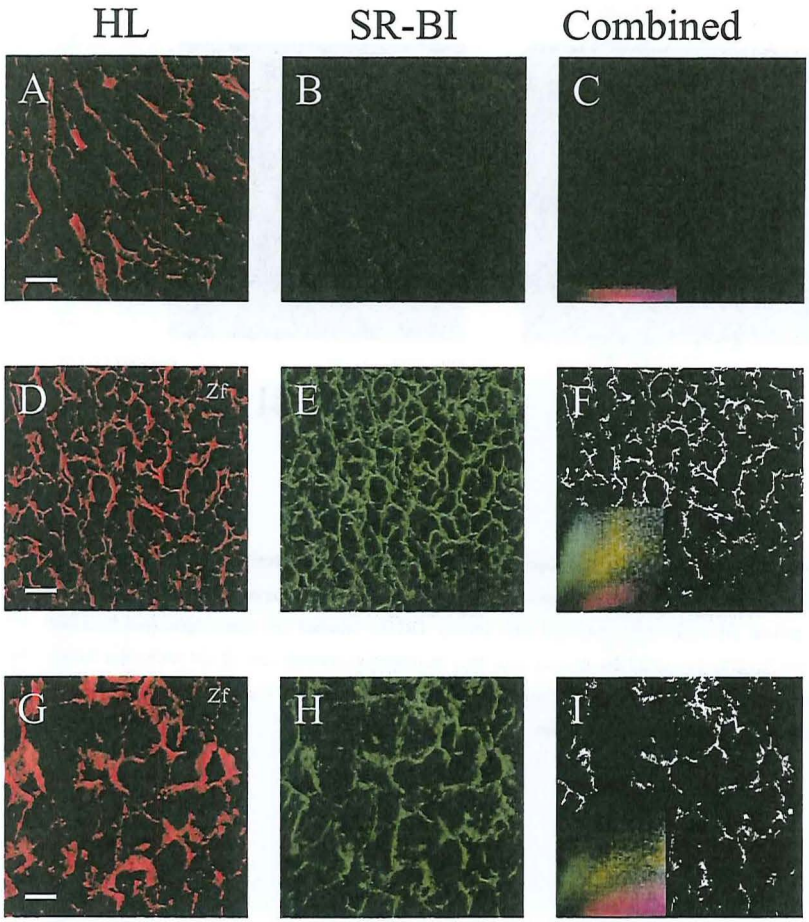

Figure 3. **Colocalization of hepatic lipase and SR-BI in rat liver and adrenal glands**

Samples were prepared as described in Experimental Procedures. Animals were in vivo injected with anti-HL antibodies and tissue cryosections were incubated with a rabbit anti-SR-BI antibody followed by simultaneous incubation with donkey anti-goat TRITC conjugated and swine anti-rabbit FITC conjugated antibodies. Panels A-C represent liver. Panels D-I represent adrenals and panels G-I represent a detail of adrenals. The left columns represent HL (red fluorescence), middle columns represent SR-BI (green fluorescence) and right columns represent the correspondent combined images (superimposed). Cytofluorograms (inset) were used in the image processing. Colocalization is seen in white. Bar: 20 μm (A-F) and 10 μm (G-I).

Based on these data one can speculate that HL could also be involved in the restoring of the cholesterol equilibrium in the membrane/cell as a result of the SR-BI action. Additionally, a recent study using PLTP/HL knock-out mice [34] showed that in liver SR-BI and HL are involved in the clearance of phospholipid/unesterified cholesterol-rich lipoproteins suggesting a cooperation between the two proteins. This suggestion is certainly not confined to liver but can also be extrapolated to steroidogenic tissues.

In conclusion, we show for the first time that SR-BI and HL colocalize in rat zona fasciculata of adrenal cortex and that colocalization at caveolae is only partial. How and to what extent both proteins interact and play a role in the cholesterol homeostasis together remains unclear. We suggest that HL and SR-BI cooperate in cholesterol uptake of these organs.

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Chapter 3.4

Opposite regulation of Hepatic Lipase and Scavenger Receptor BI by ACTH in rat liver and adrenal

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ABSTRACT

Scavenger receptor BI (SR-BI) and hepatic lipase (HL) are both involved in the uptake of high density lipoprotein (HDL) cholesteryl ester and expressed in the stereoidogenic organs and liver. In the liver HL is downregulated by corticosteroids and estrogens, while in the adrenal gland and ovary HL activity is increased by respectively ACTH and estrogens. In the case of HL, there is an opposite regulation in liver, ovary and adrenal gland. SR-BI on the other hand, is highly expressed in the adrenal gland and the regulation of SR-BI in adrenal gland is well known. In adrenals, ACTH treatment and depletion of cellular cholesterol increase expression of SR-BI. In contrast, the regulation of SR-BI in liver has not been well studied yet. We wondered whether there is an opposite regulation of SR-BI in the liver and the adrenal gland. Therefore rats were treated with Synacthen, a synthetic ACTH analogue during 1, 4 or 6 days (ACTH rats) and SR-BI mRNA expression and SR-BI protein expression was measured by respectively an competitive RT-PCR and by Western blotting. SR-BI mRNA expression in the liver was found to be very low (0.70 ± 0.08 attomol/µg total liver RNA) in comparison with the adrenal gland (55 ± 15 attomol/µg total adrenal RNA). In liver SR-BI mRNA expression was significantly lowered after 6 days of treatment (Ct 0.70 ± 0.08; 1d ACTH 0.62 ± 0.07; 4d ACTH 0.51 ± 0.10 and 6d ACTH 0.27 ± 0.13 attomol/µg total liver RNA). In adrenal gland SR-BI mRNA expression was significantly increased after 4 days of treatment (Control 55 ± 15; 1d ACTH 45 ± 20; 4d ACTH 195 ± 30 and 6d ACTH 450 ± 75 attomol/µg total adrenal RNA). Besides the SR-BI mRNA expression, also the amount of SR-BI protein in the liver was lowered after Synacthen treatment when compared with control rats (Control 100 ± 5%; 1d ACTH 58 ± 20 %; 4d ACTH 51 ± 9% and 6d ACTH 23 ± 15%). The effect of Synacthen on liver SR-BI expression was found to be mimicked by 6 days of Cortisol treatment (SR-BI mRNA Cortisol treated rats 0.30 ± 15 attomol/µg total liver RNA, SR-BI protein 42 ± 18 % relative to control rat liver). The down regulation of SR-BI mRNA in the liver was paralleled by an increase in the cholesterol/phospholipid ratio in liver microsomes indicative for a regulation of SR-BI by the cellular cholesterol levels. Despite a lowering of hepatic SR-BI and HL, liver uptake of HDL cholesteryl ester was not lowered by Synacthen treatment. (Control rats 39.3 ± 5.6 % vs 6d ACTH rats 51.0 ± 10.5 % percentage of injected dose). In order to find an explanation for this unexpected finding we studied the expression of HB_2, a putative HDL receptor by western blotting. In contrast to the decrease in SR-BI expression there was an increase in HB_2 expression upon Synacthen treatment during 6 days. In conclusion, there is an opposite regulation of HL and SR-BI in liver and adrenal. This opposite regulation does not lead to a lowered uptake of HDL cholesterylester in the liver.

INTRODUCTION

Many studies have shown that plasma high density lipoprotein (HDL) cholesterol levels are inversely related to coronary heart disease [1-3]. Elimination of excess cholesterol from the peripheral tissues back to the liver occurs via HDL and has been called reverse cholesterol transport [4]. The reverse cholesterol transport starts with the uptake of unesterified cholesterol from the peripheral cell membranes by HDL. Part of this cholesterol is esterified by lecithin:cholesterol acyltransferase (LCAT) to HDL cholesteryl esters. The HDL cholesteryl esters may be returned to the liver by several pathways including the uptake of the whole HDL particle and the selective uptake of HDL cholesteryl ester [5-7]. The steroidogenic tissues also use HDL cholesteryl esters for the synthesis of steroid hormones or suppletion of the cholesterol stores. Hepatic lipase (HL) and SR-BI have been mentioned to play an important role in the selective uptake of HDL cholesteryl esters [6,8-11]. Targeted mutation of SR-BI led to a reduction in the selective uptake of HDL cholesteryl ester by the liver [12,13]. Besides in mice with a targeted mutation in SR-BI, HDL cholesteryl uptake by the liver is reduced in the HL knock-out mice [14]. In the adrenal gland of HL knock out mice the cholesterol stores are depleted although SR-BI was upregulated indicating that HL is necessary for the SR-BI mediated cholesteryl ester uptake[15]. Recently it has been shown that HL indeed enhances the selective uptake of HDL cholesteryl ester via SR-BI in cultured cells [16]. Both HL and SR-BI are expressed in the steroidogenic tissues and in the liver. Much is known about the regulation of SR-BI in the steroidogenic tissues where it is highly expressed. In these tissues trophic hormones and steroid hormones stimulate and down regulate the expression of SR-BI, respectively [10,17-19]. SR-BI expression is also under regulation of cellular cholesterol pools. SR-BI promotor studies revealed two sterol response elements (SRE) through which sterol responsive element binding protein (SREBP) can bind and activate the SR-BI gene [20]. Besides SREBP the steroidogenic factor (SF) has also a binding sequence in the promotor of SR-BI [21]. It has been shown that the enhanced expression of SR-BI after ACTH treatment may be mediated by SF and SREBP [20]. Besides upregulation of SR-BI by ACTH there is an ACTH-induced upregulation of HL in the adrenal. This is paralleled by a down regulation of HL in the liver. Not much is known about the regulation of SR-BI in the liver. It has been shown that hepatic SR-BI is down regulated by estrogens and cholesterol feeding and upregulated by feeding rats a diet rich in polyunsaturated fatty acids [10,19,22,23]. Also, ACTH was found to slightly decrease hepatic LDL receptor and SR-BI in rats; and it is accompanied by an increase in plasma cholesterol. The uptake of cholesteryl ester from HDL by the liver is fully saturated at normal physiological plasma HDL cholesteryl ester levels [24-25]. Therefore regulation of the uptake of cholesteryl esters by the liver should have

an direct effect upon the reverse cholesterol transport. In the past we proposed that cholesteryl ester uptake in the liver may be diminished after ACTH treatment based upon the downregulation of HL in the liver and upregulation of HL in the adrenal gland [26]. Considering the cooperation between HL and SR-BI we studied whether there is also such an opposite regulation of SR-BI in the liver and adrenal. Indeed a downregulation of SR-BI in the liver was found after treatment of rats with ACTH which was parallelled by an upregulation of the microsomal cholesterol/phospholipid ratio. SREBP levels may mediate the regulation of SR-BI described here.

MATERIAL AND METHODS

Animals

Male Wistar rats (200 - 300 g) were kept under controlled conditions of humidity, light and temperature. They had free access to tap water and chow diet. The animals were fasted before use. ACTH treatment consisted of daily subcutaneous administration of 0.2 mg of Synacthen (s synthetic ACTH analogue, Ciba) per kg body weight for 1, 4 or 6 days (ACTH rats). Cortisol treatment consisted of 5 mg/kg body weight. Control rats were also fasted overnight before use and injected with saline for the same period.

Analysis of SR-BI by competitive RT-PCR

For the quantitation of rat liver SR-BI a competitive RT-PCR strategy was established. An SR-BI cDNA construct (nt 812 - 1743) was prepared from liver RNA by RT-PCR. This cDNA probe was subcloned into pBluescript after partial digestion with Apa I and XhoI resulting in a construct of 895 bp(nt 841-1736). A recombinant plasmid was selected that contained the insert in the sense orientation with respect to the T7 promotor. After multiplication of the plasmid an insert of 150 bp in the construct was deleted by digestion of the plasmid with PStI. After ligation and subsequent multiplication of the plasmid the cDNA insert was sequenced with an automated DNA sequencer (Perkin-Elmer). After linearization of the plasmid, competitor RNA was synthesized from the DNA template in vitro using T7-RNA polymerase at 40 °C for 2 h in the presence of 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 5 mM NaCl, 100 µg/ml BSA, 0.5 mM of each ribonucleotide and 4000 U/ml of RNAsin. After the in vitro transcription, the DNA was digested with 400 U/ml of RNAse-free DNAse I (Boehringer Mannheim, Germany) for 30 min at 37 °C and RNA was isolated by phenol-chloroform extraction followed by subsequent ethanol precipitation. After resuspension in TE buffer, RNA

concentration was determined by spectrophotometry at 260 nm. Quantification of SR-BI mRNA was done by mixing 0.5 µg of tissue RNA with 0.5 µg of yeast RNA containing increasing amounts of competitor RNA. Semi quantitation was done by comparing the relative intensities of the ethidium bromide-stained bands using an alpha imager and the software of the GS363 Molecular Imager System from Bio-Rad. Total tissue RNA was isolated from individual adrenals or 100 - 200 mg of liver exactly as described before [27]. The quality of the RNA preparations was judged from the ratio of 28S over 18S ribosomal RNA after denaturation and electrophoresis in a 1 % agarose/ TBE gel.

Immunoblot analysis

Membrane fractions were prepared from pulverized rat liver that had been frozen in liquid N_2 immediately after removal from the rat. Liver membranes were isolated from rat liver homogenate as described elsewhere [28]. Membrane protein (50 µg for SR-BI and 20 µg for HB_2) was separated by 7.5 % SDS-polyacrylamide gel electrophoresis under reducing conditions [29]. Proteins were transferred to a nitrocellulose-membrane and the membrane was incubated with a commercially available rabbit antibody against SR-BI or a rabbit antiserum raised against HB_2, which was kindly donated by Dr. Fidge. After washing, the membrane was incubated with horseradish peroxydase conjugated anti-rabbit immunoglobulin (1:15000). Antibody binding was detected by enhanced chemoluminescence and quantitated by GS363 Molecular Imager System from Bio-Rad.

Isolation and labeling of HDL

Human HDL (d = 1.063 to 1.21) was isolated from blood of healthy volunteers by sequential ultracentrifugation as described before [30]. HDL was passed over a Sepharose-heparin column to remove apo E containing lipoproteins [31]. HDL was labelled with a non-degradable cholesteryl ester analogue, [1α,2α-^{3}H]cholesteryl oleoyl ether (Amersham Pharmacia Biotech) using the exchange method from donor particles exactly as described before [32] with human lipoprotein deficient serum as a source of cholesteryl ester transfer protein. After labeling the HDL fraction was reisolated by gradient ultracentrifugation, dialyzed against 0.15 M NaCl and filtered through a 0.45 µm Millipore filter before use.

HDL-[^{3}H]Cholesterylether uptake in vivo

To study HDL[^{3}H]Cholesterylether uptake rats were anesthetized and 0.2 ml of the labelled HDL, corresponding to 124 nmol of total cholesterol and $1x10^6$ dpm was injected intravenously.

Animals were sacrificed 4 or 24 h after the injection of the labelled lipoprotein. Tissue samples were dissolved in Soluene-350 (Packard Instrument) for 4h at 55°C and analyzed for radioactivity. The radioactivity in the tissues was corrected for the contamination of plasma radioactivity assumed to be present at the time in the tissues.

Hepatic lipase assays

Hepatic triacylglycerol hydrolase activity was measured in adrenal or liver tissue homogenized in ice-cold phosphate buffered saline (pH 7.4) containing 10 IU/ml heparin and 1 mM benzamidine. After centrifugation (10,000 x g, 2 min, 4°C) the lipase activity was measured in the post-nuclear fraction using a gum acacia-stabilized glycerol-[^{14}C]trioleaat emulsion, pH 8.5, as substrate as described elsewhere [33]. Enzyme activities were expressed as mUnits (nmol of free fatty acids released per min).

Other assays and reagents

Membrane cholesterol content was measured using chloroform-methanol extraction of the lipids and subsequent measurement of cholesterol by a cholesterol kit (Boehringer testkit combination). Phospholipids were measured by the method of Stewart [47]. Protein was determined using the standard method of Lowry with BSA as standard [34].Statistical significances were determined by one-way-ANOVA.

RESULTS

Rats were treated with Synacthen a synthetic ACTH analogue during 1-6 days. This treatment largely affected the body, liver and adrenal weight of the animals (Table I). Already after one day of treatment the liver weight was significantly increased, while the increase in the adrenal weight did not reach statistically significance. After longer treatment adrenal weight enlarged until 3 of 4 times the initial weight. Body weight was only significantly decreased after 4 en 6 days of treatment by about 10 - 15 %. There was no change in plasma total cholesterol or plasma HDL cholesterol levels upon the treatment. HL activity in the liver on the other hand was already lowered after 1 day by about 30 % (544±40 mU/gww in control versus 402±34 mU/gww after 1 day) up to 50 % after 4 - 6 days of ACTH treatment (239±47 mU/gww and 328±60 mU/gww after 4 and 6 days, respectively). However, when HL activity is expressed per liver the decrease observed is less accentuated (Table II). Opposite to the lowering in the liver, HL activity in the

adrenal gland was enhanced 3 - 4 times after 4 - 6 days of treatment (Table II). The increase of HL activity observed per pair of adrenals is mainly due to the increase in adrenal weight.

Table I. In vivo effect of ACTH on the characteristics of the rats

ACTH (days)	0	1	4	6
Animal wt (g)	307 ± 10	306 ± 7	279 ± 4*	267 ± 5.5*
Liver wt (g)	11.5 ± 1.1	15.0 ± 1.0*	14.9 ± 0.8*	14.7 ± 1.2*
Adrenal wt (mg/pair)	50.8 ± 3.4	61.3 ± 6.6	176 ± 12*	208 ± 68*
Liver wt (% body)	3.75 ± 0.24	4.91 ± 0.27*	5.34 ± 0.27*	5.49 ± 0.30*
Plasma cholesterol (mM)	1.80 ± 0.20	2.00 ± 0.22	1.90 ± 0.33	1.60 ± 0.20
HDL cholesterol (mM)	1.19 ± 0.17	1.33 ± 0.16	1.40 ± 0.22	0.98 ± 0.17

Values represent the mean ± S.D. of 3 - 6 rats* Significantly different from control, $p<0.05$.

Table II. Effect of ACTH treatment on HL activity in rat liver and adrenal

HL activity was measured in homogenates of livers and adrenals of rats treated during 0, 1, 4 or 6 days with Synacthen (0.2 mg/kg body weight) as described in the Material and Methods.

	HL activity	
Treatment	Liver	Adrenal
(days)	(U/liver)	(mU/2 adrenals)
0	6.26±0.46	4.75±0.7
1	6.03±0.51	5.75±1.15
4	3.56±0.70	16.5±3.2
6	4.82±0.88	19.5±3.9

Values represent the mean ± S.D. of 3 - 6 rats.

Effect of ACTH on SR-BI expression in rat liver and adrenal

Competitive RT-PCR

The effect of ACTH treatment on SR-BI expression was studied. Since the expression of SR-BI in the liver could hardly be measured by northern blotting, we developed a competitive RT-PCR for SR-BI. The expression of SR-BI mRNA in control liver was found to be between 0.5 and 1 attomol/μg total liver RNA (Fig.1). We measured the relative signals for RNA and competitor and calculated a RNA/competitor curve. On basis of this curve the amount of liver SR-BI mRNA was found to be 0.70 ± 0.08 attomol/μg total liver RNA. This is much lower than the amount of SR-BI in the adrenal gland, which contained 54 ± 15 attomol SR-BI mRNA/μg total RNA. When expressed on a total liver basis the total amount of SR-BI mRNA is about three times the amount found in both adrenals (20.4 femtomol SR-BI mRNA/liver compared to 7.5 ± 2.5 femtomol/ 2 adrenals (Table III).

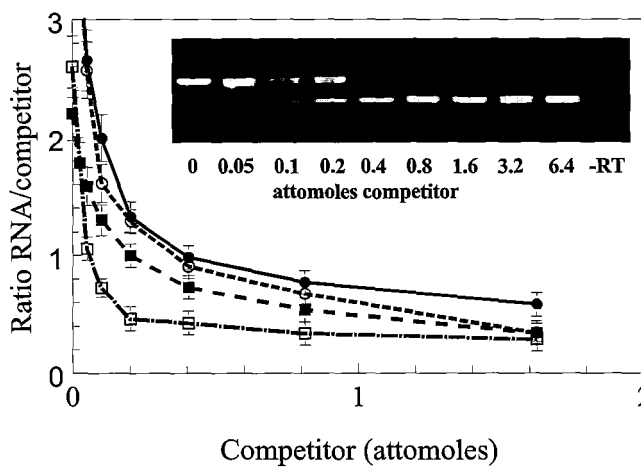

Figure 1. **Quantitative measurement of SR-BI mRNA in livers of control and Synacthen treated rats after 1, 4 or 6 days of treatment.** Competitive RT-PCR was performed on RNA isolated from control rat liver. To 0.5 μg of tissue RNA different amounts of the SR-BI competitor were added as indicated. Amplification was for 35 cycles. SR-BI gave a band of 555 bp and SR-BI competitor a band of 393 bp. Agarose gel of RT-PCR products of competitive RT-PCR (inset). The graph represents the ratio of SR-BI product/SR-BI competitor vs different amounts of SR-BI competitor in control and ACTH rats as measured by comparing the relative intensities of the ethidium bromide-stained bands. Control (•), 1d ACTH (o), 4d ACTH (■) and 6d ACTH (□). Data represent mean ± S.D. of 3 - 6 rats.

To investigate the effect of ACTH on the SR-BI mRNA expression in the liver, RNA was isolated from rats that had been treated with Synacthen during 1, 4 or 6 days. It can be seen from the results of the competitive RT-PCR that SR-BI mRNA was significantly lowered after 6 days of treatment by more than 50 % to 0.26 ± 0.11 attomoles/µg total liver RNA (Fig 1, Table III). The lowering was not significant after 1 and 4 days of treatment. Opposite to the down regulation of SR-BI in the liver there was an almost tenfold increase in the adrenal expression of SR-BI from 54 ± 15 attomoles up to 460 ± 70 attomoles SR-BI mRNA/µg total adrenal RNA after 6 days of treatment (Fig.2, Table III). When expressed per pair of adrenals and on a total liver base it can be seen that after ACTH treatment (4 - 6 d) the expression in the adrenals is even 3 - 17 times higher than in total liver. (Table III).

Table III. Effect of ACTH treatment on SR-BI mRNA in rat liver and adrenal

	SR-BI mRNA expression			
Treatment (days)	Liver		Adrenal	
	attomoles/ µg RNA	femtomoles/ total organ	attomoles/ µg RNA	femtomoles/ 2 adrenals
0d Control	0.70 ± 0.08	20.4 ± 6.5	54 ± 15	7.5 ± 2.5
1d ACTH	0.62 ± 0.10	26.5 ± 7.9	35 ± 12	6.3 ± 2.8
4d ACTH	0.51 ± 0.09	21.4 ± 7.2	200 ± 32*	50 ± 15*
6d ACTH	0.26 ± 0.11*	8.7 ± 3.2*	460 ± 70*	148 ± 24*

Values represent the mean ± S.D. of 3 - 6 rats.

*Significantly different from control $p<0.05$.

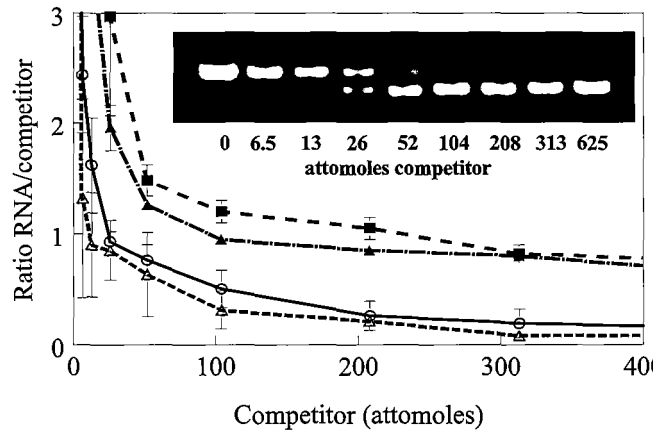

Figure 2. **Quantitative measurement of SR-BI mRNA in adrenals of control and Synacthen treated rats (ACTH rats) after 1, 4 or 6 days of treatment.** Competitive RT-PCR (inset) was performed as described in the legend to Fig.1. The graph represents the ratio of SR-BI product/SR-BI competitor vs different amounts of SR-BI competitor in control and ACTH rats as measured by comparing the relative intensities of the ethidium bromide-stained bands using an alphaimager and the software of the GS363 Molecular Imager System from Biorad. Control (o), 1d ACTH (Δ), 4d ACTH (▲) and 6d ACTH (■). Data represent mean values ± S.D. of 3 - 6 rats.

Westernblot analysis

To investigate whether the lowered mRNA expression of SR-BI in the liver also led to a lowered protein expression of SR-BI, liver membranes were isolated from control rats or from rats treated with Synacthen. Fifty μg membrane protein were loaded on the gels. After western blotting of the gels SR-BI protein was detected with respectively an antiserum against SR-BI and a second hrp-conjugated anti-rabbit immunoglobulin followed by enhanced chemoluminescence detection. It can be seen that SR-BI protein expression was unambiguously lowered after ACTH treatment (Fig 3). Quantification of the changes in intensity showed that in contrast to SR-BI mRNA expression the protein expression was already significantly lowered by about 40 % after 1 day of ACTH treatment and by 75 % after 6 days of treatment.

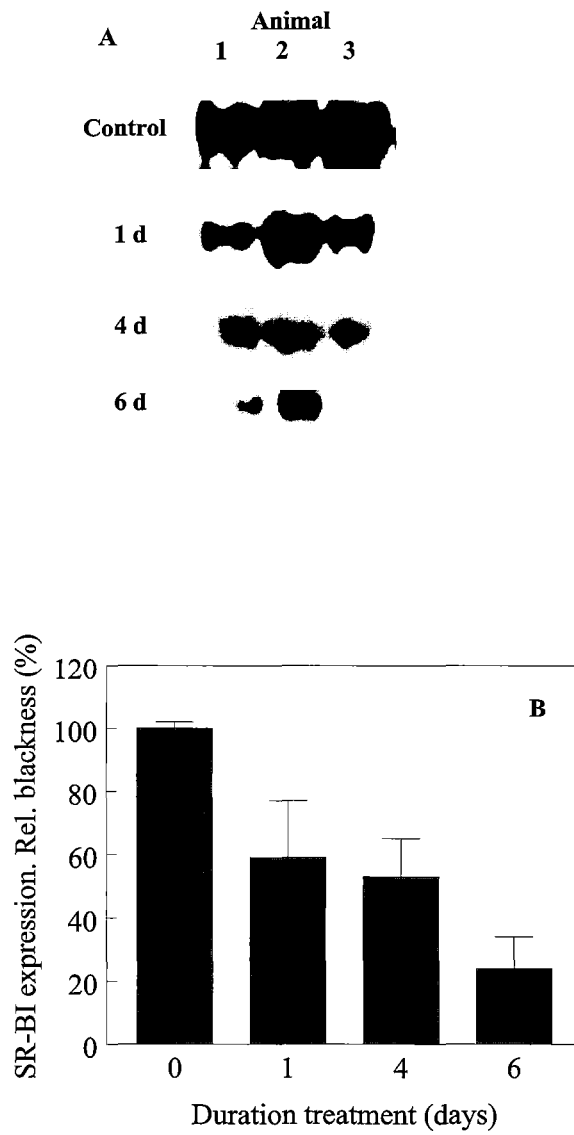

Figure 3. **SR-BI protein expression in livers of control and ACTH treated rats during 1, 4 or 6 days.** Liver membranes were isolated, subjected to SDS PAGE and blotted to nitrocellulose membranes. After incubation of the western blot with respectively a rabbit anti-body to SR-BI and a hrp-conjugated anti-rabbit antibody, SR-BI was visualized by enhanced chemiluminescence (ECL) detection (A). SR-BI bands on the blot were quantitated and compared with the relative blackness obtained in control liver membranes. (B). Data represent mean±S.D. (n=3).

Effect of Cortisol on SR-BI expression in rat liver

ACTH lowered hepatic lipase activity in the liver because of the increase in corticosteroids. To investigate whether corticosteroids were also responsible for the lowering of SR-BI expression in the rat liver, rats were treated with cortisol during 6 days. Under these conditions HL activity decreased about 34 ± 8 % (results not shown).

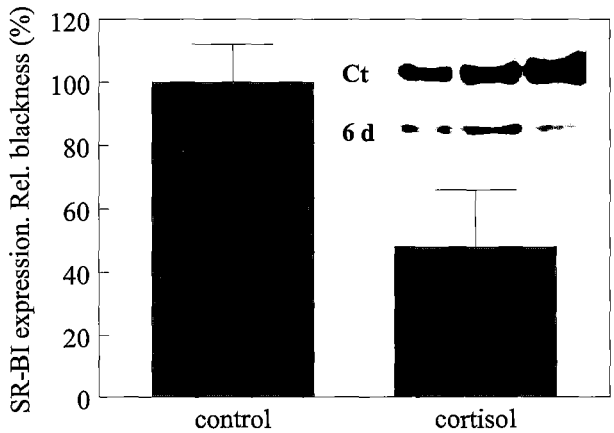

Figure 4. **Effect of cortisol treatment on SR-BI protein expression in rat livers.** Liver membranes were isolated, subjected to SDS-PAGE. Immunoblotting (inset) was performed as described in Materials and Methods. SR-BI bands obtained from membranes of cortisol treated animals were quantitated and compared with the relative blackness obtained in control liver membranes. Data represent mean±S.D. (n=3).

Fig 4 shows the SR-BI protein expression in liver. From Table IV it can be seen that cortisol led to a decrease of SR-BI mRNA expression of more than 50 % (0.71 ± 0.11 to 0.33 ± 0.10 attomoles/μg total liver RNA in control and cortisol treated rats, respectively). Similarly, the SR-BI protein expression was also decreased from 100±17 to 48 ± 18 % after 6d of treatment.

Table IV. Effect of Cortisol treatment on SR-BI mRNA and SR-BI protein in rat liver

	SR-BI expression		
	mRNA		protein
	attomoles/ μg RNA	femtomoles/ total organ	relative expression (%)
Control	0.71 ± 0.11	22.0 ± 3.5	100 ± 17
cortisol (6d)	0.33 ± 0.10*	6.75 ± 2.2*	48 ± 18*

Values are given as the mean ± S.D. of 4 rats.

* Significantly different from control $p<0.05$.

Effect of ACTH and Cortisol on liver cholesterol/phospholipid ratio

Because SR-BI expression is sensitive to intracellular cholesterol levels, at least in adrenals, we studied whether the observed decrease of SR-BI coincides with a change in the intracellular cholesterol levels in the liver. Liver microsomes were isolated from control, ACTH en cortisol treated rats and cholesterol was measured. To correct for the amount of microsomes isolated, we expressed the cholesterol values as cholesterol/phospholipid ratio. Fig 5 shows that the cholesterol/phospholipid ratio was significantly increased after 6 days of ACTH treatment, from 0.118 ±0.004 (control) to 0.160 ± 0.019 (ACTH). After cortisol treatment the cholesterol/phospholipid ratio was significantly increased from 0.108 ± 0.0045 to 0.178 ± 0.025 in control and treated rats, respectively (data not shown).

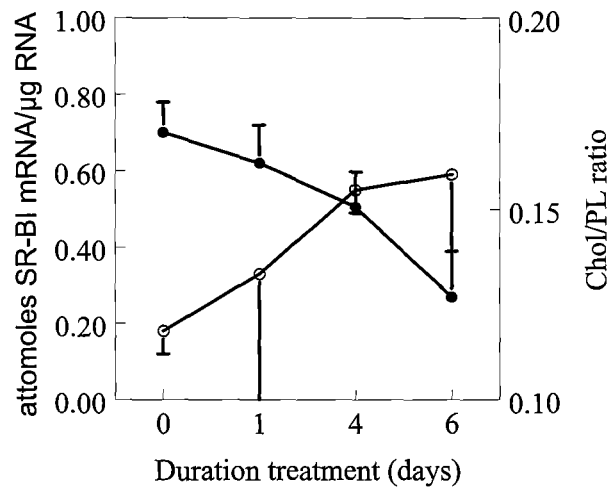

Figure 5. **Effect of ACTH on SR-BI mRNA and Cholesterol/Phospholipid ratio in liver microsomes.** Cholesterol (Chol) and Phospholipid (PL) were measured in liver microsomes isolated from control and Synacthen treated rats as described in Materials and Methods. SR-BI mRNA (•) and Chol/PL ratio (o). Values are means ± S.D. of 3 - 6 rats.

Effect of ACTH on liver and adrenal HDL cholesteryl ester uptake

In order to estimate whether the lowering of SR-BI and HL by ACTH resulted in a reduced uptake of HDL cholesterylether in the liver, [^{3}H]-labelled HDL-cholesterylether was intravenously injected in control rats and in another group of rats that have been treated with ACTH during 6 days. HDL-[^{3}H]cholesterylether was used to prevent intracellular hydrolysis. 4 or 24 hr after injection of HDL cholesterylester, rats were killed and the amount of cholesterylester taken up by the liver was determined.

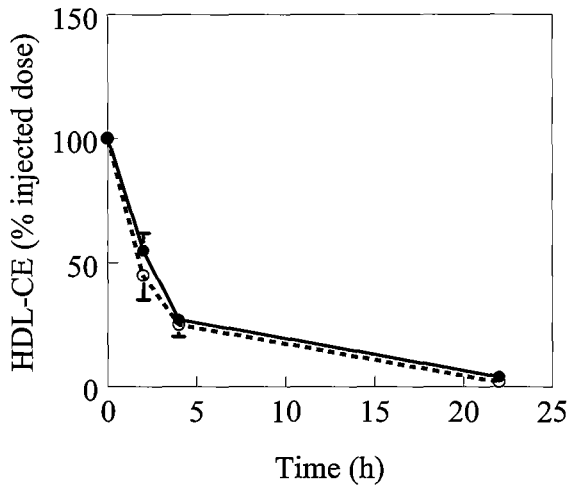

Figure 6. **Effect of ACTH on serum decay of HDL-[^{3}H]Cholesterylether in the rat.** 0.2 ml labelled HDL, corresponding to 124 nmol total cholesterol and 1x10^6 dpm, were injected intravenously. Blood was withdrawn at the indicated time points and the amount of label present in the serum was measured. Control (•) and ACTH (o). Data are expressed as percentage of the injected dose ± S.D. of 6 rats.

From the plasma decay of HDL [^{3}H]-cholesterylether (Fig 6) it can be seen that HDL cholesterylether is not removed at a slower rate because of ACTH treatment. Also the liver HDL cholesterylether uptake was not significantly lowered by ACTH treatment (Table V).

In fact, HDL-cholesterylether.uptake increased with ACTH treatment. The percentage of the HDL cholesterylether that have been taken up by the liver after 4 h was significantly enhanced by ACTH treatment, by about 30%. This is obviously much less than the increase in the adrenal uptake that was enhanced more than ten times. Because HDL cholesterylester levels were lowered in ACTH rats, we corrected the cholesterylether uptake by the altered specific activity of HDL cholesterylether by dividing the radioactivity that has been taken up by the specific activity of the cholesterylether. It can be seen from Table IV that the HDL cholesterylether uptake is then no longer increased in the liver upon the treatment, while in the adrenal gland the increase was still obvious. The HDL cholesterylether uptake/gram tissue, however, was only slightly increased upon the ACTH treatment probably because of the large increase in the adrenal weight.

Table V. Effect of ACTH treatment on HDL-[^{3}H]cholesteryl ether uptake in rat liver and adrenal

	HDL-CE uptake		
	Liver		
	% injected dose /liver	µmol/ g.w.w	µmol/ total liver
Control	39.3 ± 5.6	0.62 ± 0.08	7.17 ± 0.77
ACTH (6d)	51.0 ± 10.5*	0.63 ± 0.08	7.57 ± 0.87
	Adrenal		
	%injected dose/organ	µmol/ g.w.w.	nmol/ 2 adrenals
Control	0.58 ± 0.11	0.139 ± 0.008	6.21 ± 0.18
ACTH (6d)	7.24 ± 1.58*	0.404 ± 0.007*	75.8 ± 1.16*

Values represent the mean ± S.D. of 3 - 6 rats.

* Significantly different from control value, $p<0.05$.

Effect of ACTH on the HB_2 protein expression in rat liver

Besides specific uptake by SR-BI, HDL cholesteryl esters can be taken up by means of other HDL receptors such as HB_2. To investigate whether HB_2 expression was affected by ACTH treatment, liver membranes were isolated from control and from rats treated with Synacthen. Twenty µg membrane protein were loaded on the gels. After western blotting of the gels HB_2 protein was detected with an antiserum against HB_2 and a second horseradish peroxydase (hrp)-conjugated anti-rabbit immunoglobulin followed by enhanced chemoluminescence. It can be seen in Fig 7 that the HB_2 protein expression was more than doubled by ACTH treatment.

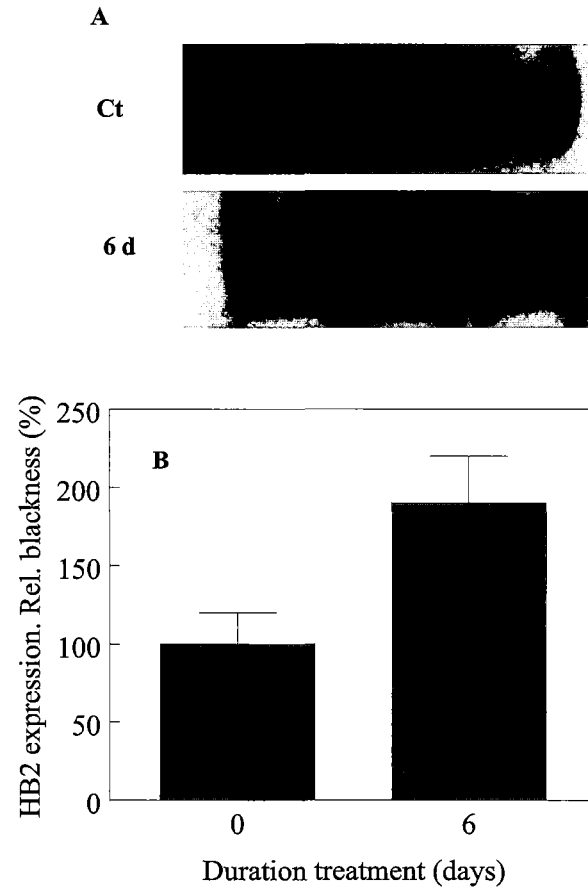

Figure 7. **HB_2 protein expression in livers of control and ACTH-rats after 6 days of treatment.** Liver membranes were isolated, subjected to SDS PAGE and blotted to nitrocellulose membranes. After incubation of the western blot with respectively a rabbit antibody to HB_2 and a hrp-conjugated anti-rabbit antibody, HB_2 was visualized by enhanced chemiluminescence (ECL) detection.(A). HB_2 bands on the blot were quantitated and compared with the relative blackness obtained in control liver membranes. (B). Results represent mean±S.D. (n=3).

DISCUSSION

Previously we have found an opposite regulation of HL in adrenal and liver by ACTH treatment in the rat [26]. HL is believed to play a role in the HDL-cholesterol uptake and HDL cholesterol has been shown to be an important substrate for adrenal steroidogenesis at least in rodents [35,36]. We suggested before that an enhanced HL activity in the adrenal and a lowered HL activity in the

liver may lead to a redirection of the flux of HDL cholesteryl-ester from the liver to the adrenal gland. Besides HL, SR-BI has been proposed to be essential for the uptake of HDL-cholesterylester [6,10,11]. Therefore we wondered whether there was also an opposite regulation of SR-BI in adrenal and liver by ACTH. SR-BI expression in the adrenal has been shown to be enhanced by ACTH and depletion of cellular cholesterol pools [18]. Nothing is known about the effect of ACTH on the SR-BI expression in the liver. Based on the hypothesized redirection of the flow of HDL-cholesteryl ester from the liver to the adrenal one would expect a lowered expression. However, an enhanced expression cannot be excluded because inhibition of HL activity, at least in the adrenal by an antibody against HL, led to an increase in the expression of SR-BI. Moreover SR-BI expression was also increased in the adrenal gland in HL knockout mice. In order to see whether SR-BI was up- or down- regulated under our conditions, we measured both HL activity and SR-BI expression in liver and adrenal gland. The SR-BI expression in liver was very low. Especially after ACTH treatment, the expression of liver SR-BI mRNA was too low to obtain reliable results by measurement with a nuclease protection assay, as used in many studies. Therefore we developed a competitive RT-PCR to be able to measure the liver SR-BI mRNA expression quantitatively. To our knowledge this is the first report that quantitatively measured the amount of SR-BI mRNA in liver. It can be calculated that an expression of 0.7 attomoles of SR-BI mRNA corresponds to about 7 copies of SR-BI mRNA per liver cell. Also in other studies measuring the relative amount of SR-BI, a low liver expression was found. Also in the hamster only a faint signal of SR-BI was obtained in control animals by using a nuclease protection assay to measure the relative amount of liver SR-BI mRNA [23]. In the rat or mice little or no detectable immunostaining was found in the liver using a anti-SR-BI antibody [10,37]. This result was confirmed in the rat (data not shown). This is rather due to a low level of expression and probably not caused by inaccessibility of the peptide epitope to the antibody in this tissue, because in sections of mouse livers with adenovirus mediated overexpression of SR-BI a high level of immunofluorescence was found.

When we compare the expression of SR-BI in liver and adrenal, it appears that the level in liver is about 10 times lower than the level in adrenal. Based on the whole organ, however, liver has more than three times as much SR-BI mRNA compared to the adrenal. The rather low level of SR-BI in liver is even more lowered after treatment with ACTH. After 4 - 6 days of treatment the amount of SR-BI in liver is even 50 times lower when compared to the adrenal. Based upon the whole organ the expression of SR-BI in the adrenals of ACTH rats is also higher than in liver, despite the much larger organ weight of the liver. Such an opposite regulation of both HL and SR-BI in ACTH rats could serve to redirect the HDL cholesteryl uptake away from the liver to

the adrenal, in order to supply the adrenal gland with sufficient substrate to maintain steroidogenesis. The increase in adrenal SR-BI expression may be a direct effect of ACTH but also may be partly due to a decrease in the adrenal cholesterol [18]. The SR-BI promoter contains two sterol responsive elements (SRE) [20]. Besides SRE's the SR-BI promoter contains Sp1 sites and SF-1 sites [21,38]. The Sp1 family members are essential for transcription of the SR-BI gene since mutations in any of these sites decreased efficient transcription. SF-1 and SREBP act synergistically to induce SR-BI expression in the adrenal gland. The mechanism responsible for the decrease in SR-BI expression in the liver is not known. So far in liver a down regulation of SR-BI expression has been found by high doses of estrogens and by feeding rats a high cholesterol diet [10,11,19]. An upregulation of SR-BI expression has been found by feeding rats a diet rich in polyunsaturated fatty acids or a Vitamin E-depleted diet [23,39]. The downregulation of SR-BI is accompanied by a enhanced cholesterol/ phospholipid ratio in liver microsomes after 4-6 day of treatment. SR-BI expression is regulated by SREBP levels and downregulated by cholesterol. SREBP expression is also downregulated when cholesterol levels are increased. In this way, it may well be that the observed downregulation of SR-BI by ACTH or Cortisol treatment is due to a lowered amount of SREBP after an enhanced cholesterol/phospholipid ratio. To our surprise this opposite regulation of SR-BI does not lead to a redirection of the flow away from the liver to the adrenal gland as measured by the uptake of ^{3}H-labelled HDL-cholesterylether. The relative amount of cholesterylester taken up by the adrenal was increased more than 10 fold by the ACTH treatment. The absence of a decrease in the uptake of HDL cholesterylester despite a downregulation of SR-BI and HL in the liver in the ACTH treated rats raises the possibility of alternative pathways for the uptake of HDL-cholesterylester, independent of SR-BI and/or HL. An SR-BI independent uptake of HDL-cholesterylester has been proposed before [10, 25, 40]. Landschultz et al [10] did not observe a fall in the uptake of HDL in estrogen treated rats despite a lowering in the SR-BI expression in the liver, suggesting the presence of this SR-BI-independent mechanism. Woollett and collaborators [25] came to the same suggestion in the hamster. They observed that LDL cholesterol, which is also able to bind to the SR-BI receptor, did not compete with the HDL cholesterylester clearance in a more than 30-fold excess, while the HDL cholesterylester clearance was saturated at normal plasma HDL concentrations. Finally, Silver et al [40] observed a 50% decrease in the selective HDL-cholesterylester uptake in ob/ob mice that was normalized by treatment with leptin. The decrease in selective uptake was not accompanied by a lowered SR-BI expression. Therefore they suggested that also in mice an alternative pathway may exist for the selective uptake of HDL-cholesteryl ester. Such an alternative pathway for the uptake of HDL-cholesteryl ester may be mediated by HB_2. The

alternative HDL receptor, HB_2 was first described by Tozuka [41] as an HDL binding protein in rat and human liver. Recently in porcine hepatocytes two HDL binding sites were established, SR-BI being the low affinity binding site and HB_2 being the high affinity HDL binding site [42]. HB_2 may also be a functional HDL receptor since cells overexpressing HB_2 showed an increase in HDL binding and uptake [43]. Milliat and collaborators [44] suggested that the HB_2 receptor may be of physiological importance since they found that the increase in the plasma level of HDL in diabetic, hypercholesterolemic Ricoh rats was accompanied by an similar, but opposite decrease in hepatic HB_2 level. Bocharov et al [45] stated that a high-affinity HDL binding site was upregulated in cultured rat hepatocytes by glucocorticoids. Since HB_2 is the high affinity HDL binding site, our finding of an upregulation of HB_2 in ACTH treated rats is in line with the observation of Bocharov [45]. HB_2 expression was found to be lowered in livers of simvastatin-treated rats [46]. Liver cholesterol is lowered by simvastatin, suggesting that liver cholesterol levels control the expression of HB_2. Therefore, the upregulation of HB_2 expression in ACTH treated rats may be due to the increase in cholesterol/phospholipid ratio in liver microsomes in these rats. The increase in HB_2 expression in livers of ACTH treated rats could be an explanation for the relatively enhanced HDL-cholesteryl ester uptake in the livers of these rats, compensating for the lowered uptake of HDL-cholesteryl ester as a consequence of the lowering of SR-BI. Finally our studies showed an opposite regulation of both HL and SR-BI in liver and adrenal. The upregulation in the adrenal does lead to an increase in the uptake of HDL-cholesteryl ester confirming the importance of HL and SR-BI for the uptake of HDL-cholesterol in the adrenal. However, in the liver the lowered expression of SR-BI and HL does not lead to a decrease in the uptake of HDL-cholesterylester suggesting that in liver besides HL and SR-BI, HB_2 may be involved as an alternative pathway for the uptake of HDL-cholesterol.

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Chapter 4.

General Discussion

4. GENERAL DISCUSSION

The aim of this thesis was to study the expression and regulation of the hepatic lipase gene and the role of HL in cholesterol homeostasis in rat adrenal glands and ovaries. Hepatic lipase is present in steroidogenic tissues where it plays an important role in the metabolism of lipoproteins cholesterol, mainly HDL. The origin of the lipase found in those organs remains controversial. Also, how the lipase activity may be involved in the cholesterol homeostasis is not completely understood yet. In the present study efforts were made to better understand this matter.

Expression and regulation of the LIPC gene in steroid hormone producing tissues

Hepatic lipase besides in the liver is also present in rat adrenal glands and ovaries. The HL form present in steroid hormone producing tissues is named liver- or l-type lipase. The full-length of HL mRNA could not be detected in adrenals and ovaries [1] suggesting that the lipase would be secreted from the liver and transported to these organs, though no lipase activity could be found in the circulation in rats. In adrenal glands, the HL mRNA differs from that of the liver since the two first exons are absent [1]. In chapter 2.1. and 2.2. we showed that in ovaries and adrenals the rat HL gene is transcribed into a variant mRNA form and regulated by hormones. This variant HL mRNA is probabily under control of an alternative promoter that we identified in the 5'- untranslated region of the HL gene. This alternative promoter has a very low activity that can be due to the absence of an unequivocal TATAbox, which is important but not essential for transcription. Interestingly, we found a possible translation product of the variant mRNA with the expected molecular weight. This protein was transiently induced in parallel with the mRNA upon stimulation by tropic hormones. These findings strongly suggest that during short-term stimulation of rat adrenal glands, an alternative HL gene product is formed which is regulated by an alternative promoter. Since this product lacks the two first exons and thus the signal peptide sequence it probably remains intracellular [1] (chapter 2.1.). The function of this protein is still unknown and we have not been able to assign any catalytic activity. Since the N-terminal part of HL protein encoded by exons 1 and 2 is missing, the catalytic activity would probably differ from that of hepatic lipase or l-type lipase. It is remarkable however, that the translation would have started at the AUG found in the longest open reading frame in exon 4 which is found upstream of the catalytic triad. Therefore, this protein may have esterase activity [1]. The fact that the variant HL mRNA and 47 kDa protein is found in steroidogenic tissues and is induced by

corticotropic and gonadotropic hormones suggests a role in cholesterol metabolism in those tissues. This hypothesis is further supported by the existence of binding sites for transcription factors in the alternative promoter which are mostly present in promoters of genes associated with cholesterol metabolism. Moreover, the transient expression observed upon stimulation of steroidogenesis coincides with the reduction of intracellular cholesterolester content and parallels the increase of de novo cholesterol synthesis [2]. Under these conditions, the endogenous cholesterol level becomes limited. Taken together, these observations suggest a role for the variant HL gene product in the intracellular cholesterol homeostasis in steroidogenic tissues. This role may be structural or catalytical. When the intracellular cholesterol supply is depleted, the extracellularly localized l-type lipase is increased and it may facilitate the uptake of circulating HDL-cholesterol (Fig. 1). In this way, the intracellular variant form and the extracellular l-type lipase may be involved in an acute and prolonged stimulation of steroidogenesis and subsequent need of cholesterol for hormone synthesis, respectively. In the liver, the variant HL mRNA is also present but its function and regulation was not investigated in this study.

From our results we cannot completely ensure that hepatic lipase activity found in rat ovaries and adrenals is a translation product of the variant HL mRNA or transported from the liver. We approached this question by investigating the functional molecular mass in rat liver, adrenals and ovaries (chapter 2.3.). Using a radiation inactivation technique we found that the functional molecular mass of hepatic lipase in rat liver, adrenals and ovaries is different. In liver, hepatic lipase is functional as a monomer (about 63 kDa) and in adrenals and ovaries as a dimer (about 117 kDa). Although the target mass in adrenals and ovaries is higher than in liver, the size of the structural unit is smaller, 51 kDa. These are unexpected results if it is predicted that the l-type lipase activity found in steroidogenic tissues is derived from the liver. On the other hand, it is also unlikely that the catalytic activity results from the translation product of the variant HL mRNA. This intracellular protein is not expected to have a triacylglycerol hydrolase activity similar to that of HL and the results here described are based on triacylglycerol hydrolase activity. One possibility is that the hepatic lipase from liver can undergo post-translational modification in liver or during its transport to the adrenals and ovaries, explaining the difference in functional units. The dimeric form of the lipase found in steroid hormone producing tissues could contribute to differences in substrate specificity and physiologic function in these tissues compared with liver.

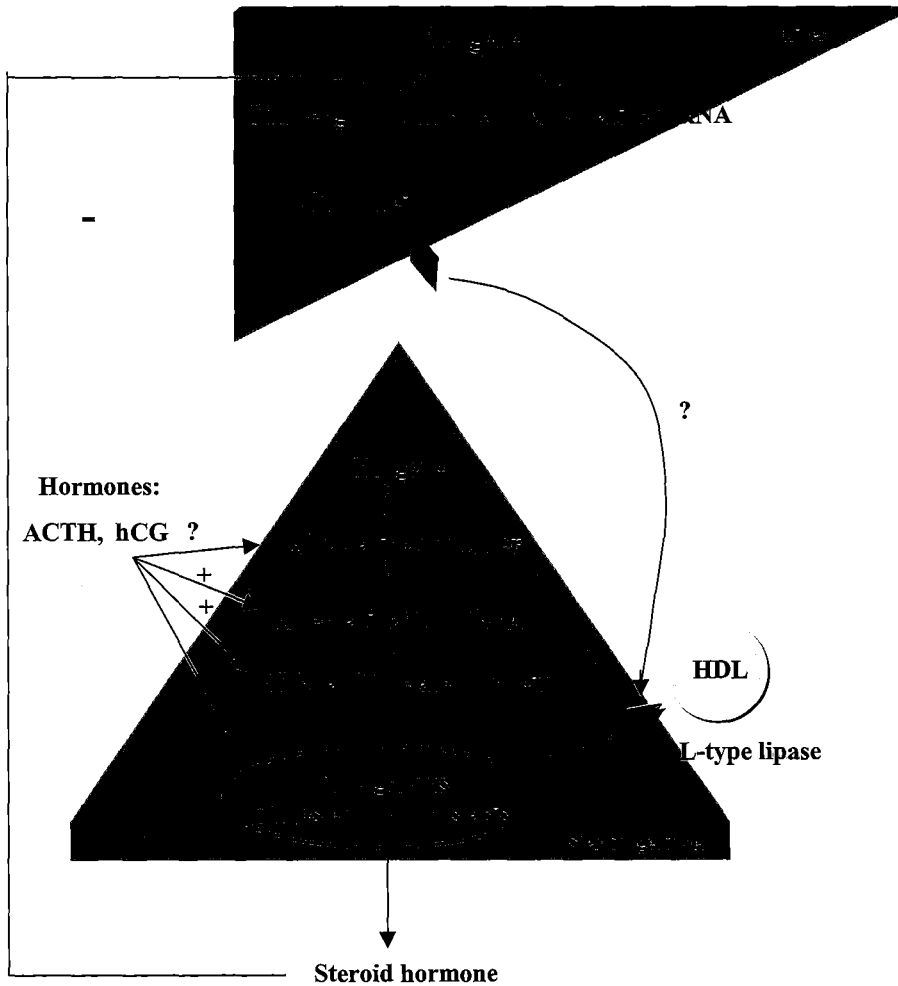

Figure 1. **HL gene expression, location and regulation in rat liver and steroidogenic tissues.** (+) represents upregulation, (-) represents downregulation, (?) represents mechanisms that are not yet completely understood.

Role of hepatic lipase in cholesterol homeostasis

Steroidogenic tissues utilize cholesterol for steroid hormone synthesis. During a prolonged hormone production the most important cholesterol provision is lipoprotein-cholesterol. In the rat HDL-cholesterol is the main source. Jansen and collaborators [3] suggested that HL may modulate the unesterified cholesterol uptake from HDL particles. In this model, HL

hydrolizes phospholipids from the HDL particle, promoting the uptake of cholesterol by the cell. More recently, SR-BI was also suggested to play an important role in the HDL-cholesterol esters uptake by the cell. In chapter 3. we studied the role of HL in cholesterol homeostasis in adrenal glands and ovaries in relation to SR-BI.

First, we studied whether HL modulates the HDL binding to rat adrenal membranes (chapter 3.1.). We found that HDL binding is partly heparin sensitive and that HL mediates this binding. The effect of HL on HDL binding to adrenal plasma membranes was more accentuated after stimulation with ACTH which increases HL activity in adrenals. This is in line with the fact that HL found in rat adrenal glands is heparin-releasable and is suggested to play a role in HDL cholesterol uptake to be used for hormone synthesis [4]. During ACTH stimulation the expression of the putative HDL receptor, SR-BI is also increased. Our hypothesis that HL and SR-BI together or independently mediate HDL binding is further supported by the work of Ji and collaborators [5]. These authors found that in cells overexpressing HL part of the HDL binding and HDL-cholesterol esters uptake was mediated by HL and that it could be disrupted by heparinase. The binding of HDL that is not HL dependent might involve HDL binding to its receptor SR-BI. This is supported by the work of Lambert and collaborators [6]. These authors showed that in cells overexpressing HL, the HDL binding was enhanced and when this overexpression was simultaneous with SR-BI overexpression also the HDL-CE uptake was increased. The effect of HL was abolished by heparin. This suggests that HL modulates the HDL binding to adrenal membranes and SR-BI the HDL-CE uptake by the cells. The role of HL is also very important after ACTH stimulation when large amounts of cholesterol are needed for hormone production. Under these conditions an interaction between HL and SR-BI would be of importance.

We further approached this question by studying the effect of HL on HDL-cholesteryl ethers uptake by rat adrenals in vivo, in controls and in ACTH stimulated animals. In chapter 3.2. we show that the effect of HL on HDL-cholesteryl ether uptake is indirect. Inactivation of HL in vivo resulted in increased SR-BI expression and [^{3}H]-HDL-cholesteryl ether uptake in control rats. After ACTH treatment, HL inhibition did not affect the SR-BI expression or the HDL-CEth uptake. Our results suggest that HL activity in adrenal glands is an important modulator of SR-BI expression and subsequently, SR-BI is responsible for HDL-CEth uptake. In line with this, gene-targeted inactivation of HL in mice was associated with increased expression of SR-BI, but in adrenals the intracellular cholesterol esters stores remained partly depleted [7].

This suggests that SR-BI alone is not enough to compensate for the loss of HL activity in cholesterol homeostasis. HL and SR-BI may interact to ensure an optimal cholesterol homeostasis. We hypothesize that HL is important in the modulation of unesterified cholesterol fluxes between HDL particles and cells, and it prevents the efflux of cholesterol from cells to HDL, as previously described [8,9]. SR-BI expression is supposed to be regulated by intracellular cholesterol levels [7]. In this way, it is possible that in vivo inhibition of HL activity alters the unesterified cholesterol flux between HDL and the adrenocortical cell. Subsequently, SR-BI expression is increased to compensate for this process. As a result, HDL-CEth uptake is expected to be increased. Based on these observations one can suggest that HL and SR-BI probably interact and are involved in a mechanism to ensure sufficient cholesterol supply to the cells. When HL and SR-BI directly interact they would colocalize. Our study shows that HL and SR-BI colocalize in zona fasciculata of rat adrenal cortex but this colocalization is partial (Chapter 3.3.). HL and SR-BI also exist separated from each other. These observations indicate that a direct interaction may exist but that HL and SR-BI also may act independently. Furthermore, in a direct interaction HL can mediate the binding/bridging of HDL to SR-BI on the cell surface prior to the HDL-cholesteryl ester uptake via SR-BI. Alternatively, hepatic lipase can modulate the HDL particle by hydrolyzing the phospholipids from the lipoprotein surface facilitating the cholesterol uptake via SR-BI. Figure 2 represents a model of the possible involvement of HL and SR-BI in the cholesterol homeostasis in steroidogenic tissues.

Finally, we studied whether HL and SR-BI regulation in rat adrenal glands and liver are similar. In chapter 3.4. we showed that ACTH treatment decreases the expression of both HL and SR-BI in liver, contrary to what is observed in adrenals. Furthermore, the uptake of HDL-CEth remains unaffected in rat liver under hormone treatment. Our results suggest that the interaction between HL and SR-BI is more important in rat adrenals than in liver. Besides HL and SR-BI, the HDL binding protein HB_2 is probably responsible for the mediation of HDL-cholesterol uptake in liver and can explain the increase in HDL-CEth uptake even when HL and SR-BI are decreased.

The exact role of hepatic lipase and SR-BI in steroidogenesis needs further investigation. However, from the results described in this thesis, we suggest that both independently and/or in combination, are crucial for an optimal maintenance of the intracellular cholesterol homeostasis in the rat steroidogenic cell.

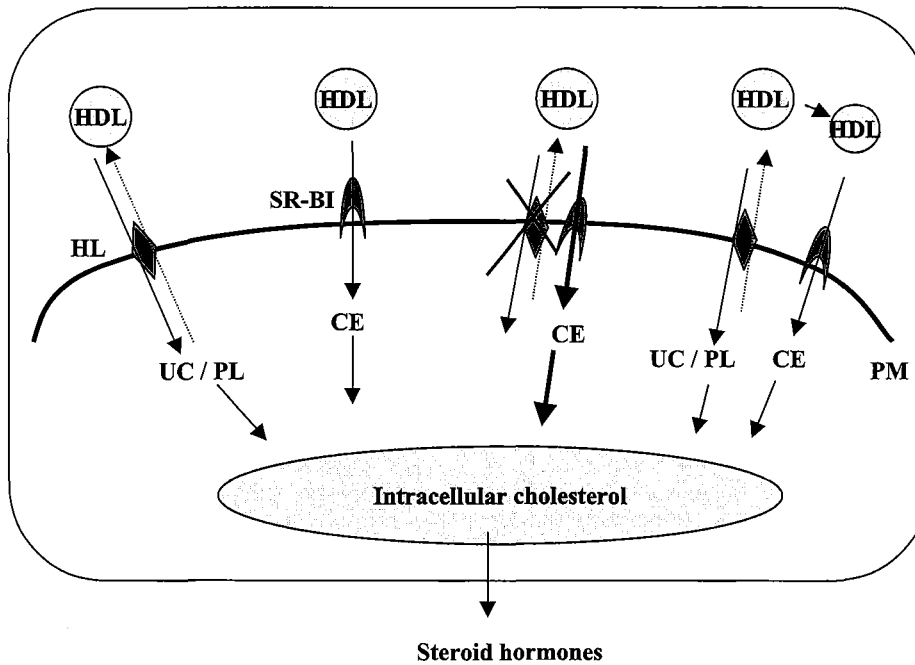

Figure 2. Postulated models of the role of hepatic lipase (HL) and scavenger receptor class B type I (SR-BI) in cholesterol homeostasis in steroidogenic tissues. PM, plasma membrane; UC, unesterified cholesterol; PL, phospholipids; CE, cholesterol esters; HDL, high density lipoproteins.

Also in humans HL and SR-BI may be of crucial importance in cholesterol metabolism. Together they mediate the delivery of sufficient lipoprotein cholesterol to the cell and they are involved in the maintenance of the intracellular cholesterol homeostasis, but, they also can offer alternative routes when one of them is dysfunctional. HL mediates a more efficient HDL-cholesterol uptake via SR-BI. This is supported by the observation that in HL knock-out mice SR-BI expression is increased but cholesterol stores in adrenals are impaired suggesting that HL is essential for an efficient cholesterol supply [7]. Furthermore, HL knockout mice produce lower levels of progesterone and have reduced litter size even when SR-BI is available [10]. It also seems that the composition of membrane lipids is essential in this process (K. Schoonderwoerd, personal communication). SR-BI was also suggested to alter the plasma membrane cholesterol distribution with consequences to the intracellular cholesterol homeostasis [11] and possible abnormalities in the membrane of oocytes in females. In fact, SR-

BI knockout female mice are infertile mainly due to an abnormal HDL metabolism [12]. This suggest that a deficiency in SR-BI may also contribute to some form of human infertility, certainly when HL is also dysfunctional and no alternative route is available for the metabolism of HDL.

In conclusion, hepatic lipase and SR-BI are important factors in cholesterol metabolism. Together they ensure an optimal cholesterol supply for the cell under a variety of conditions.

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Summary

A review about hepatic lipase is made in **chapter 1**. Aspects as characterization of hepatic lipase **(chapter 1.2)** and its role in cholesterol homeostasis **(chapter 1.3)** are described.

Regulation and expression of the LIPC gene in steroid hormone producing tissues

In **chapter 2.1** HL gene expression is studied in rat ovaries of mature cyclic females and immature female rats treated with PMS-hCG to induce superovulation. Using RT-PCR and competitive RT-PCR a HL transcript lacking exon 1 and 2 was detected and quantified. Hepatic lipase gene expression was transiently induced by PMS-hCG with a maximum at 2 days after treatment. Using pulse-labelling techniques, a 47 kDa HL-immunorelated protein was similarly induced after 2 days of hormone treatment. L-type lipase activity was increased only when HL mRNA and de novo synthesized 47 kDa protein reached basal levels. We concluded that in rat ovaries HL gene is expressed into a variant mRNA which lacks the two first exons and is translated into a 47 kDa HL-related protein. The mRNA and the protein are transiently induced by gonadotropic hormones and precede the HL (l-type lipase) activity.

In rat ovaries and adrenal glands the HL gene is exclusively transcribed into a variant mRNA where the two first exons are substituted by an intron-like sequence. In **chapter 2.2** we identify an alternative promoter in intron-2 of rat hepatic lipase gene which is active in rat liver and steroidogenic tissues. The 5'-end of the variant mRNA was located immediately upstream of exon 3 with a putative transcription start site at 465 bp upstream of exon 3. The promoter region lacks an unequivocal TATA box but contains several potential regulatory sites. The alternative promoter has a very low activity when compared to the normal rat HL promoter region. The variant transcript would be translated into a C-terminal part of the HL protein of about 47 kDa, but an appropriate translation startsignal is lacking. Instead several other potential start codons were found. Similar to rat ovaries after PMS/hCG treatment, the variant HL mRNA and the 47 kDa protein was transiently induced by corticotropic hormones (ACTH) preceding the l-type lipase activity. In rat adrenal glands the promoter can be regulated independently of the normal HL gene promoter.

It is not clear from our results whether the l-type lipase activity found in steroidogenic tissues is the same as that found in liver. In **chapter 2.3** the functional molecular mass of hepatic lipase in rat liver, adrenals and ovaries is studied. Using radiation inactivation we showed that the functional unit of hepatic lipase in liver is a monomer with a functional molecular mass of

about 63 kDa. In adrenal glands and ovaries hepatic lipase activity have a functional molecular mass of about 117 kDa, the functional unit of which is a dimer of a 51 kDa as determined by western-blot. The difference in functional molecular mass between liver and steroid hormone producing organs may be responsible for differences in physiologic functions of HL in those tissues.

HL and SR-BI in cholesterol homeostasis

In **chapter 3.1.** the role of hepatic lipase on HDL binding to rat adrenal glands is investigated. ^{125}I-HDL specific binding to adrenal membranes was shown to be partly heparin sensitive. The decrease in HDL specific binding to membranes pretreated with heparin was completely restored by addition of purified rat hepatic lipase. The effect of hepatic lipase was more pronounced in ACTH treated rats than in controls. Also SR-BI expression was several-fold increased by ACTH. Our results suggest that HL modulates the binding of HDL to rat adrenals and that this may occur simultaneously with or precede the binding of the lipoprotein to SR-BI.

HL and SR-BI are both involved in HDL-cholesterol(esters) uptake in cholesterol utilizing tissues. In **chapter 3.2** we investigate the effect of inhibition of hepatic lipase activity on SR-BI expression and HDL-[^{3}H]cholesteryl ether (CEth) in adrenal glands of controls and ACTH treated rats. In control rats, acute in vivo inhibition of hepatic lipase activity by antibodies increased adrenal SR-BI expression (mRNA and protein) several-fold and resulted in 41% more accumulation of HDL-[^{3}H]CEth within 4 h. ACTH treatment significantly increased HL activity, SR-BI expression and HDL-[^{3}H]CEth uptake in adrenal glands. Inhibition of HL activity did not further induce expression of SR-BI or HDL-[^{3}H]CEth uptake by the adrenals. We conclude that in rat adrenal glands changes in HL activity can influence SR-BI expression but it is not necessary for SR-BI-mediated HDL-cholesteryl esters uptake.

In **chapter 3.3** the colocalization of hepatic lipase and SR-BI in rat adrenals is biochemically and cytochemically investigated. We also studied whether this occurs at the caveolae. Colocalization of HL and SR-BI is entirely found at the sinusoids of the zona fasciculata of adrenals. A minority of the localization occurs at the caveolae and HL and SR-BI can also be found separated of each other. Our results suggest that HL and SR-BI can function

synergistically or in parallel with implications in the cholesterol homeostasis of the steroidogenic cell.

In **chapter 3.4** the regulation of HL and SR-BI in rat liver and adrenal glands is studied. ACTH treatment decreased HL and SR-BI expression in liver in contrast to what is observed in adrenal glands. The down regulation of SR-BI expression in liver was paralleled by an increase in the cholesterol/phospholipid ratio in liver microsomes indicating a regulation of SR-BI by cellular cholesterol levels. The effect of ACTH was mimicked by cortisol treatment. The HDL-[^{3}H]CEth uptake by the liver was unaffected after down regulation of HL and SR-BI after ACTH treatment. In an attempt to explain this observation we studied the expression of the HDL binding protein -2 (HB_2) under the same conditions. HB_2 expression was increased by ACTH, this suggests that in liver it may offer an alternative pathway to deliver HDL-cholesterol when SR-BI and HL are less abundant or dysfunctional.

Finally, in **chapter 4.** the results presented in this thesis are further discussed. Models to explain our observations and to elucidate the role of HL and SR-BI in the cholesterol homeostasis in steroidogenic tissues are proposed.

Samenvatting

Hoofdstuk 1 is een review over lever lipase. Aspecten zoals karakterisatie van lever lipase (**hoofdstuk 1.2**) en zijn rol in de cholesterol homeostase (**hoofdstuk 1.3**) worden beschreven.

Regulatie en expressie van het LIPC gen in steroïdhormoon producerende organen

In **hoofdstuk 2.1** werd lever lipase bestudeerd in rat ovaria van volwassen cyclische vrouwtjes en van onvolwassen vrouwtjes die behandeld waren met PMS-hCG om superovulatie te induceren. Met behulp van RT-PCR en competitieve RT-PCR werd een HL transcript, dat exon 1 en 2 miste, gedetecteerd en gekwantificeerd. De lever lipase gen expressie werd tijdelijk verhoogd tot een maximum na 2 dagen van behandeling met PMS-hCG. Met behulp van de pulse labelling techniek werd gevonden dat een 47 kDa HL immuno gerelateerd eiwit gelijktijdig werd geïnduceerd na 2 dagen van hormoon behandeling. De L-type lipase activiteit werd pas geïnduceerd als het HL mRNA en het de novo gesynthetiseerde 47 kDa eiwit weer het basaal niveau hadden bereikt. We concluderen dat in rat ovaria het HL gen als een variant mRNA tot expressie komt, dat de eerste twee exonen mist en wordt getransleerd tot een 47 kDa HL-gerelateerd eiwit. Het mRNA en het eiwit worden tijdelijk geinduceerd doormiddel van gonadotrope hormonen. Deze inductie gaat vooraf aan de HL (l-type lipase) activiteit.

In ovaria en bijnieren van de rat wordt het HL gen uitsluitend tot expressie gebracht in de vorm van een variant mRNA waar de eerste twee exonen zijn vervangen door een intron achtige DNA sequentie. In **hoofdstuk 2.2** identificeren we een alternatieve promoter in intron 2 van het rat lever lipase gen die actief is in rattenlever en steroïdogene weefsels. Het 5' deel van de mRNA variant was gelokaliseerd direct upstream van exon3 met een transcriptie startplaats gelokaliseerd op 465 bp upstream van exon 3. In de promoter regio is geen duidelijke TATA box aanwezig maar het bevat enkele potentiële regulerende DNA sequenties. De alternatieve promoter heeft een erg lage activiteit in vergelijking met de normale rat HL promoter. Het variant transcript zou worden getransleerd in een C-terminaal deel van het HL eiwit van ongeveer 47 kDa, maar een geschikt start signaal is niet aanwezig. In plaats daarvan werden er verschillende andere potentiële start codons gevonden. Zoals in ratten ovaria werd het variant HL mRNA en het 47 kDa eiwit tijdelijk geïnduceerd doormiddel van corticotrope hormoon (ACTH) voorafgaand aan de l-type lipase activiteit. In rattenbijnieren kan de promoter onafhankelijk van de normale HL promoter worden gereguleerd.

Uit onze resultaten is het niet duidelijk of de l-type lipase activiteit die gevonden wordt in steroïdogene weefsels dezelfde is als die gevonden wordt in de lever. In **hoofdstuk 2.3** is de functionele massa van lever lipase in rattenlever, bijnieren en ovaria bestudeerd. Met behulp van stralings inactivatie hebben we aangetoond dat het functionele deel van lever lipase in de lever een monomeer is met een functioneel molecuul gewicht van ongeveer 63 kDa. In bijnieren en ovaria heeft lever lipase een functioneel molecuul gewicht van ongeveer van ongeveer 117 kDa waarvan het functionele deel een dimeer is van 51 kDa zoals vastgesteld met behulp van westernblots. Het verschil in functionele moleculaire massa tussen lever en steroïd hormoon producerende organen is mogelijk de oorzaak van de verschillende fysiologische functies van HL in die organen.

HL en SR-BI in cholesterol homeostase

In **hoofdstuk 3.1** is de rol van lever lipase in HDL binding aan ratten bijnieren bestudeerd. De ^{125}I-HDL specifieke binding aan membranen die waren voorbehandeld met heparine, werd volledig hersteld na toevoeging van gezuiverd ratten lever lipase. Het effect van lever lipase was meer uitgesproken bij de ratten behandeld met ACTH dan bij de controle ratten. Ook was de SR-BI expressie enkele malen verhoogd na ACTH behandeling. Deze resultaten suggereren dat HL de binding van HDL aan ratten bijnieren moduleert en dat dit mogelijk gelijktijdig plaats vindt of voorafgaat aan de binding van het lipoproteïne aan SR-BI.

HL en SR-BI zijn beide betrokken bij de HDL-cholesterol(ester) opname van cholesterol verbruikende weefsels. In **hoofdstuk 3.2** hebben we het effect onderzocht van de remming van lever lipase activiteit op SR-BI expressie en HDL-[^{3}H]cholesteryl ether (CEth) in bijnieren van controle ratten en ratten behandeld met ACTH., Een snelle in vivo remming van lever lipase doormiddel van antilichamen in controle ratten, verhoogde de SR-BI expressie in bijnieren (mRNA en eiwit) vele malen en resulteerde binnen 4 uur in een 41% hogere accumulatie van HDL-[^{3}H]CEth. Behandeling met ACTH verhoogde de HL activiteit, SR-BI expressie en HDL-[^{3}H]CEth opname door de bijnieren significant. Remming van HL activiteit induceerde de SR-BI expressie en HDL-[^{3}H]CEth opname door de bijnieren niet extra. We concluderen dat in rattenbijnieren veranderingen van HL activiteit de SR-BI expressie kan beïnvloeden maar HL activiteit is niet noodzakelijk voor HDL-cholesteryl ester opname via SR-BI.

In **hoofdstuk 3.3** is de co-lokalisatie van lever lipase en SR-BI in ratten bijnieren biochemisch en cytochemisch bestudeerd. Ook werd bestudeerd of er co-lokalisatie is in de caveolae. HL en SR-BI zijn voornamelijk geco-lokaliseerd in de sinusoiden van de zona fasciculata van de bijnieren. In de caveolae is een klein deel geco-lokaliseerd. HL en SR.BI kunnen ook onafhankelijk van elkaar voorkomen. Onze resultaten suggereren dat HL en SR-BI samen of in parallel, een rol spelen in de cholesterol homeostase in steroïd producerend cellen.

In **hoofdstuk 3.4** is de regulatie van HL en SR-BI in rattenlever en bijnieren bestudeerd. Behandeling met ACTH verlaagde in lever de HL en de SR-BI expressie, dit in tegenstelling tot de situatie in de bijnieren. De down regulatie van de SR-BI expressie in lever verliep omgekeerd evenredig met de cholesterol/fosfolipide ratio in lever microsomen. Dit wijst op een regulatie van SR-BI door cholesterol niveaus. Het effect van ACTH werd nagebootst door middel van cortisol behandeling. De HDL-[^{3}H]CEth opname door de lever bleef onveranderd na down regulatie van HL en SR-BI na ACTH behandeling. In een poging om deze observatie te verklaren hebben we de expressie bestudeert van het HDL binding protein-2 (HB_2) onder dezelfde condities. HB_2 expressie werd verhoogd doormiddel van ACTH. Dit suggereert dat in de lever mogelijk een alternatieve route aanwezig is om HDL-cholesterol aan te leveren als er niet genoeg SR-BI en HL beschikbaar is of als ze niet functioneel zijn.

Als laatste worden in **hoofdstuk 4** de resultaten in dit proefschrift bediscussieerd. Met modellen worden onze bevindingen verklaard en de rol van HL en SR-BI in de cholesterol homeostase in steroïdogene weefsels toegelicht.

Dankwoord

DANKWOORD

HET IS AF! In de afgelopen jaren waren er velen die een bijdrage hebben geleverd om dit proefschrift tot stand te laten komen. Niet iedereen zal ik met naam noemen, maar iedereen was even belangrijk.

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CURRICULUM VITAE

De auteur van dit proefschrift is geboren op 12 januari 1965 te Lisboa, Portugal. Na haar studie Biochemie aan de Faculdade de Ciências van de Universidade de Lisboa, kwam ze eind 1991 naar Nederland waar ze ging werken als wetenschappelijk onderzoeker op de afdeling Biochemie van de Faculteit der Geneeskunde van Erasmus Universiteit Rotterdam. Op deze afdeling verrichte ze meerdere projecten onder de supervisie van Prof. Hans Jansen. In de periode van 1991-1992 werkte ze mee aan het project "Hepatic lipase in normocholesterolemic men with atherosclerotic manifestations". Dit werd gesubsidieerd door de Nederlandse Hart Stichting. Het volgende project waar ze aan werkte was (1992-1997) "Functionality of steroid hormonal regulation of hepatic lipase". Dit werd gedeeltelijk gefinancierd door de Praxis XXI- JNICT te Portugal. In 1999 betrof het project "Rol van atorvastatine in hepatische lipase en SR-BI expressie" gesubsidieerd door Pfizer. In 2000 is ze voor twee maanden werkzaam geweest op de afdeling Cellular Biology of the Faculty of Biology, University of Barcelona, Spain, in een project gesubsidieerd door Biomed-2. Haar laatste project aan de universiteit (2001-2002) was een onderdeel van de DIALOG studie. Het project "Clinical relevance of genetic predisposition and disturbed lipid metabolism in pathogenesis of type 2 Diabetes and cardiovascular complications" heeft ze werd gesubsidieerd door het NWO.

Een deel van de resultaten van de verschillende projecten is beschreven in dit proefschrift.

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