HEPARIN RELEASABLE LIVER-TYPE LIPASES OF THE RAT

APPLICATION OF MONOCLONAL ANTIBODIES

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HEPARINE MOBILISEERBARE LEVER-TYPE LIPASEN VAN DE RAT

TOEPASSING VAN MONOCLONALE ANTILICHAMEN

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voor mijn ouders
voor Marion

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LIST OF ABBREVIATIONS

VLDL	Very low density lipoprotein
IDL	Intermediate density lipoprotein
LDL	Low density lipoprotein
HDL	High density lipoprotein
LPL	Lipoprotein lipase
HMG-CoA	Hydroxy-3-methylglutaryl coenzyme A reductase
ACAT	Acyl-CoA: cholesterol acyltransferase
LCAT	Lecithin: cholesterol acyltransferase
CETP	Cholesterolester transfer protein
NEM	N-ethylmaleimide
TPCK	N-tosyl-L-phenylethylchloromethyl ketone
PITC	Phenylisothiocyanate
PMSF	Phenylmethylsulfonylfluoride
SDS	Sodium dodecyl sulphate
PBS	Phosphate buffered saline
BSA	Bovine serum albumin
ELISA	Enzyme linked immunosorbent assay
DNA	Deoxyribonucleic acid
Tris	Tris[hydroxymethyl]-aminomethane
IgG	Immunoglobulin G

CHAPTER I

INTRODUCTION

SHORT HISTORY

The study on extracellularly located lipases started with an observation made by Hahn (1) in 1943 while studying erythrocyte circulation in dogs. After the intravenous injection of the polyanion heparin, used as an anticoagulant, alimentary lipemia in one of the animals had disappeared three to five minutes later. In 1955, Korn (2) was the first to isolate the factor responsible for this clearing phenomenon.
Korn extracted the heparin activated enzyme from acetone powders of rat heart capable of clearing a coconut oil emulsion and he named this enzyme "Lipoprotein Lipase". A similar lipase activity was also observed in adipose tissue, spleen and aorta. All these lipases were inhibited by protamine sulphate (a cationic, arginine rich, small protein which is associated with nucleic acids) and also by high concentrations of sodium chloride. However, in the same paper Korn also reported a lipase derived from the liver that could not be inhibited by a preincubation with 1 M sodium chloride. From a more extensive study on this liver enzyme by Spitzer and Spitzer (3) it became clear that the liver enzyme and the lipases from other tissues showed different biochemical characteristics. Based on these and other studies (4-7), it has now been accepted that upon the in vivo administration of heparin, a number of lipases are released into the circulation. In 1972 La Rosa et al. (8) established the lipase activity in postheparin rat plasma as triacylglycerol hydrolase activity originating from the liver (indicated as liver lipase) and from extrahepatic tissues such as adipose tissue, heart, kidney, lung and spleen (indicated as lipoprotein lipase). With respect to enzyme activity, several differences between the heparin-releasable lipases from hepatic and extrahepatic origin were described:
i. Triacylglycerol hydrolase activity originating from the liver is resistant to inhibitors of lipoprotein lipase such as

protamine sulphate (300 µg/ml), sodium chloride (1.0-3.0 M) and pyrophosphate (10 nmol/ml) (3,5,8).
ii. In contrast to lipoprotein lipase from adipose tissue, the liver enzyme does not significantly promote the hydrolysis of triacylglycerol from particles such as chylomicrons or very low density lipoproteins (see section plasma lipoproteins).
By the introduction of immunological methods in the study on the lipolytic activity of postheparin rat plasma, Jansen et al. (9) showed that liver lipase and lipoprotein lipase are indeed different proteins. A polyclonal antibody directed against a lipase purified from rat postheparin plasma was found to inhibit the liver enzyme only. Despite the profound differences between liver lipase (E.C. 3.1.1.3) and lipoprotein lipase (E.C. 3.1.1.34), these enzymes show a number of similarities. In this thesis about the salt-resistant heparin releasable rat lipase, attention will therefore sometimes also be paid to lipoprotein lipase.
In the research on the liver enzyme a similar lipase activity was also identified in the adrenal gland and ovary (10). From the presence of the enzyme in three different steroid producing tissues as well as information derived from other studies that will be discussed in this thesis, it has been hypothesized that the salt-resistant heparin releasable lipase may be involved in cholesterol homeostasis. Recently, in a lipid lowering intervention study in male patients with advanced coronary artery disease, the activity of liver lipase correlated most strongly with regression of atherosclerosis (11-13).

IN VITRO ENZYME ACTIVITY OF LIVER LIPASE

The lipolytic activity of rat liver lipase towards a variety of substrates makes it difficult to define the substrate specificity. In vitro studies on the enzyme, isolated from rat liver heparin containing perfusates or postheparin rat plasma, demonstrated a number of enzyme activities.

1. Monoacylglycerol hydrolyzing activity
   - long chain monoacylglycerols such as 1- or 2-monopalmitoyl-, monooleoyl- or monolinoleoylglycerol emulsified in defatted bovine serum albumin (14-16).
2. Diacylglycerol hydrolyzing activity
   - dipalmitoylglycerol emulsified in gum arabic (14,15).
3. Triacylglycerol hydrolyzing activity
   - trioleoylglycerol emulsified in gum arabic (14-18) or the water-soluble tributyroylglycerol (19,20).
4. Phospholipase A_1 activity
   - phosphatidylethanolamine and phosphatidylcholine preferably with an unsaturated fatty acid at the 2' position of the glycerol head group such as arachidonic acid or linoleic acid (6,16,18,21,22).
5. Monoacylglycerol transferase activity (to form diacylglycerol and glycerol) (14,15,23).
6. Acyl-CoA hydrolyzing activity
   - long chain acyl-CoA such as palmitoyl-CoA (24).

Jansen et al. (17) described the enzyme kinetics of rat liver lipase (purified by Sepharose-heparin affinity chromatography) with trioleoylglycerol and phosphatidylcholine as the substrates. Although the maximal rate for the hydrolysis of phosphatidylcholine was much lower compared with the V_{max} for the hydrolysis of trioleoylglycerol (116 mU/mg *versus* 2390 mU/mg), the K_M for this substrate turned out to be the lowest (0.2 mM *versus* 6.2 mM). These experiments are in good agreement with those reported by Ehnholm et al. (18) who observed an approximately 25-fold higher triacylglycerol hydrolase activity than phospholipase A_1 activity (with phosphatidylcholine as the substrate) with the human enzyme of postheparin plasma. They also reported that the capacity of the enzyme to hydrolyze triacylglycerol was about the same as its phospholipase A_1 activity with phosphatidylethanolamine as the substrate (ratio 1.0-1.3). Based on the lower K_M for phospholipids, these *in vitro* studies favour a role of the enzyme in

the catabolism of phospholipids *in vivo*, since moreover the surface of lipoproteins consists of phospholipids. It has also been shown that liver lipase is capable to completely hydrolyze triacylglycerols (producing glycerol and free fatty acids), which contrasts lipoprotein lipase, that has generally been accepted to be a triacylglycerol hydrolase (25). Lipoprotein lipase only hydrolyzes fatty acids from the sn-1 and sn-3 position of a triacylglycerol (26) thereby creating a toxic non-ionic detergent (sn-2 monoacylglycerol) that could affect cellular membranes (27). Isomerization of the 2-monoacylglycerol to either 1- or 3-monoacylglycerol (28) and the subsequent breakdown by a monoacylglycerol hydrolase in blood (29,30) may avoid such damage.

Besides the chemical composition, the three dimensional structure of a substrate, which determines the accessibility of the enzyme to the molecules, is also very important for enzyme activity. For instance an emulsion of dipalmitoylphosphatidylcholine (stabilized by detergent) can serve as a substrate for liver lipase (31), but in a monolayer system the same molecules cannot be hydrolyzed by the enzyme (32). Therefore it has to be realized that although studies on the *in vitro* action of the solubilized enzyme on artificial substrates are instructive, they do not consider all the parameters that may be important for the *in vivo* function of the enzyme. To be able to understand the function of liver lipase *in vivo*, the localization of the enzyme and the organization and metabolism of triacylglycerols and phospholipids are discussed next.

LOCALIZATION OF LIVER LIPASE

In man and most animals (with the exception of the mouse) (33), only a low amount of active liver enzyme can be detected in normal plasma. However, upon heparin administration, most of the liver lipase activity is released into the bloodstream.

Perfusion of a liver with heparin results in an instantaneous release of lipase activity which suggests an extracellular localization of the enzyme (34). Most of the lipase activity has been shown to be associated with liver plasma membranes from where it is released by heparin (35).

It has been reported that after the enzyme is synthesized and secreted by liver parenchymal cells (31), the lipase is bound and stabilized by isolated non-parenchymal cells (36). Using immunofluorescence and electronmicroscopy, Kuusi et al. (37,38) observed an endothelial localization of the enzyme in the liver. It is therefore believed that following synthesis and secretion of the enzyme by the liver parenchymal cell, liver lipase is bound by the liver endothelium (Fig. 1.1).
It is not known whether a specific receptor for liver lipase is involved in the interaction of the enzyme with the endothelium. With respect to lipoprotein lipase a possible interaction of the enzyme with glycosaminoglycans was suggested (39). *In vitro* liver lipase is bound by Sepharose-heparin which is an ideal tool in purification procedures (40-43). This interaction is disrupted by 0.75 M NaCl which indicates an ionic binding between the enzyme and heparin. The enzyme is also released from the endothelium by means of a high salt (1 M NaCl) perfusion through the liver (unpublished result). This is suggestive for an interaction *in vivo* similar as with heparin *in vitro*. Heparan sulphate proteoglycans have been described in the liver (44). Three forms have been purified from liver: *i*. an integral membrane protein, *ii*. a (heparin releasable) membrane protein and *iii*. a form that is believed to be associated with lysosomes (44). The heparin displacable

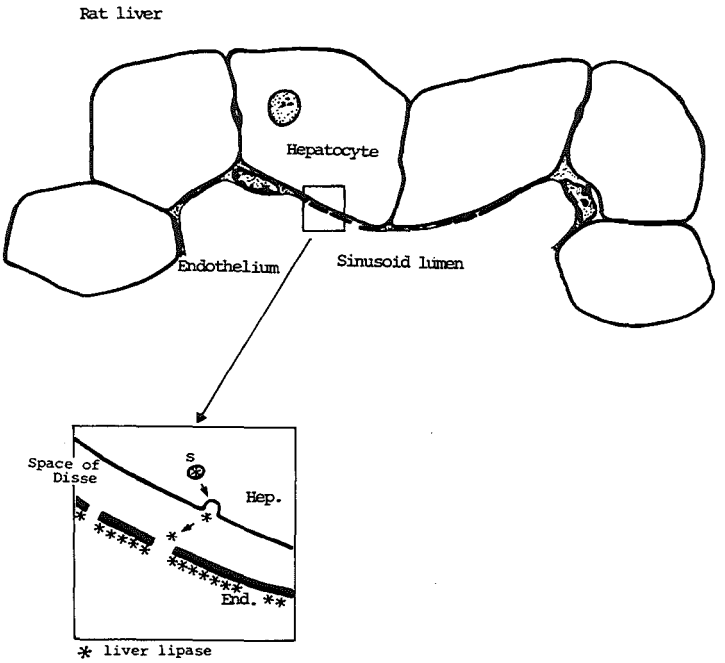

Figure 1.1
Localization of liver lipase.
After synthesis of liver lipase by the hepatocyte, this secretory protein will most probably leave the cell by the process of exocytosis. The apical membrane of the liver parenchymal cell with its numerous microvilli is in close contact with the endothelium (not shown in this figure) which may make it easy for the enzyme to pass the space of Disse before binding on the endothelium. (s = secretory granule, Hep. = hepatocyte, End. = endothelium).

and membrane intercalated forms have been shown to be largely restricted to the apex of the liver parenchymal cell (45) and were not observed onto the endothelium. In view of the localization described above these heparan sulphate proteoglycans cannot provide the binding sites for the enzyme. The lipases that have been found in the adrenal gland and in the ovary (10) are also rapidly released from the organs by heparin. In the rat ovary, dissected corpora lutea were shown to contain more than 95% of all lipase activity (46). These results also suggest a predominant extracellular localization of the lipase in these organs.

PLASMA LIPOPROTEINS

In vivo, triacylglycerol and phospholipids are organized together with free cholesterol, cholesterolester and protein in complex water-soluble structures that are called lipoproteins. The essential composition of lipoproteins consists of a hydrophobic globular core built up with triacylglycerols and cholesterolesters surrounded by a monolayer of phospholipids. These phospholipids, together with free cholesterol and proteins (called apolipoproteins) (47) are directed with their hydrophylic sites to the hydrophylic environment (48-50). Based on the density at which these lipoproteins float during ultracentrifugation, several classes have been divided (Table 1.1).

INTESTINUM DERIVED LIPOPROTEINS

The lipid constituents of these particles are derived from the food consumed: triacylglycerol, phospholipids and cholesterol, after absorption by the intestinal epithelium. In the intestinum, a remodellation of the absorbed fats together with cholesterolesters and apolipoproteins (apo) (apo B-48 and apo A) (51) takes place to form the "chylomicrons" (Fig. 1.2).

Table 1.1

Composition of human plasma lipoproteins

Lipoproteins	Chylomicrons	VLDL	IDL	LDL	HDL
Density range (g/ml)	<0.950	0.950-1.006	1.006-1.019	1.019-1.063	1.063-1.210
Apolipoproteins	A-I, A-II, A-IV, B-48, C, E	B-100, C, E	B-100, E	B-100	A-I, A-II, C, E
Triacylglycerol	78	57	39	12	2
Cholesterol (Free cholesterol + cholesterolester)	12	20	32	49	24
Phospholipid	7	13	17	18	26

Triacylglycerol, cholesterol and phospholipid are given in percentage dry weight (**Eisenberg, S. and R.I. Levy** (1975) Adv. Lipid Res. **13**:1-89).

These lipoproteins, diameter 800-5000 Å, with a low density are secreted into the mesenteric lymph and subsequently enter the blood. Here they are partly catabolyzed by lipoprotein lipase that is located on the endothelial surface lining the capillaries of muscle, heart and adipose tissues. Lipoprotein lipase is activated by apo C-II that is located on the surface of the chylomicron (25). In concert with apo C-II binding, lipoprotein lipase hydrolyzes triacylglycerol from the chylomicron's core thereby inducing its shrinkage and successive depletion of excess surface material leaving a smaller particle with a higher density: "Chylomicron remnant". Part of the fatty acids, liberated from the triacylglycerol, enter the adjacent cells where they are either oxidized or reesterified for storage, while the remaining part binds to albumin to be transported to other organs. The excess surface material is rich in phospholipids and free cholesterol and is bound to high density lipoprotein (HDL). The chylomicron remnant (300-800 Å) is able to pass the fenestrated endothelium of the liver and enters the space of Disse where it binds to a receptor on the liver parenchymal cell. The fenestrated endothelium can be regarded as a structural barrier (a sieve) excluding particles with a diameter of 1000 Å or more (52). The interaction of the remnant with the receptor is mediated by apo E on the surface of the particle (apo E and apo C-II are derived from HDL particles during circulation) (53). After binding, the remnant particle is taken up by the parenchymal cell by receptor mediated endocytosis (54) followed by the complete breakdown of the particle in the lysosomal compartment. The metabolism of exogenous fat (the removal of triacylglycerol by LPL and the uptake of the cholesterolester-rich remnants by the liver) is highly efficient in man. The half-time for the clearance of chylomicrons and their remnants from the plasma is four to five minutes, which illustrates the efficiency of the liver to remove exogenous cholesterolester.

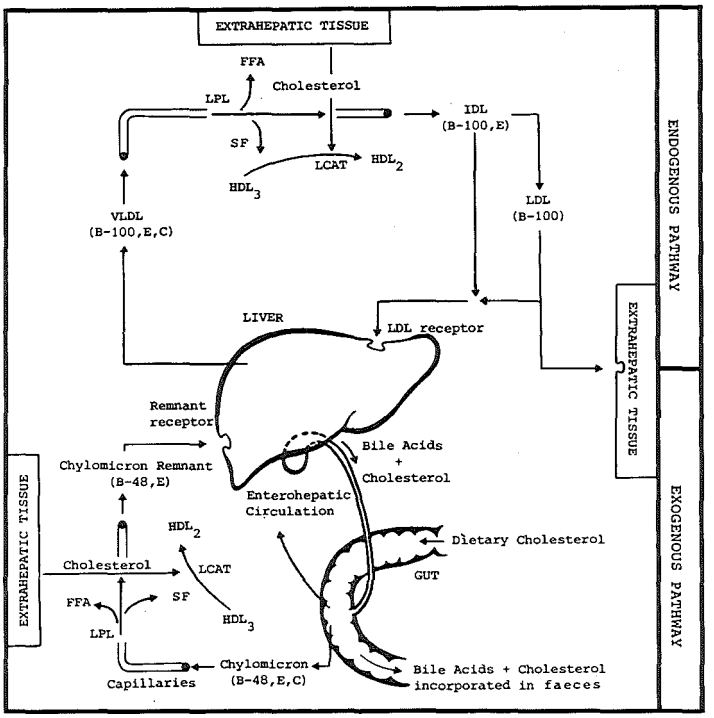

Figure 1.2
Lipoprotein metabolism in man.
The exogenous pathway is initiated by intestinal uptake of dietary fat. It enters the circulation as "chylomicrons". Hydrolysis of the core-triacylglycerol by lipoprotein lipase (LPL) in the capillaries liberates free fatty acids (FFA) and results in the release of surface fragments (SF) that are accepted by high density lipoproteins (HDL_3). Free cholesterol, derived from extrahepatic tissues by HDL_3 is esterified by lecithin:cholesterol acyltransferase (LCAT) which lowers the density of the particle (HDL_2). Lipid secreted by the liver (endogenous pathway), involving VLDL is degraded in a similar way. Remnant particles from both pathways are taken up by the liver or (in case of intermediate density lipoprotein, IDL) are partly further degraded into low density lipoprotein (LDL) particles.

LIVER DERIVED LIPOPROTEINS

Very low density lipoproteins (VLDL) (diameter 300-800 Å) are secreted by the liver (Fig. 1.2). These particles consist of triacylglycerol synthesized in the liver, cholesterolester, phospholipid, free cholesterol, apo B-100, apo E and C apolipoproteins) (51,55). In cholesterol fed animals, cholesterolesters replace much of the normally predominant triacylglycerol in the core without affecting the size of the particle (51,56). Evidently, the liver has the capacity to package and secrete VLDL from available nonpolar lipids. Cholesterol is derived from the receptor mediated uptake of cholesterol-rich particles or from de novo cholesterol synthesis in the liver (57). In the extrahepatic tissues, a similar but less efficient hydrolysis of VLDL triacylglycerol by lipoprotein lipase takes place as has been described for the chylomicron (the half time of VLDL is 1-3 h) (59). Again the density of the particle is changed, excess surface material is accepted by HDL and a new lipoprotein class indicated as intermediate density lipoprotein (IDL) or VLDL remnant, is introduced.
Most IDL particles will bind with the LDL (apo B-100,E)-receptors on the liver parenchymal cells and are then degraded as was also the case for the chylomicron remnant. Some particles however undergo further lipolysis whereby most of the remaining triacylglycerol is removed. At last, a small particle (diameter 220 Å) with almost pure cholesterolester in the core and only apo B at the surface has been formed: low density lipoprotein (LDL) (56). Extrahepatic tissues (53,57) and the liver can obtain cholesterol from LDL via a high affinity LDL (apo B-100,E) receptor in the process of receptor mediated endocytosis as has been discovered by Brown and Goldstein (54,58,59). Both exogenous and endogenous lipids are catabolyzed by three corresponding mechanisms.
i. The catabolism of triacylglycerol-rich particles by lipoprotein lipase in extrahepatic tissues. ii. The acceptance of

excess surface material (formed during the hydrolysis of the core triacylglycerols) by HDL. iii. The receptor mediated uptake of remnant particles in the liver. In the liver, most of the cholesterol is converted into bile acids which after secretion into the bile canaliculus will enter the upper intestine where dietary fats are emulsified prior to their absorption. A part of these bile acids will leave the body with faeces. Since no enzymes are present for the degradation of the sterol nucleus of the cholesterol molecule, this is the most prominent way to eliminate cholesterol (57). Most of the intestinal cholesterol as well as the bile acids are reabsorbed to be taken up by the liver to complete the socalled enterohepatic circulation. This recycling of cholesterol and bile acids limits the liver's need for cholesterol. Deprivation of the liver from intestinal cholesterol and bile acids by interruption of this cycle activates the liver to synthesize cholesterol (part of) which is again converted into bile acids. This may be accompanied by the oral administration of a bile acid binding drug, such as cholestyramine.

CHOLESTEROL HOMEOSTASIS IN THE LIVER

The concentration of free cholesterol in the liver is under stringent control. The increase of intracellular free cholesterol that results from the lysosomal breakdown of cholesterolesters obtained after the receptor-mediated uptake of cholesterol-rich particles induces a number of effects to lower its concentration. i. The ability of the cell to synthesize cholesterol is regulated through 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), that catalyzes the rate determining step in de novo cholesterol synthesis. ii. The intracellular storage of cholesterol into cholesterolester is stimulated by activation of the enzyme acyl-CoA:cholesterol acyl transferase (ACAT). iii. The delivery of more exogenous cholesterol by means of the cellular uptake of LDL

is blocked by inhibition of the synthesis of LDL receptors. Such inhibition takes place on the level of transcription of the DNA coding for the receptor protein. iiii. The liver can lower the concentration of free cholesterol by the activation of cholesterol-7α-hydroxylase to stimulate the synthesis of bile acids. The three key enzymes in cholesterol metabolism, HMG-CoA, ACAT and cholesterol-7α-hydroxylase are all regulated by reversible phosphorylation and dephosphorylation (60,61). In most other tissues, mechanisms i, ii and iii are important cellular tools to regulate the cellular concentration of free cholesterol. Just like the liver, the adrenal gland and the ovary have a fourth way to use cellular cholesterol for steroid hormone production.

ORIGIN AND METABOLISM OF HIGH DENSITY LIPOPROTEINS

In man, the high density lipoproteins can be divided into two main subclasses with solvent densities between 1.063-1.125 $^{g}/ml$ (HDL_2) and 1.125-1.210 $^{g}/ml$ (HDL_3) (62-64). The mature spherical HDL particle evolves from a nascent particle with a discoidal structure produced by the liver (65) and the intestine (66) during enrichment with cholesterolester in the circulation. The key enzyme in this process of maturation is lecithin:cholesterol acyl transferase (LCAT, E.C. 2.3.1.43) that catalyzes the transfer of an acyl chain from the 2 position of phosphatidylcholine to the hydroxyl group of cholesterol, yielding lysophosphatidylcholine and cholesterolester (67,68). LCAT is activated by apo A-I (69,70) (and apo C-I) (70,71) on the surface of the HDL particle and inhibited by apo A-II (69,71). During VLDL-triacylglycerol hydrolysis by lipoprotein lipase in vitro, particles are formed that are morphologically indistinguishable from nascent discoidal particles produced by the liver and the intestine (72). It has been postulated by Tall and Small (73) that after hydrolysis of triacylglycerols by LPL, bilayer buds are formed on the

surface. These buds easily break off in sheets and are rich in phospholipid and free cholesterol. Such surface fragments fused with HDL_3 (or alone) accumulate cholesterolester through the action of LCAT which changes the solvent density of the particle into the HDL_2 density range. In humans (and rabbits) cholesterol ester enrichment of HDL is followed by a bidirectional exchange or a transfer of cholesterolester between HDL and other lipoproteins catalyzed by cholesterolester transfer protein(s) (CETP) (74). Rats, pigs and dogs do not possess such transfer protein(s) in their plasma (64). Up till now it is not clear whether different proteins are involved in the bidirectional flux of cholesterolester or in the exchange of cholesterolester and triacylglycerol. Through the action of cholesterolester transfer protein(s), LCAT derived cholesterolester in HDL is transferred to LDL, IDL and VLDL. In man, a high percentage (up to 80%) of this cholesterolester is recovered in LDL (75). When this unidirectional transfer of cholesterolester is followed by hepatic uptake of IDL and/or LDL, this process contributes to "reversed" cholesterol transport from peripheral tissues to the liver (Fig. 1.3). Recently it has been shown by Tall (76), that cholesterolester transfer proteins may also catalyze a straight transfer of lipoprotein derived cholesterolester to the liver, independent of a receptor mediated uptake of the particles. After incubation of human hepatoma cells (Hep G2) with HDL and a 74 kD purified protein with cholesterolester transfer activity, Tall was able to demonstrate HDL-derived cholesterolester enrichment of the cells. Both systems are involved in reversed cholesterol transport, as shown in Fig. 1.3.

IN VIVO ENZYME ACTIVITY OF LIVER LIPASE

Although a lot of research has been carried out on the physiological function of liver lipase, the complete elucidation of the in vivo enzyme substrate(s) has not been accompli-

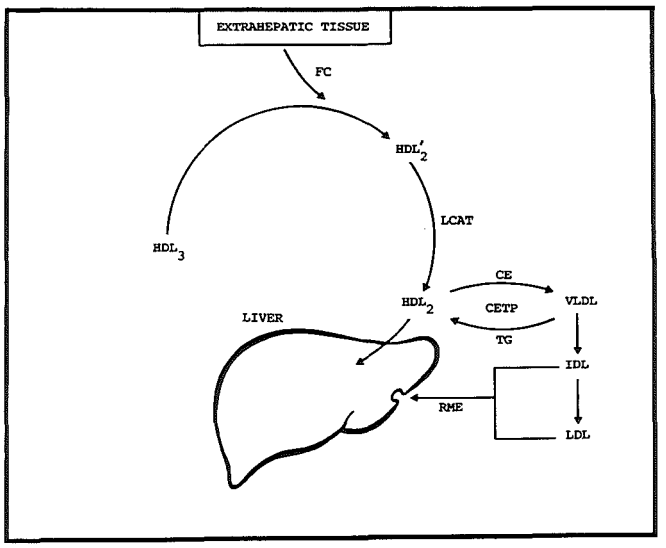

Figure 1.3
Reversed transport of cholesterol from the extrahepatic tissues to the liver in man.
Free cholesterol (FC) that is taken up by HDL_3 and subsequently converted into cholesterolester (CE) by LCAT is exchanged for triacylglycerol (TG) from VLDL (or other triacylglycerol-rich lipoproteins). By this way HDL derived cholesterolester is transferred via VLDL into the IDL and LDL classes. The receptor-mediated endocytosis (RME) of both IDL and LDL by the liver completes this reversed transport of cholesterol (in cholesterolester). As still more evidence is being presented about the existence of a specific HDL receptor, the uptake of HDL_2 particles by the liver may offer a direct route of reversed cholesterol transport. CETP = cholesterolester transfer protein.

shed. The inhibition or stimulation of liver lipase activity in man and in animals has been shown to be accompanied by a change in HDL subclasses suggesting a role of the enzyme in HDL metabolism. Antibodies have proved to be potential tools for in vivo inhibition of liver lipase activity. A short-term treatment (1 h) of a rat with a polyclonal antibody against rat liver lipase (77) did increase only the HDL_2 (78) phospholipid levels, while after a 4 h treatment significant rises in the level of HDL_2 free cholesterol, LDL phospholipids and total VLDL and IDL were observed. Increased values of other lipoprotein classes were also found by Murase and Itakura (79) and Grossner et al. (80) after a long-term (3-4 h) treatment of rats with anti-liver lipase antibodies. The initial change in lipoprotein components (within 5-30 min) after antibody administration, reported in a similar experiment by Landin et al. (81,82), even showed a higher rise in both HDL-phospholipids. From these results obtained after the in vivo inhibition of liver lipase by antibodies, it was concluded that a short-term enzyme blockade initially affects the HDL class followed by marked changes in other lipoprotein classes. In the study on the physiological function of liver lipase in humans, the enzyme activity was shown to be modulated by the action of androgens, progestins and estrogens. After the administration of androgenic (anabolic) steroids, a reduction in HDL_2 cholesterol in both athletes and sedentary subjects was observed (83). The decrease in HDL cholesterol was suggested to be the result of an increase in liver lipase activity (84). A similar relationship between liver lipase and HDL_2 was also observed after administration of progestins (norgestrel) in women (85,86). Estrogens (estradiol) on the other hand inhibit liver lipase activity (87,88) which is accompanied by an increase in the level of HDL_2 (85,89). Further evidence for a role of liver lipase in the metabolism of human HDL_2 was obtained by Kuusi (90) in a study on young healthy men where he observed a highly significant negative correlation between the concentra-

tion of HDL_2 phospholipid, HDL_2 cholesterol, HDL_2 protein on the one hand, and liver lipase activity in postheparin plasma on the other, whereas no such correlation could be observed between HDL_3 and liver lipase activity. The results presented so far and *in vitro* studies (91-93) favour a role of liver lipase in the hydrolysis of HDL_2 phospholipids. By this way, the enzyme is involved in the metabolism of lipoproteins and in cholesterol homeostasis.

i. The decrease of phospholipids in the surface of HDL_2 induces a flux of free cholesterol from the HDL particle to the underlying tissues (94-97). The enzyme therefore offers a third route of reversed cholesterol transport of HDL_2 cholesterol to the liver (Fig. 1.4). This might even be the most important pathway in animals without cholesterolester transfer proteins. Indeed, it has been shown in the rat that inhibition of liver lipase by antibodies *in vivo* induces *de novo* cholesterol synthesis in the liver (98). *ii*. The enzyme could regulate the conversion of HDL_2 into HDL_3 (64,85,90,94,99) thereby completing the interconversion of HDL subfractions involving cholesterolester transfer protein, lipoprotein lipase and lecithin:cholesterol acyltransferase (55, Fig. 1.4).

In spite of the large amount of information that favours a role of liver lipase in HDL metabolism, several scientists describe the enzyme as being important in the catabolism of IDL particles (51,63). For instance, in an epidemiological study on 117 human subjects, Nozaki et al. (100) observed an inverse correlation between both lipoprotein lipase and liver lipase on the one hand and IDL cholesterol and IDL phospholipid on the other. These correlations are difficult to understand regarding the generally accepted role of LPL in the hydrolysis of VLDL triacylglycerol. In the same group, however, a significant negative correlation between the HDL_2 to HDL_3 cholesterol ratio and liver lipase activity was observed which again is in agreement with the proposed role of liver lipase in the breakdown of HDL_2.

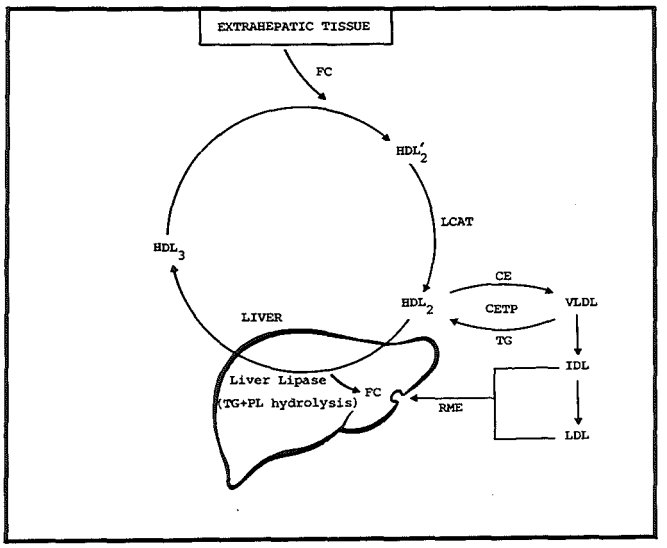

Figure 1.4
In vivo function of liver lipase in man.
HDL_2-phospholipid and triacylglycerol (derived from the action of CETP) are submitted to lipolysis during their passage through the liver. The preferred hydrolysis of HDL-phospholipids by the enzyme liver lipase (which initiates a flux of membraneous free cholesterol (FC) from the particle to the liver tissue) will be followed by the breakdown of core-triacylglycerol. This lipolytic action by liver lipase could be an important event in the circulation of HDL subfractions for the continuation of the process of reversed cholesterol transport to the liver.

ATHEROSCLEROSIS AND LIVER LIPASE

In man, the dynamics of lipoproteins, carrying cholesterol (derived from the endogenous and exogenous pathways) between the several tissues are under stringent control (101). An impaired lipoprotein metabolism caused by acquired or inborn defect(s) may have important consequences for the individual (102, 103). In the United States, fifty percent of all deaths is related to "atherosclerosis", which is generally accompanied by a disorder(s) in lipid metabolism causing the accumulation of cholesterol-rich plaques in the wall of arteries. Plaque formation during decades might suddenly inhibit the blood flow after subsequent blood platelet aggregation in an artery, causing tissue ischemia. Coronary artery obstruction may lead to a heart attack and cerebral artery obstruction to a stroke. Ross and Glomset (104,105) have proposed a model for the pathogenesis of atherosclerosis, including initial endothelial damage (106) followed by the penetration of LDL particles, blood platelets and, later on, of monocytes. These monocytes are transformed into macrophages that (together with proliferating smooth muscle cells) accumulate cholesterol derived from LDL (107, 108). More recently, a study with the non human primate as an animal model further supported the model of Ross and Glomset (119).
Such cholesterol-rich cells (called "foam cells") initiate the formation of an atheroma resulting in the narrowing of an artery. A disordered lipoprotein profile, cigarette smoking, hypertension (109), stress (type "A" behavior) (110) and positive family history of coronary heart disease are risk factors for the development of atherosclerosis. With respect to plasma lipoproteins, a high concentration of LDL-cholesterol is generally believed to be the main risk factor for coronary artery disease (111,112) while the concentration of HDL-cholesterol seems to be negatively correlated to this process (113-116). Therefore, for the development of atherosclerosis, factors

involved in the formation and catabolism of lipoproteins are important in our understanding of the mechanisms.

Goldstein and Brown showed that familial hypercholesterolemia can be explained by the defective removal of LDL from the circulation, due to mutation(s) in the gene coding for the LDL-receptor (58,59). I feel that defects in apolipoprotein structure might even cause a much greater incidence in the development of atherosclerosis. These proteins are involved in almost all reactions that can affect lipoprotein metabolism. Therefore, research is being carried out on genetic polymorphisms of the DNA coding for apolipoproteins (117). An example of a genetic polymorphism is the so-called restriction fragment length polymorphism, which refers to an altered pattern of gene fragments observed when DNA from different individuals is digested with a restriction endonuclease (118). Recently, information has also been presented on the possible link between lipolysis and atherosclerosis. In a study on posthepatin plasma lipases in men who had undergone coronary arteriography to assess atherosclerosis (11), liver lipase activity was shown to be lowered in patients with atherosclerosis compared to a group of control patients. In the "Leiden Intervention Trial" (12), patients with stable angina pectoris were studied by coronary arteriography before and after a 2 years during intervention with a vegetarian lipid lowering diet (enriched in polyunsaturated fatty acids) and lipolytic enzymes were measured. After the dietary intervention, liver lipase activity was found to be the variable that correlated most strongly with regression of atherosclerosis! Patients with an initially high ratio total cholesterol/HDL-cholesterol that showed regression or no progression of coronary atherosclerosis after the intervention, were normal in liver lipase activity, whereas patients with an observed progression of the disease had low liver lipase values (13). These results suggest that not only a high HDL-cholesterol but also a normal liver lipase activity are important to establish a non athero-

sclerotic condition. From the biochemical point of view, a normal process of reversed cholesterol transport may require the action of liver lipase for the proper catabolism of HDL_2 particles (carrying cholesterol derived from the tissues to the liver) and the turnover of HDL_2 into HDL_3 for the continuation of this process.

THE AIM OF THE STUDY

From the introduction, it will be clear that liver lipase is involved in the metabolism of lipoproteins. The exact function(s) of the enzyme in lipoprotein metabolism, however, still remains to be elucidated which of course is necessary for the understanding of a possible relationship between liver lipase activity and regression of atherosclerosis (13). Basic research on the enzyme is needed to reveal the properties of the protein itself. In this study, research is carried out on the molecular structure, the localizaton and the function of the lipase from the liver and the liver-type lipases that are present in the adrenal gland and in the ovary of the rat. Earlier, antibodies against the liver enzyme had been developed. Such antibodies must be monospecific for the liver enzyme, well characterized and be usable in sufficient amounts. Because of these considerations and others (see below), the isolation of a monoclonal antibody against the rat liver enzyme has been undertaken.

As compared with the conventional polyclonal antisera, a monoclonal antibody may have certain advantages. In theory, a hybridoma cell line can be a long lasting source of a well-characterized antibody. Inhibition of lipase activity by a monoclonal antibody might reveal how the enzyme is capable in hydrolyzing different substrates. When using a polyclonal antiserum for enzyme inhibition, all activity is completely blocked, most probably because of the interaction of several antibodies with the lipase. A monoclonal antibody may be

useful in localization studies as the problem of cross-reactivity is often less compared with a polyclonal antibody. For the production and isolation of monoclonal antibodies, immunological assays must be developed and adapted for the reliable, efficient and quick detection of anti-liver lipase antibodies in hundreds of hybridoma supernatants.

An immunoprecipitation assay and an enzyme linked immunosorbent assay that were developed for the screening of antibodies against rat liver lipase are described in chapters II and III. In chapter III, monoclonal antibodies against the rat liver enzyme are introduced. These antibodies were characterized and tested for cross-reactivity with other lipases. Immunoprecipitation of phospholipase A_1 activity of liver lipase by the monoclonal antibodies together with protein modification and mild proteolytic degradation of the enzyme, are presented in chapter IV. These techniques were used to study the capability of the enzyme in performing both phospholipase A_1 and triacylglycerol hydrolase activity.

In chapter V the separation of different preparations of monoclonal antibodies by Fast Protein Liquid Chromatography are shown. Ascites preparations were used for <u>in vivo</u> inhibition of rat lipase activity. Until now, the localization of the enzyme in extrahepatic tissues was not published. Chapter VI presents the results of an immunolocalization study of the heparin releasable lipases in the rat liver, adrenal gland and ovary. The characterization of a lipase that was identified in the medium of an established human hepatoma cell line (Hep G2) is shown in chapter VII.

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CHAPTER II

TWO DIFFERENT SCREENING ASSAYS FOR THE DETECTION OF ANTIBODIES AGAINST RAT LIVER LIPASE

For the initial screening of immunized mice on the production of antibodies that are directed against rat liver lipase, two different immunological assays were used. First of all, the sera of five immunized mice (and two non-immunized mice) were tested in immunoprecipitation, using a second anti-mouse antibody (from rabbit) and Sepharose-protein A as the precipitating agent. Protein A is a molecule of molecular weight 42 kD, made by most strains of Staphylococcus aureus that binds the F_c portion of certain IgG subclasses of several species including the rabbit. A schematic presentation of this assay is shown in Fig. 2.1.
Five µl of the serum of all immunized mice (1-5) precipitated a total amount of 63-78% of the salt-resistant triacylglycerol hydrolase activity in 10 µl postheparin rat plasma as compared with the serum of non-immunized mice (B_1 + B_2) (Fig. 2.2).
All five hybridoma cell lines described in chapter III were obtained after isolation of spleen cells from mouse 3 (Fig. 2.2) for the hybridization procedure.

The serum of one of the immunized mice was used for optimalization of a micro enzyme-linked immunosorbent assay. A schematic presentation of this assay, using a second anti-mouse antibody (from sheep) linked with β-galactosidase is shown in Fig. 2.3 (see also Materials and Methods of chapter III).

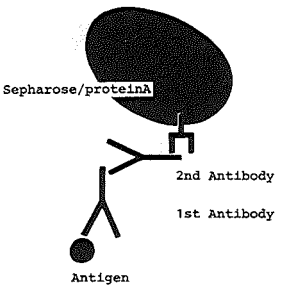

Figure 2.1
Immunoprecipitation assay.

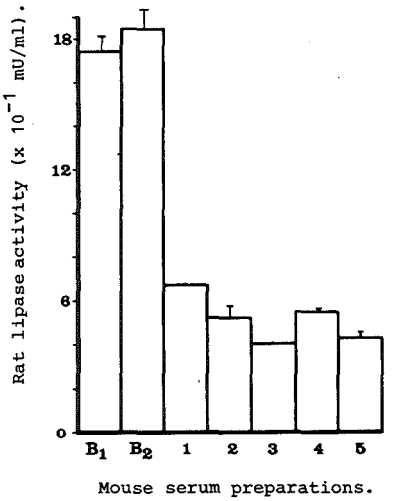

Figure 2.2
Immunoprecipitation of salt-resistant triacylglycerol hydrolase activity of 10 µl postheparin rat plasma with 5 µl of the serum from two non-immunized mice (B_1 and B_2) and 5 immunized mice (1-5).

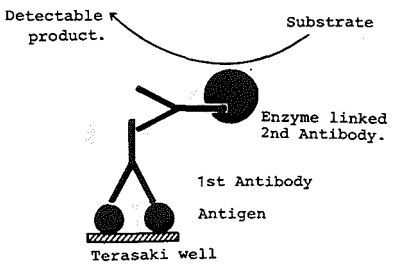

Figure 2.3
Enzyme linked immunosorbent assay.

Immobilization of the antigen (liver lipase, purified by Sepharose-heparin affinity chromatography) on the wells of Terasaki trays was carried out with poly-L-lysine, heparin, a combination of both or without a binding reagent (Fig. 2.4).

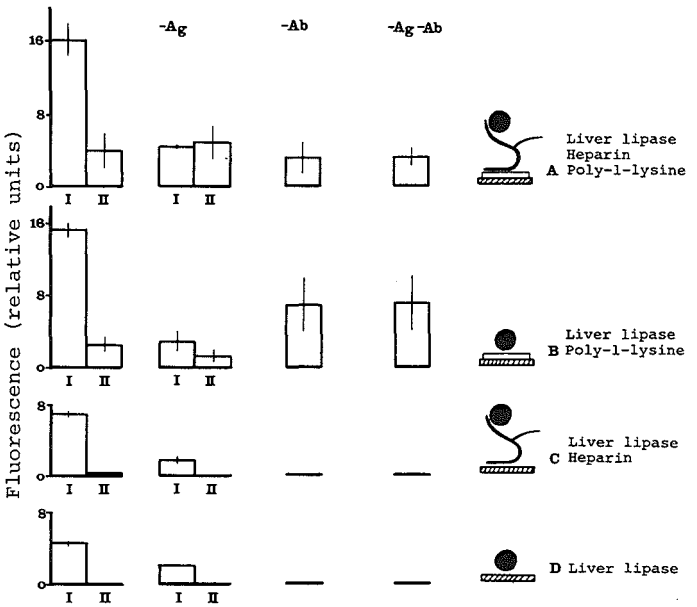

Figure 2.4
Four different ways of immobilization of rat liver lipase on the Terasaki trays were tested for optimilization of the ELISA.
5 µl of a purified sample of liver lipase was used as the antigen (Ag) and 5 µl of the serum of an immunized (I) or a non-immunized mouse (II) was used as the antibody (Ab). In this assay, controls were included by leaving out the antigen (-Ag), the antibody (-Ab) or both (-Ag,-Ab). The trays were precoated with A: 25 µl poly-L-lysine (0.1 mg/ml, 1h, 20°C) followed by heparin (20 µl, 15 h, 4°C) or with B: poly-L-lysine alone or C: heparin alone or were not precoated: D.

It is clear from this figure that poly-L-lysine as a binding reagent results in higher signals but also increases the nonspecific binding of the first and especially the second antibody with the well (situation B, Fig. 2.4). Heparin alone (situation C, Fig. 2.4) gives a better result than without the use of a binding reagent (situation D, Fig. 2.4) with less nonspecific binding (-Ag). Heparin has a stabilizing effect on enzyme activity (see ref. 36, chapter I) and heparin-like structures may also be involved in the immobilization of the lipase onto the liver endothelium *in vivo*. Because of these reasons and the fact that less nonspecific binding is observed in situation C (Fig. 2.4), Terasaki trays have further always been precoated with heparin when using this enzyme-linked immunosorbent assay as a screening assay for the detection of monoclonal antibodies against rat liver lipase.

CHAPTER III

MONOCLONAL ANTIBODIES AGAINST SALT-RESISTANT RAT LIVER LIPASE. Cross-reactivity with lipases from rat adrenals and ovaries

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(1986) Biochim. Biophys. Acta **875**:286-292

ABSTRACT

To obtain monoclonal antibodies against rat salt-resistant liver lipase, mice were immunized with enzyme purified from heparin-containing rat liver perfusates. Hybridomas were screened for antibody production by means of an enzyme linked immunosorbent assay (ELISA) and an immunoprecipitation assay. Five hybridoma cell lines secreting antibodies against rat liver lipase indicated as A, B, C, D and E, have been obtained. All antibodies possess gamma one (γ_1) heavy chains and kappa (K) light chains. The antibodies precipitate salt-resistant lipase from rat postheparin plasma, are positive in ELISA, inhibit liver lipase activity and bind monospecifically with the enzyme as shown by immunoblotting. The monoclonal antibodies showed no significant reactivity with human liver lipase. The salt-resistant lipases of rat adrenals and ovaries are also precipitated by the monoclonal antibodies directed against the liver enzyme. Therefore the heparin-releasable lipases of the liver, adrenals and ovaries possess identical epitopes.

INTRODUCTION

Hepatic triacylglycerol hydrolase also referred to as "liver lipase", is a salt-resistant lipase that is found in the liver of many if not all species (1). The rat enzyme is synthesized and secreted by parenchymal liver cells and binds, *in vitro*, to isolated non-parenchymal (endothelial) liver cells in a saturable manner (2). *In situ*, the lipase has been located at the vascular endothelium by immunocytochemical techniques using polyclonal antibodies (3). Therefore it is likely that the enzyme, after secretion by the parenchymal cells, is transported to the endothelium where it exerts its physiological function. Indeed, the binding of injected polyclonal antibodies to the lipase at the liver endothelium

results in marked changes in serum lipoprotein levels (1). An enzyme with the same characteristics as liver lipase has been found in rat ovaries and in the adrenal gland of a number of species (4). Both the ovarian and the adrenal lipases are resistant to high NaCl concentrations in vitro, are eluted from Sepharose-heparin columns by the same salt concentration as liver lipase and react with polyclonal antibodies raised against the liver enzyme, indicating a close relationship between the enzymes in different tissues. Polyclonal antibodies against the liver lipase (5), are frequently used to identify the enzyme. Nevertheless, monoclonal antibodies were raised, as they have certain advantages over a polyclonal antibody. For instance, in immunocytochemistry less bulky complexes with the enzyme (and less cross-reactivity) may be expected to be formed. During studies in which antibodies are administered in vivo non-specific steric hindrance will be lessened. Moreover the monospecificity of polyclonal antibodies even if raised against highly purified enzymes is often difficult to assess. The monoclonal antibodies against rat liver lipase were raised by the technique developed by Köhler and Milstein (6). Their cross-reactivity with salt-resistant lipases from various tissues is presented.

MATERIALS AND METHODS

Immunization of mice

Five female BALB/c mice were primed intraperitoneally with rat liver lipase (100 mU lipase activity each) emulsified in an equal volume of complete Freund's adjuvant (DIFCO laboratories, Detroit, U.S.A.) (200-400 µl total). Liver lipase was isolated according to Jensen and Bensadoun (7), except that Triton N101 was not removed. The sample was thoroughly dialyzed against 0.9% NaCl before injecting into the mice. Six weeks after priming, the mice were boostered intraperitoneally with rat liver lipase (160-200 mU) emulsified in incomplete

Freund's adjuvant (DIFCO). Two weeks after the first booster, the serum of the mice was tested for antibody production by means of an enzyme linked immunosorbent assay (ELISA) and an immunoprecipitation assay. One month later the mice were boostered again (intravenously) with rat liver lipase (120 mU, 300 µl). After 3 days the spleens of 2 positive mice were removed to be used in the hybridization procedure.

Monoclonal antibody production

The fusion procedure was carried out as described earlier (8, 9) using Sp2/0-Ag-14 as the mouse myeloma cell line (10). Eleven days after fusion, supernatants from all the wells were screened on antibody production by means of an ELISA. Wells that scored positive in ELISA were also tested in an immunoprecipitation assay. Hybridomas in wells that scored positive in both tests were cultured in 24 well plates (Costar, 205 Broadway, Cambrigde, U.K.). in regular culture medium (RPMI medium supplemented with 15% (^{v}/v) fetal calf serum) and in flasks (Costar) in 5 ml medium. Supernatants of the cultured hybridomas were tested several times in ELISA and immunoprecipitation. To reduce the risk of overgrowth by non-producing variants, cells from 5 different wells were plated as soon as possible 3 or 4 weeks after fusion at 2, 5 and 10 cells per well on a feeder layer of spleen cells (part of the cells was frozen). Cell cultures were considered as monoclonal when more than 37% of the wells in a 96 well plate had no proliferating hybridomas and most of the cells were positive in ELISA and immunoprecipitation (9). Further subclonation yielded identical results.

Enzyme linked immunosorbent assay

Liver lipase was partially purified from heparin-containing rat liver perfusates by means of Sepharose-heparin affinity chromatography, concentrated by $(NH_4)_2SO_4$ precipitation and dialysed against 50 mM Tris buffer, pH=7.2 (7). This prepara-

tion was used as the antigen and was immobilised on the wells of Terasaki trays (60 well plates, Falcon, Cockeysville, U.S.A.), using heparin as a binding reagent. After the immobilization of the lipase and several washing procedures, the enzyme is still active in hydrolyzing palmitoyl-CoA which can be visualized in vitro (11). The lipase can only be washed from the heparin under 0.75 M salt conditions. Wells were precoated with 20 µl heparin (5000 IU/ml) (Tromboliquine, Organon, Oss, The Netherlands) overnight at 4°C. Five µl of the antigen were incubated in the wells for 1 h at room temperature, followed by a washing procedure consisting of washing twice with phosphate buffered saline (0.8% (^{w}/v) NaCl, 0.02% (^{w}/v) KCl, 0.143% (^{w}/v) $Na_2HPO_4.2H_2O$ and 0.02% (^{w}/v) KH_2PO_4) supplemented with 0.02% (^{w}/v) gelatine and twice with the same buffer with gelatine and 0.1% (^{v}/v) Tween-20. Five µl of the serum from the immunized mice diluted several times as indicated or 15 µl of hybridoma supernatant were added as the first antibody and incubated for one h at room temperature followed by washing 3 times with the washing buffers as described above. Sheep anti-mouse immunoglobulins, linked with β-galactosidase (Amersham International PLC, Amersham, Buckinghamshire, U.K.) were used as the second antibody (12). Five µl sheep anti-mouse β-galactosidase (in a dilution of ^{1}/300 in phosphate buffered saline) were incubated for 1 h at room temperature followed by a washing procedure as described above. The artificial substrate, 4-methylumbelliferyl galactopyranoside was used. Five µl 0.025% (^{w}/v) of this substrate was incubated for 1 1/2 h in a humidified atmosphere at 37°C, the reaction was terminated with 5 µl 1 M Na_2CO_3. Fluorescence in the individual wells of the Terasaki trays was then quantitatively analysed using a scanning inverted micro fluorometer, connected to a digital voltmeter and a desk-top calculator (13). A well is called ELISA-positive when the fluorescence exceeds 10-times the background.

Antibody subclass typing

Antibody subclass typing was carried out by means of an ELISA. First of all, wells were coated with rat anti-mouse monoclonal antibodies specific for immunoglobulin heavy chain classes, followed by an incubation with hybridoma supernatant. Mouse monoclonal antibodies were detected using a β-galactosidase conjugated anti kappa light chain antibody. The substrate 4-methylumbelliferyl galactopyranoside was added and fluorescence in the individual wells was detected as described above.

Immunoprecipitation

A mixture of antigen (liver lipase preparations from different origin) and antibody (supernatants of the 5 clones or serum of the immunized mice) was incubated for 1 h on ice. As a second antibody, whole rabbit anti-mouse immunoglobulins (Ig, Nordic Immunology, Tilburg, The Netherlands) were used. 10 µl from a stock solution of 1 ml of lyophilized antibody dissolved in phosphate buffered saline were added and incubated for 1 h on ice. 50 µl of a 1:1 suspension of protein A-Sepharose 4B (Pharmacia, Uppsala, Sweden) in phosphate buffered saline was used as the precipitant. Incubation was performed for 1 1/2 h with gentle rotation at 4°C. Samples were centrifuged and lipase activity was determined in 100 µl of the supernatants. As controls supernatants of 2 hybridoma cell lines (C_1 and C_2) were used. C_1 is negative in both an ELISA and an immunoprecipitation assay with rat liver lipase while C_2 is as positive in an ELISA with rat liver lipase as the 5 monoclonal antibodies A, B, C, D and E. A polyclonal antibody against rat liver lipase (from goat) that completely precipitates the rat enzyme, served as a reference. Liver lipase is not inhibited by protein A-Sepharose, rabbit anti-mouse antibodies or the tissue culture medium.

The immunoprecipitation of postheparin plasma lipase was carried out as follows: 10 µl postheparin rat plasma was

diluted (1:1) with phosphate buffered saline and incubated with 1, 2.5, 5, 10, 30 or 50 µl hybridoma supernatant (buffer was added to a total volume of 70 µl) and tested in immunoprecipitation. C_1 (50 µl) served as a control. Human postheparin plasma (20 µl) was used undiluted.

Immunoblotting

SDS-polyacrylamide gel electrophoresis of rat liver lipase was carried out on a 3% stacking gel and a 10% resolving gel as described by Laemmli (14). Proteins were visualized by silverstaining. Liver lipase was isolated from rat liver perfusates by Sepharose-heparin affinity chromatography, concentrated by ammonium sulphate precipitation and dialyzed against 50 mM Tris-HCl, pH=7.2. After electrophoresis, proteins were electroblotted onto nitrocellular paper according to the method described by Towbin et al. (15). After blotting, nonspecific protein binding sites were blocked with 2% ($^{w}/v$) BSA in 0.9% NaCl, pH=8 for 3 h.

The blot was divided into separate lanes and each lane was incubated for 1 h with hybridoma supernatant, followed by an incubation with ^{125}I-labeled anti-mouse whole antibodies from sheep (Amersham, U.K.) as a second antibody. As a reference, one lane was incubated for 1 h with a purified immunoglobulin fraction of a polyclonal antibody from rabbit against rat liver lipase followed by an incubation with ^{125}I-labeled protein A (Amersham, U.K.). During each labeling procedure, the blots were thoroughly washed with phosphate buffered saline, 0.05% Tween 20. The immunoblots were dried and mounted on top of Kodak XAR 5 films and developed after 3 days.

Enzyme inhibition assay

Rat postheparin plasma (10 µl) was incubated with 50 µl hybridoma supernatant on ice for 1 h and tested for salt-resistant lipase activity.

Isolation of salt-resistant ovarian lipase and adrenal lipase

An acetone powder of 80 ovaries from normal rats was homogenised in 15 ml 10 mM Na phosphate buffer (50% (^{v}/v) glycerol, 5 IU heparin/ml, pH=7.0) on ice and centrifuged. The supernatant was loaded on a column of Sepharose-4B to which heparin was covalently bound (Sepharose-heparin, 8.5 x 0.5 cm). After washing the column with sodium phosphate buffer, a linear salt gradient of 0.0 - 2.0 M NaCl, in the same buffer as described above with a total volume of 40 ml was applied. Fractions (1.0 ml) were collected at 4°C and 100 µl of the fractions were tested for salt-resistant lipase activity immediately (salt concentrations in the fractions were determined by conductivity measurements). 100 µl of the pooled peak fractions, were tested in the immunoprecipitation assay. Twelve rat adrenals were homogenised in 3 ml sodium phosphate buffer containing 5 IU heparin/ml on ice and centrifuged. The supernatant (15 µl) was tested in the immunoprecipitation assay.

Biochemical measurements

Lipase activity was measured as triacylglycerol hydrolase activity, using an artificial tri[^{3}H]-oleoylglycerol emulsion in the presence of 1.0 M NaCl (16) (assay was during 30 min at 30°C). Lipase activities are given in mU/ml (1 mU represents 1 nmol free fatty acid released from the substrate in 1 min at 30°C).

RESULTS

Monoclonal antibody production

Two weeks after the first booster (8 weeks after priming), the serum of all 5 immunized mice contained antibodies against rat liver lipase. In an immunoprecipitation assay, 5 µl of the serum from the mice precipitated 63-78% of the salt-resistant lipase activity in 10 µl of postheparin rat plasma

(the serum of 2 non-immunized mice served as controls, not shown) . Compared with the controls, the serum of all 5 mice was strongly positive in ELISA using a partially purified rat liver lipase preparation as the antigen. Fig. 3.1 shows 3 typical curves obtained with the serum of 1 control and 2 positive mice. These 2 positive mice were further used for monoclonal antibody production.

After fusion, ELISA positive results were only obtained with 1 mouse (Fig. 3.1, □). The serum of this mouse is strongly positive in ELISA even after a 2000 times dilution in phosphate buffered saline (Fig. 3.1) and precipitated the enzyme in postheparin rat plasma for 78% (not shown). Hybridomas from 5 different wells, positive in ELISA and immunoprecipitation after recloning twice, were derived from this mouse.

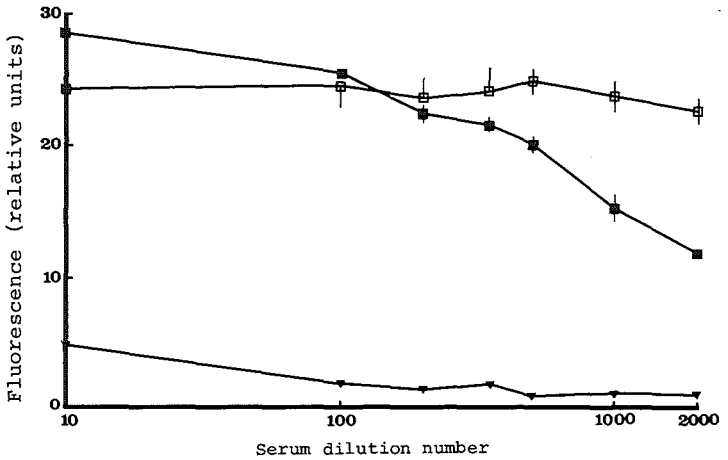

Figure 3.1
Enzyme linked immunosorbent assay of the serum of immunized mice with rat liver lipase (mouse 2 (□) and mouse 3 (■)) and a non-immunized mouse (▼). Measurements were carried out in triplicate. The serum dilution number is given on a logarithmic scale.

For simplicity, the 5 monoclonal antibodies from the 5 hybridoma cell lines: 6C2.6B6.1E2, 5F3.8E5.3C3, 5F9.2C4.5C6, 4F3.3F10.7B5 and 5C10.5F4.9B8 are indicated as A, B, C, D and E, respectively. These 5 monoclonal antibodies and control C_2 all possess gamma one (γ_1) heavy chains and kappa (K) light chains.

Reactions of the monoclonal antibodies

Binding with salt resistant liver lipase.

Fig. 3.2 shows an immunoprecipitation experiment of postheparin rat plasma with different amounts of hybridoma supernatants A, B, D and E. The results obtained with C were identical with those obtained with D and are therefore not given in the figure. The control (0 µl hybridoma supernatant) repre-

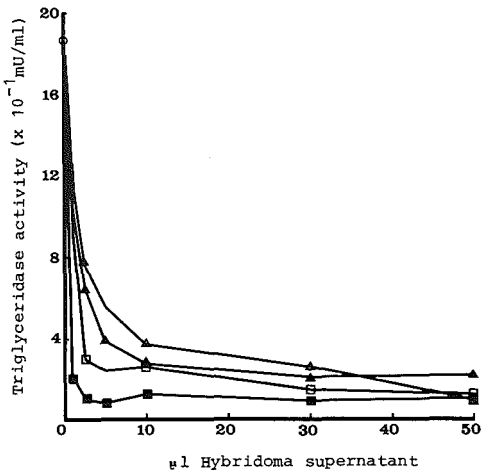

Figure 3.2
Immunoprecipitation of salt-resistant triacylglycerol hydrolase activity of 10 µl rat postheparin plasma with several amounts of hybridoma supernatant (A (■), B (□), D (△) and E (▲), phosphate buffered saline was added to a total volume of 70 µl. Hybridoma supernatant C_1 (50 µl) was used as a control (in the figure (O), at 0 µl hybridoma supernatant).

sents the result of 50 µl control hybridoma supernatant C_1 in the same experiment. It is seen that 1 µl A almost completely precipitates the enzyme activity, while 1 µl of the other supernatants precipitate the enzyme activity for 50% (B and E) or less (C and D). By increasing amounts of A, B, D and C (not shown) the lipase activity is precipitated for 94%. Addition of more than 10 µl E gives no further lowering of enzyme activity so that maximally 88% is precipitated by this antibody. The salt-resistant lipase activity of postheparin human plasma was not precipitated by an amount of antibody found to be optimal with rat plasma (50 µl) of any of the clones (not shown). After isolation of the monoclonal antibodies on an anion exchange mono Q (Pharmacia) column by means of Fast Protein Liquid Chromatography, identical results were obtained in immunoprecipitation (unpublished data). To study to what extent the antibodies are able to inhibit enzymatic activity, an enzyme inhibition assay with 10 µl rat postheparin plasma and 50 µl hybridoma supernatants was performed. All monoclonal antibodies inhibited lipase activity to different extents whereas enzyme activity is almost completely inhibited by the polyclonal antibody (Table 3.1). C and D always inhibited enzyme activity less (about 38%) than A (85%) and B and E (about 75%). Immunoblotting of a partly purified sample of liver lipase from rat liver perfusates with the 5 monoclonal antibodies and a polyclonal antibody from rabbit is shown in Fig. 3.3. While polyacrylamide gel electrophoresis shows at least 13 major and minor protein bands (Fig. 3.3) after immunoblotting only a 58 kD and sometimes a 53 kD protein band are visualized. From other experiments it is known that rat liver lipase is completely inhibited by the rabbit polyclonal antibody.

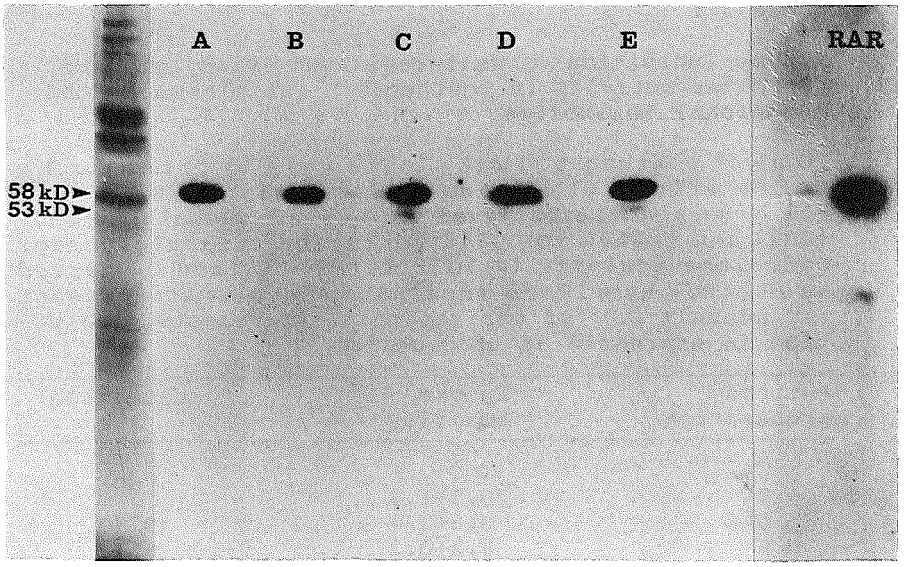

Figure 3.3
Immunoblotting of a partly purified sample of rat liver lipase using the 5 monoclonal antibodies A, B, C, D and E and a polyclonal antibody against rat liver lipase from rabbit (RAR) as the first antibody. As a second antibody, ^{125}I-labeled anti-mouse whole antibodies were used. The polyclonal antibody was detected by ^{125}I-labeled protein A. The gel was visualized by silverstaining.

Cross-reactivity with salt-resistant ovarian and adrenal lipase

A salt-resistant lipase from rat ovaries was isolated by Sepharose-heparin affinity chromatography (4). An immunoprecipitation assay using 100 µl of the most active fractions and 50 µl hybridoma supernatant is shown in Table 3.2. Compared with the controls C_1 and C_2, the monoclonal antibodies precipitate salt-resistant lipase activity from ovarian origin. The polyclonal goat anti-rat liver lipase that is shown to precipitate the ovarian and the adrenal enzyme served as a refe-

Table 3.1

Enzyme inhibition of salt-resistant triacylglycerol hydrolase activity in postheparin rat plasma by the monoclonal antibodies.

10 µl of postheparin rat plasma was incubated for 1 h on ice with 50 µl hybridoma supernatant A, B, C, D, E and a goat anti-rat liver lipase polyclonal antibody (GAR) and tested for salt resistant triacylglycerol hydrolase activity (x 10^{-1} mU/ml). C_1 and C_2 served as control hybridoma supernatants. Results present the means ± S.D. of two separate experiments. Inhibition is expressed as percentages.

Antibody preparation	Enzyme activity	Inhibition
C_1	20.0±0.9	0
C_2	28.9±1.4	
GAR	0.8±0.1	97±0
A	4.2±0.3	85±1
B	6.9±0.6	76±2
C	17.0±1.6	41±6
D	18.7±0.2	35±1
E	7.8±0.3	73±1

rence.

An immunoprecipitation of salt-resistant lipase from adrenals (Table 3.2) using an equal amount of enzyme activity as was used with the ovarian lipase, shows similar results. The adrenal lipase is precipitated by all monoclonal antibodies and the goat anti-rat liver lipase polyclonal antibody. Inhibition assays on the lipase activity of ovarian- and adrenal lipase preparations gave similar results as in the immunoprecipitation tests (not shown).

Table 3.2

Immunoprecipitation of salt-resistant rat ovarianand rat adrenal triacylglycerol hydrolase activity.

As described in the text, 50 µl hybridoma supernatant A, B, C, D, E and a goat anti-rat liver lipase polyclonal antibody (GAR) were incubated with rat ovarian and rat adrenal lipase in an immunoprecipitation assay. C_1 and C_2 served as control hybridoma supernatants. Results present the means ± S.D. of experiments in duplicate.

Antibody	Precipitation of lipase activity	
	Ovarian lipase	Adrenal lipase
C_1	0[a]	0[a]
C_2	0[a]	0[a]
GAR	89±9	81±15
A	93±2	81±4
B	87±9	98±23
C	78±2	70±9
D	67±2	66±2
E	82±13	79±2

[a] At 0% precipitation, enzyme activity is 0.47 mU/ml.

DISCUSSION

Five hybridomas secreting antibodies against rat liver lipase have been obtained. The serum of the mouse whose spleen was used in the fusion procedure was most strongly positive in ELISA and immunoprecipitation. Differences in immunotitration with the 5 monoclonal antibodies obtained (Fig. 3.2) may be caused either by differences in binding affinity of the antibodies for rat liver lipase and/or by differences in antibody concentration in the hybridoma supernatants. Antibody E which does not precipitate enzyme activity to the same extent as A, B, C and D (Fig. 3.2), probably has a lower binding affinity for the antigen.

In contrast to the monoclonal antibodies raised against dog liver lipase recently described by Chuang et al. (17), all our monoclonal antibodies bind and directly inhibit liver lipase activity. If this is not due to a species difference it would mean that their and our antibodies are directed against different epitopes on the lipase molecule.

Due to the great excess of antibody that is used in the enzyme inhibition assay (Table 3.1), a lowering of liver lipase activity in post heparin rat plasma cannot be explained by precipitation. From these results we conclude that at least 3 monoclonal antibodies against rat liver lipase with a different specificity have been obtained. Inhibition of activity of the rat enzyme by the monoclonal antibodies may be due to steric hindrance caused by the antibodies either in the process of binding of the enzyme with its micellar substrate or by interference with its active site for lipase activity. After immunoblotting a 58 kD protein band is recognized by all monoclonal antibodies and the polyclonal antibody (Fig. 3.3). Monoclonal antibodies C and E as well as the polyclonal antibody (which is difficult to see on the photograph) also bind with a 53 kD protein band. Recently Cisar and Bensadoun (18) described similar molecular weights of rat liver lipase after purification and SDS gel electrophoresis. The results with the immunoblotting show (1) a monospecific binding of the monoclonal antibodies and the polyclonal antibody with the lipase and (2) homology between the 58 kD and the 53 kD protein band. Therefore the 53 kD protein band most probably will be a proteolytic breakdown product of the 58 kD protein band. The monoclonal antibodies showed no significant cross-reactivity with salt-resistant lipase in human postheparin plasma, which indicates that they are raised against other epitopes. The goat anti-rat liver lipase polyclonal antibody, however, strongly precipitates the human enzyme (unpublished data), indicating homologous epitopes on the enzymes from human and rat origin.

From the characteristics that are described in the Introduction and the results obtained with the 5 monoclonal antibodies in immunoprecipitation of the salt-resistant lipase from rat adrenals and ovaries (Table 3.2) it is concluded that these enzymes and the liver enzyme have epitopes in common. It is tempting to speculate that these enzymes are identical. Therefore it is interesting to know whether these enzymes are synthesized and secreted by the different organs or whether they are all derived from the liver. We expect that the availability of monoclonal antibodies against rat liver lipase will facilitate the study on this enzyme in vivo. For this purpose, the use of Fab (papain) fragments or $F(ab')_2$ (pepsin) fragments could be better tools for obtaining a partially lowering of enzyme activity than the polyclonal antibody.

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CHAPTER IV

STRUCTURAL MODULATION OF SALT-RESISTANT RAT LIVER LIPASE ALTERS THE RELATIVE PHOSPHOLIPASE AND TRIACYLGLYCEROL HYDROLASE ACTIVITIES

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ABSTRACT

This chapter demonstrates that structural modification of the heparin-releasable salt-resistant lipase of rat liver (liver lipase) alters its relative capacity to hydrolyze phospholipid and triacylglycerol emulsions. Enzymatic activities were modified by immunoinhibition and proteolysis by selective amino acid agents. Binding of 3 different monoclonal antibodies resulted in a lower extent of inhibition of phospholipase A_1 than of triacylglycerol hydrolase activity. Degradation of the enzyme by trypsin under mild conditons led to a decrease of both enzyme activities in a different way. Triacylglycerol hydrolase activity was less affected than the phospholipase A_1 activity. Visualization of the proteolysis of the purified enzyme by immunoblotting revealed the actual breakdown of a 58 kD protein into a 53 kD protein band and subsequently in a 48 kD one. Incubation of the purified enzyme by N-tosyl-L-phenylethylchloromethylketone (acting on cystein or histidine) or N-ethylmaleimide (a sulfhydryl reagent) did not influence either enzyme activity. On the other hand, after the selective modification of lysine residue(s) by phenylisothiocyanate, the phospholipase A_1 activity was stimulated by 68% whereas the triacylglycerol hydrolase activity was completely lost.

The role of a lysine residue(s) in the activity of the enzyme towards phospholipid and triacylglycerol emulsions is discussed.

INTRODUCTION

The liver of many, if not all, vertebrates (1) contains a heparin-releasable lipase, commonly referred to as hepatic triglyceridase or liver lipase. Evidence has been presented for a role of this enzyme in lipoprotein metabolism. After the in vivo inhibition of the enzyme activity for instance by

using anti-liver lipase antibodies (2,3) or by the administration of estrogens (4,5), significant changes were observed in serum lipoprotein levels suggesting a role of the enzyme in the conversion of HDL subfractions (4,6,7). Since a similar enzyme (8,9) was reported in the adrenal gland and in the ovary, a mechanism was proposed (7) by which the hydrolytic action of the enzyme on HDL phospholipids would promote in the flux of free cholesterol between the HDL particle and the underlying tissue in favour of the three organs mentioned. The extracellular localization of the enzyme in the liver (10) was recently also established in the corpus luteum (11,12) and in the zona fasciculata in the adrenal cortex (11) indicating a role of the enzyme in progesterone synthesis and in glucocorticoid production (see also chapter VI). It is not clear whether the enzyme is also important for in vivo triacylglycerol hydrolysis. In vitro, the enzyme has a high K_m for the triacylglycerol substrate (13). Collagenase treatment of rat liver lipase by Kuusi et al. (14) resulted in a drastic decrease in triacylglycerol hydrolase activity without effect on its monoacylglycerol hydrolase activity. Jensen et al. (15) and Shirai et al. (16) confirmed these results using monooleoylglycerol (15) and tributyroylglycerol (16) respectively. Jensen et al. (15) did not observe any change in the phospholipase activity after the proteolytic digestion, whereas Shirai et al. showed that the capability of the enzyme to bind to dipalmitoylphosphatidylcholine vesicles was lost. These results suggest the existance of a hydrophobic binding site on the molecule for triacylglycerol micelles apart from the catalytic centre. The aim of the present study is to investigate the effects of structural alteration of the enzyme on the hydrolysis of phosphatidylcholine and triacylglycerol emulsions by rat liver lipase. For this purpose, three different methods were used.

i. Immunoinhibition of phospholipase A_1 activity and triacylglycerol hydrolase activity with monclonal antibodies.

ii. The effect of mild tryptic digestion on both enzyme activities.
iii. Protein modification with amino acid reactive agents and the effect on enzyme activities. By the selective modification of a single amino acid, complete inactivation of some enzymes has been reported (17,18). The influence of three protein modifiers were studied to elucidate the role of certain amino acids in both phospholipase A_1 and the triacylglycerol hydrolase activities. N-Tosyl-L-phenylethylchloromethylketone (TPCK), which is known to react with either histidine (18) or cysteine (17,19), N-ethylmaleimide, a sulfhydryl reagent, specific for cysteine (19,20) and phenylisothiocyanate which binds the ε-amino group of lysine at an alkaline pH (21).

MATERIALS AND METHODS

Monoclonal antibodies

A panel of 5 different monoclonal antibodies against salt-resistant rat liver lipase was used in this study. The characteristics of these antibodies that were indicated as A, B, C, D and E and a control (indicated as C_1) with respect to immunoprecipitation and immunoinhibition of triacylglycerol hydrolase activity of the enzyme was reported earlier (9).

Determination of triacylglycerol hydrolase activity

A mixture of 20 µCi tri[^{3}H]oleoylglycerol (Amersham, Buckinghamshire, U.K.) 1.0 Ci/mmol and 34 mg trioleoylglycerol was taken to dryness and emulsified by sonication in 2.5 ml 5% (^{w}/v) gum acacia (BDH Chemicals, U.K.) containing 8 mM $NaHCO_3$. The emulsion was mixed with 3 ml 10% (^{w}/v) defatted bovine serum albumin, 0.5 ml 1 M Tris-HCl pH 8.5 and 1.5 ml 5 M NaCl. 150 µl of the mixture was incubated with 100 µl sample for 30 min at 30° C. The free fatty acids formed were extracted as described by Belfrage and Vaughan (22). Triacylglycerol hydrolase activity was determined as mU/ml (1 mU represents 1 nmol

free fatty acid released from the substrate in 1 min at 30°C).

Determination of phospholipase A_1 activity

Labeling of phosphatidylcholine was performed by incubation of rat liver microsomes with [9,10-^{3}H]oleic acid, ATP, $MgCl_2$ and coenzyme A as described by Van den Bosch et al. (23). In a similar procedure, using phosphatidylethanolamine as a substrate, Groot et al. (24) showed that 98.6% of the acyl label was associated with position 1 of the lipid. Radioactive phosphatidylcholine was purified from rat liver microsomes according to established methods (24). The lipid concentration was determined according to Stewart (25).
A mixture of 0.4 µCi 1-[9,10-^{3}H]oleoylphosphatidylcholine and 250 nmol phosphatidylcholine (also from rat liver microsomal origin) was evaporated under N_2 and emulsified by sonication in 0.25 ml water. The emulsion was mixed with 50 µl 55 mM $CaCl_2$, 50 µl 1 M Tris pH 8.50 and 150 µl water. 50 µl of the mixture was incubated with 100 µl sample for 15 min at 30° C. The reaction was terminated with 1 ml of a mixture of heptane, isopropranol and 10 N H_2SO_4 (40:40:0.1, v/v). After the addition of 200 µl water, vigorous stirring and centrifugation, the water phase was incubated with silicic acid to remove all phosphatidylcholine. After centrifugation, the radioactivity was determined in 200 µl of the sample by liquid scintillation counting. Phospholipase A_1 activity was determined in mU/ml.

Purification of rat liver lipase

Liver lipase was isolated from 8 rats by means of <u>in vitro</u> perfusion of the liver at 6° C with a Tyrode buffer gassed with 95% O_2 - 5% CO_2 containing 30% (^{v}/v) glycerol, 10 IU/ml heparin (Tromboliquine, Organon, Oss, The Netherlands) and 100 µM phenylmethylsulfonylfluoride (PMSF, Merck, Darmstadt, F.R.G.). To avoid proteolytic degradation of the enzyme during the isolation procedure, PMSF was included in all buffers. PMSF (a serine protease inhibitor) does not have any

effect on both the triacylglycerol hydrolase activity and the phospholipase A_1 activity of liver lipase. The perfusates were loaded on a Sepharose-heparin column and the enzyme was eluted with a linear salt gradient from 0.0-2.0 M NaCl as described by Jensen (26). The enzyme was concentrated by $(NH_4)_2SO_4$ precipitation and dialysed against 5% ($^v/v$) glycerol in 50 mM Tris buffer, pH 7.2 without PMSF. (350 mU/ml triacylglycerol hydrolase activity, 17.5 mU/ml phospholipase A_1 activity (PLase), TGase/PLase = 20/1). During the determination of the effect of pH on the hydrolysis of trioleoylglycerol and phosphatidylcholine by liver lipase, the enzyme was tested for both enzyme activities by using the following buffers: citrate-phosphate buffer pH 3.95, Tris-maleate buffer pH 5.20, pH 5.85, pH 6.85, pH 7.40, pH 8.05, pH 8.70 and glycine-NaOH buffer pH 9.80 and 10.60.

Immunoprecipitation

A mixture of 5 µl purified liver lipase and an increasing amount of anti-liver lipase monoclonal antibodies A,B,C,D and E (0, 25, 50, 75 or 100 µl hybridoma supernatants) was incubated for 1 h on ice. Hybridoma supernatant is about 1.5 mg/ml. Whole rabbit anti-mouse immunoglobulins (Nordic Immunology, Tilburg, The Netherlands) were used as a second antibody (10 µl from a stock solution of 1 ml of lyophilized antibody (10 mg/ml) dissolved in phosphate-buffered saline were incubated for 30 min on ice). 50 µl of a 1:1 suspension of protein A-Sepharose 4B (Pharmacia, Uppsala, Sweden) in phosphate-buffered saline were used as the precipitant. Incubation was performed for 30 min with gentle rotation at 4° C. Samples were centrifuged and phospholipase A_1 activity was determined in 100 µl of the supernatants. As a control, mouse IgG_1 antibodies that do not bind with liver lipase (C_1) were used. Phospholipase A_1 activity is not inhibited by protein A-Sepharose 4B, the second antibody or the tissue culture medium.

Immunoinhibition

In the immunoinhibition assay, a mixture of 5 µl purified liver lipase and 75 µl (hybridoma supernatant) of the monoclonal antibodies A, B, C, D or E was incubated for 1 h on ice and subsequently tested for phospholipase A_1 activity. Again an identical incubation with 75 µl hybridoma supernatant C_1 served as a control.

Proteolytic degradation of liver lipase

An amount of 384 µl purified liver lipase and 100 µl 0.1% ($^w/_v$) trypsin were incubated at 37° C. The reaction was terminated after 0 sec, 15 min, 30 min, 45 min, 60 min and 75 min by mixing 78 µl of the reaction mixture with 50 µl 0.1% ($^w/_v$) trypsin inhibitor (trypsin/trypsin inhibitor = 1/3). The samples were kept on ice and tested for both triacylglycerol hydrolase activity and phospholipase A_1 activity. An identical experiment without trypsin but with trypsin inhibitor incubated for 75 min at 37° C served as a control.
A similar experiment was carried out and the samples were prepared for protein blotting. Liver lipase and trypsin were incubated on ice for 0 sec, 20 sec, 1.5 min, 3 min, 6 min and 10 min. Again an identical inhibition of liver lipase without trypsin but with trypsin inhibitor for 10 min on ice served as a control. After SDS-polyacrylamide gel electrophoresis, the proteins were blotted onto nitrocellulose paper. Liver lipase was identified on the blot using a mixture of the anti-liver lipase monoclonal antibodies and ^{125}I-labelled anti-mouse whole antibodies (9).

Modification of liver lipase

5 µl purified liver lipase and 75 µl 25 mM Tris-HCl pH 7.6 were incubated with 20 µl N-tosyl-L-phenylethylchloromethylketone (TPCK) or N-ethylmaleimide (BDH Chemicals, U.K.), dissolved in methanol at a final concentration of 50 µM, 100 µM, 200 µM or 300 µM (TPCK was a generous gift from Dr. B.

Kraal, Department of Biochemistry, State University of Leiden). After 75 min on ice, the reaction was terminated by the addition of 25 µl 20 mM 2-mercaptoethanol and triacylglycerol hydrolase and phospholipase A_1 activities were measured. Identical incubations with 20 µl water or 20 µl methanol served as controls. Modification of liver lipase with phenylisothiocyanate (lot nr. 26921, Pierce, Rockford, U.S.A.) was carried out with 5 µl purified enzyme, 85 µl glycine-NaOH buffer pH 9.5 and 0 µl, 0.125 µl, 5 µl or 10 µl PITC in a total volume of 100 µl for 45 min at 22° C.

RESULTS

pH-dependency of phospholipase A_1 and triacylglycerol hydrolase activity of purified rat liver lipase

The effect of pH on the hydrolysis of a phosphatidylcholine emulsion or a trioleoylglycerol emulsion is shown in Fig. 4.1. Both enzyme activities show a pH curve with a maximum at a pH of about 9.5. At a physiological pH, the phospholipase A_1 activity is about 80% of the maximal enzyme activity at pH 9.5 whereas more than 50% of the maximal triacylglycerol hydrolase activity is found.

Effect of monoclonal antibodies on phospholipase A_1 activity

To study the effect of five monoclonal antibodies on the phospholipase A_1 activity of purified rat liver lipase it is important to know the binding capacity of the enzyme for the several antibody preparations. Fig. 4.2 shows the results of the immunoprecipitation of 5 µl purified rat liver lipase by an increasing amount of hybridoma supernatants A and E. The results with hybridoma supernatant B, C and D were identical with those obtained with A and are therefore not given in this figure. 50 µl hybridoma supernatant A, B, C or D is shown to be sufficient for 98% precipitation of the phospholipase A_1 activity of 5 µl purified liver lipase that was used in the

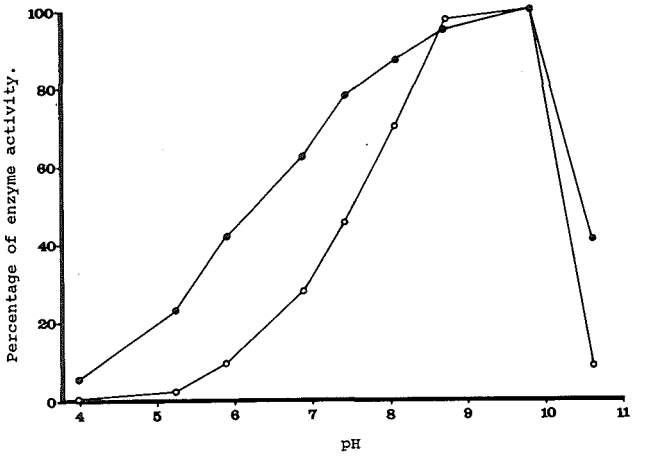

Figure 4.1
A purified sample of rat liver lipase was tested for phospholipase A_1 activity (●) and triacylglycerol hydrolase activity (○) at different pH's. 100% activity is 349 mU/ml triacylglycerol hydrolase and 16.5 mU/ml phospholipase A_1 activity. Measurements were carried out at least twice.

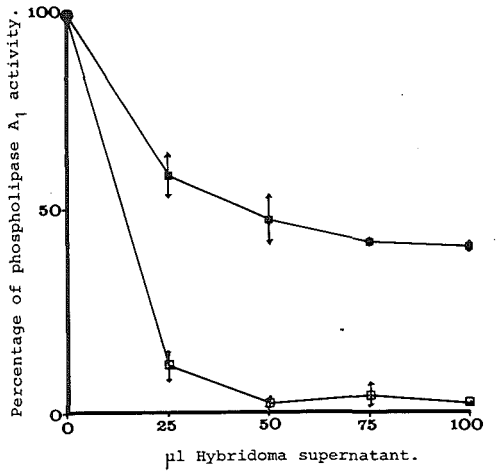

Figure 4.2
Immunoprecipitation of phospholipase A_1 activity of 5 µl purified rat liver lipase with an increasing amount of hybridoma supernatant A (□) and E (■). Hybridoma supernatant C_1 (100 µl) was used as a control (in the figure (●), at 0 µl hybridoma supernatant). 100% represents 13.6 mU/ml phospholipase A_1 activity. Measurements were carried out at least twice.

assay. Monoclonal antibody E on the other hand surprisingly shows only 59% precipitation of the phospholipase A_1 activity with 75 µl hybridoma supernatant or more. In other words: 41% of the phospholipase A_1 activity of the purified enzyme is not recognized by monoclonal antibody E. An identical amount of the purified enzyme (5 µl) was tested for phospholipase A_1 activity in an immunoinhibition assay, using an excess amount (75 µl) of hybridoma supernatant A, B, C, D or E (Table 4.1). It is shown in this table that all monoclonal antibodies inhibit phospholipase A_1 activity of the purified enzyme to a certain degree. Whereas monoclonal antibodies C and D inhibit both the phospholipase A_1 and triacylglycerol hydrolase activity (Table I, ref. 9) in a similar way, antibodies A, B and E show different results. Immunoinhibition of phospholipase A_1 and triacylglycerol hydrolase activity was also tested with an emulsified mixture of both substrates (see Materials and Methods) to exclude the possibility that the differences in immunoinhibition obtained with both enzyme activities could be related to the different micellar substrates used. The results obtained were similar as with those reported in Table 4.1 (not shown).

Table 4.1
Enzyme inhibition of phospholipase A_1 activity of purified rat liver lipase

5 µl of a purified sample of rat liver lipase was incubated for 1 h on ice with 75 µl hybridoma supernatant A, B, C, D, E and tested for phospholipase A_1 activity. C_1 served as control hybridoma supernatant. Results are given as means ± S.D. of three separate experiments in triplicate. Inhibition is expressed as percentage.

Antibody preparation	Enzyme activity mU/ml	Inhibition %
C_1 (control)	16.0±0.8	0
A	11.4±0.5	29±3
B	12.2±0.5	24±3
C	9.1±0.8	43±5
D	11.4±1.8	29±11
E	10.6±2.1	34±13

Proteolytic digestion of rat liver lipase

The results of the proteolytic degradation of liver lipase and its consequences for enzyme activity are presented in Figs. 4.3 and 4.4. Both the triacylglycerol hydrolase and the phospholipase A_1 activities of purified rat liver lipase are lowered by trypsin treatment of the enzyme (Fig. 4.3). However, the phospholipase A_1 activity is more sensitive to proteolysis than the triacylglycerol hydrolase activity. To study the actual breakdown of the molecule, the degradation by trypsin was followed by western blotting (Fig. 4.4). Immunoblotting of the enzyme revealed a 58 kD enzyme form as was reported earlier (9). Fig. 4.4 shows that after a 1.5 min incubation of liver lipase with trypsin on ice, 100% of the

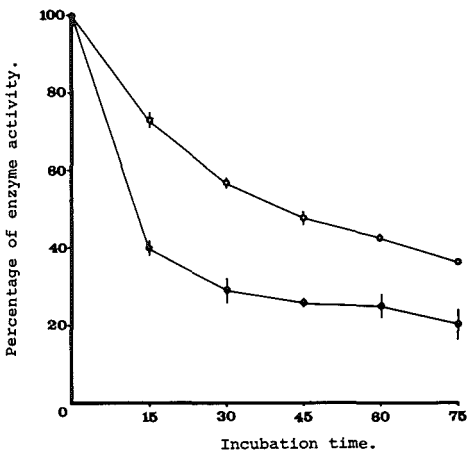

Figure 4.3
Proteolytic digestion of purified rat liver lipase by trypsin. The degradation was carried out at 37° C and was terminated by means of trypsin inhibitor at the time intervals indicated. 100% phospholipase A_1 activity (●) is about 0.17 mU/ml and triacylglycerol hydrolase activity (○) is 12.8 mU/ml. Measurements were carried out at least twice.

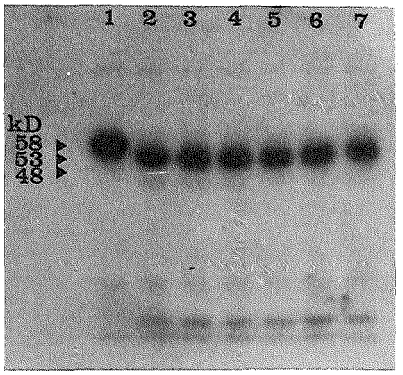

Figure 4.4
Immunoblotting of trypsin digested purified rat liver lipase. The enzyme was detected using a mixture of the monoclonal antibodies (A-E) and a ^{125}I-labelled anti-mouse antibody (as the second antibody). Liver lipase and trypsin were incubated on ice for 0 sec (lane 7), 20 sec (lane 6), 1.5 min (lane 5), 3 min (lane 4), 6 min (lane 3) and 10 min (lane 2). An identical incubation for 10 min without trypsin (lane 1) served as a control.

enzyme is degraded into a smaller 53 kD form (lane 5). After an incubation for 10 min on ice, the intensity of the 48 kD band increased at the expense of a 53 kD band (lane 2). Incubations for 15 min or longer at 37° C (not shown) result in a rapid decrease of both the 53 and the 48 kD band indicating a further breakdown of the enzyme which however cannot be detected anymore by the monoclonal antibodies.

Modification of liver lipase

TPCK modification (Fig. 4.5) and N-ethylmaleimide modification (Fig. 4.6) of purified rat liver lipase did not have any effect on phospholipase A_1 or triacylglycerol hydrolase activities. On the other hand, after modification of lysine residue(s) by phenylisothiocynate marked changes were observed in both enzyme activities tested (Fig. 4.7). Although this modification rapidly decreases the capability of the enzyme to hydrolyse a triolein emulsion, the phospholipase A_1 activity is even initially stimulated upto 168% of the control value.

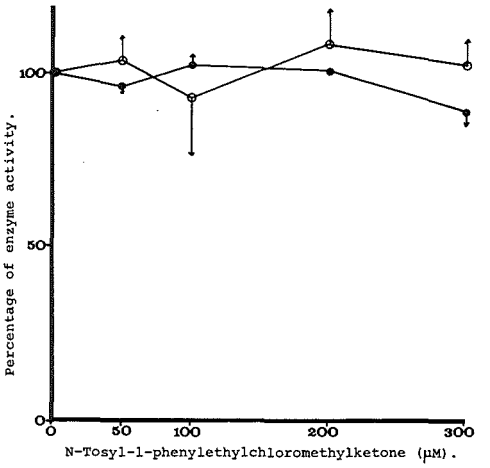

Figure 4.5
Modification of purified rat liver lipase by TPCK. 5 μl of the enzyme was incubated with 20 μl TPCK (in methanol) for 75 min on ice in a total volume of 100 μl. The reaction was terminated with 25 μl 20 mM 2-mercaptoethanol. The enzyme was tested for phospholipase A_1 (●) activity (100% = 9.6 mU/ml) and triacylglycerol hydrolase (O) activity (100% = 195 mU/ml). Measurements were carried out in duplicate.

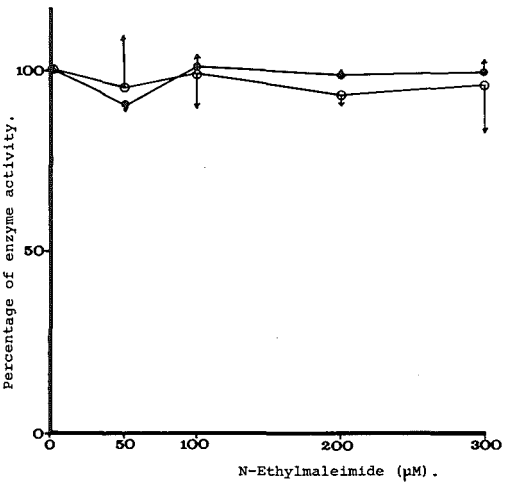

Figure 4.6
Modification of purified rat liver lipase by N-ethylmaleimide. The reaction was carried out as described in the legend of Fig. 5. The enzyme was tested for phospholipase A_1 (●) activity and triacylglycerol hydrolase (O) activity.

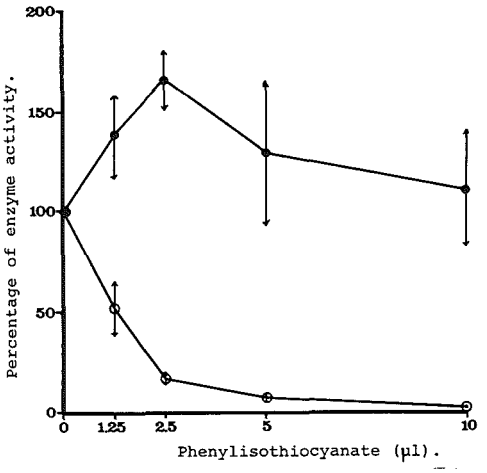

Figure 4.7
Modification of purified rat liver lipase by phenylisothiocyanate 5 µl of the enzyme was incubated with 0, 0.125, 5 or 10 µl PITC in a glycine-NaOH buffer pH 9.50 in a total volume of 100 µl for 45 min at 22° C. The enzyme was tested for phospholipase A_1 (●) activity and triacylglycerol hydrolase (○) activity. Measurements were carried out at least three times.

DISCUSSION

In the study on the function of the salt-resistant heparin releasable liver lipase, evidence has been presented by several groups (2-5,27,28), along different lines, for the role of the enzyme in the metabolism of HDL. Besides these studies that support the hypothesis of the role of the enzyme in the degradation of HDL phospholipids, an effect on human HDL_2 triacylglycerol was also reported (27,29,30). Goldberg et al. (31), on the other hand, presented data in the Cynomolgus monkey, suggesting a possible function of the enzyme in the metabolism of intermediate density lipoproteins. Nozaki et al. (32) demonstrated that in humans under pathological conditions intermediate density lipoprotein concentrations are inversely correlated with human liver lipase. From these studies the question arises, whether liver lipase is besides as a phospho-

lipase also important as a triacylglycerol hydrolase *in vivo*. Information about the action of the enzyme on both phospholipid and triacylglycerol as the *in vitro* substrate might be helpful for a better understanding of the physiological action of liver lipase. The activity is *in vitro* most frequently tested at the optimal alkaline pH. It is shown in Fig. 4.1 however that at a physiological pH, in spite of the higher triacylglycerol hydrolase activity (that is always observed *in vitro*), enzyme activity is changed in favour of the phospholipase A_1 activity (ratio triacylglycerol hydrolase/phospholipase A_1 = 10 vs 20).

Based on the almost complete precipitation by the monoclonal antibodies A, B, C and D of both the triacylglycerol hydrolase activity (94%, ref. 9) and the phospholipase A_1 activity (98%) in a purified sample of the enzyme, it is concluded that liver lipase as a single protein is capable in performing both activities. In the presence of an excess of monoclonal antibody E on the other hand, triacylglycerol hydrolase activity and phospholipase A_1 activity were precipitated for 88% (9) and 59% respectively (Fig. 4.2). This suggests the presence of a form of liver lipase that represents about 6% of the triacylglycerol hydrolase activity and about 39% of the phospholipase A_1 activity of the total precipitable amount of activity by the antibodies A, B, C and D. This heterogeneity is not reflected in different molecular weights studied by immunoblotting of the same purified sample using the different monoclonal antibodies. On the other hand, the complete precipitation of enzyme activity by the four different monoclonal antibodies A, B, C and D, shows a strong homology that would suggest for just a minor difference(s) between the two forms described above. The nature of the enzyme form with a relatively higher phospholipase A_1 activity is not clear yet. Besides the effect of the antibodies on the triacylglycerol hydrolase activity (9), it is shown in this chapter that all antibodies are also able to inhibit its

phospholipase A_1 activity (Table 4.1). It is important to note that while monoclonal antibodies C and D inhibit both activities similarly, binding of antibodies A, B or E results in pronounced differences. When using different enzyme preparations (the purified enzyme or the enzyme in postheparin plasma) or an emulsified mixture of both substrates, identical results were obtained. Inhibition of enzyme activities may be the result of the binding of a 160,000 dalton IgG_1 antibody to the enzyme, thereby interfering with the hydrolyzing action of the enzyme or with the interaction with the micellar substrate. These results strongly suggest a difference between the phospholipase A_1 activity and the triacylglycerol hydrolase activity, possibly related to structural differences in the enzyme molecule.

Proteolytic digestion of liver lipase by trypsin decreased both enzyme activities (Fig. 4.3). However, the phospholipase A_1 activity was more sensitive to tryptic digestion of the enzyme than the triacylglycerol hydrolase activity which again strongly suggests a difference between both enzyme activities as discussed above. The results obtained with the phospholipase A_1 activity disagree with a study of Jensen et al. (15) but support the finding of Shirai et al. (16) of a loss of the capability of the enzyme to bind dipalmitoylphosphatidylcholine vesicles after tryptic digestion. Tryptic digestion of the enzyme seems therefore to destroy the binding site(s) of the enzyme for both micellar substrates thereby decreasing its activity. Immunoblotting of the trypsin-digested enzyme did reveal that the 53 kD protein which was also observed by others (9,33,34), results from proteolysis of a 58 kD protein what was already suggested before (9) (Fig. 4.4). The use of proteolytic inhibitors during the isolation of the enzyme is therefore highly recommended. These results show that when using the partially degraded enzyme in the study on the function of liver lipase, the phospholipase A_1 activity of the enzyme will be highly underestimated.

From the results obtained with the protein modification with TPCK and N-ethylmaleimide (Figs. 4.5 and 4.6), it can be concluded that neither cysteine nor histidine residue(s) are directly involved in the catalytic activity of the enzyme towards phospholipids or triacylglycerols. Neutralization of the positive charge on the ε-amino group of a lysine residue(s) and the introduction of the hydrophobic phenyl group by the modification with phenylisothiocyanate on the other hand did strongly influence both enzyme activities (Fig. 4.7). The introduction of a phenyl group in a hydrophylic region might irreversibly disturb the correct interaction of the hydrophobic micelle with the enzyme, resulting in a complete loss of triacylglycerol hydrolase activity. The complexity of the interactions of the enzyme with the different substrates may be even larger if natural substrates (lipoproteins) containing possible cofactors (apolipoproteins) are involved. This will be studied in future experiments.

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CHAPTER V

IN VIVO INHIBITION OF RAT LIVER LIPASE ACTIVITY WITH MONOCLONAL ANTIBODIES. PURIFICATION OF DIFFERENT ANTIBODY PREPARATIONS WITH FAST PROTEIN LIQUID CHROMATOGRAPHY

ABSTRACT

Immunotitration of salt-resistant rat liver lipase with monoclonal antibodies in the presence or absence of 15 µl normal rat serum, revealed that normal rat serum does not contain an enzymatically inactive antibody precipitable form of the lipase(s). Injection of ascites fluid obtained from two different hybridoma cell lines (producing monoclonal antibodies against rat salt-resistant liver lipase) into male rats turned out to inhibit both the liver and the adrenal lipase activity.

The two different monoclonal antibodies (A + C) inhibited the salt-resistant triacylglycerol hydrolase activity for 85% and 42% respectively in the liver and for 81% and 36% in the adrenal gland. The different antibody preparations that have been used, were purified by Fast Protein Liquid Chromatography and polyacrylamide gelelectrophoresis. A 20% ($^{w}/v$) Na_2SO_4 precipitation of hybridoma supernatant resulted in the precipitation of about 10% of the total amount of protein including all antibodies. By this way, the antibodies were concentrated and freed from a 81 kD protein and albumin (68 kD).

INTRODUCTION

Antibodies have been used in the study of the salt-resistant lipases in rat liver, adrenal gland and ovary (1,2). Similar enzymes are also present in the human and probably most other vertebrata (3). These enzymes have been suggested to play a role in the metabolism of lipoproteins to meet the need for cholesterol used for steroid production (bile acid, glucocorticoid and progestin) in the liver, adrenal gland and ovary respectively (4-7). Because of their extracellular localization (8), the salt-resistant lipase activity is easily released from the three organs after the <u>in vivo</u> administration of the polyanion heparin (except for mice (9), liver

lipase activity is normally not found in the blood). In this chapter, a monoclonal antibody, directed against rat liver lipase, will be used to detect whether inactive enzyme is present in normal rat serum. To obtain the high titre of antibodies that is required for in vivo inhibition of lipase activity, ascites preparations were made from two different hybridoma cell lines. The results obtained with in vitro and in vivo inhibition of enzyme activity are compared and discussed. Different antibody preparations (hybridoma supernatant and ascites fluid) were purified by anion-exchange fast protein liquid chromatography. In addition, the effect of a 20% Na_2SO_4 precipitation on the antibody concentration of hybridoma supernatant was also tested.

MATERIALS AND METHODS

Monoclonal antibodies

Five different monoclonal antibodies against the salt resistant rat liver lipase were obtained (2). These antibodies were indicated as A, B, C, D and E and have been characterized with respect to immunoprecipitation and in vitro immunoinhibition of lipase activity, their antibody class and their cross-reactivity with other lipases. The monoclonal antibodies cross-reacted with the liver type lipases that are present in the adrenal gland and in the ovary as was shown by immunoprecipitation experiments (2).

Fast Protein Liquid Chromatography

For the purification of antibodies, several antibody preparations were separated by ion exchange chromatography on a 1 ml anion exchange (mono Q) column of the Fast Protein Liquid Chromatography system of Pharmacia (Uppsala, Sweden). Sample preparation before loading on the column consisted of extensive dialysis against a buffer containing 20 mM Tris-HCl, pH=8.0 (buffer A) followed by filtration through a 0.22 µm

filter or centrifugation for 1 h at 100,000 g (to avoid column obstruction by particles). After injection of 0.5 ml sample on the column (using a 0.5 ml sample loop), the column was washed for 5 min with buffer A (flow = 1 ml/min). A linear salt gradient was then applied which started with buffer A at t=5 min and ended with 30% (^{v}/v) of buffer B (20 mM Tris-HCl, 1 M NaCl, pH=8.0) at t=20 min. At the end of a complete run, the column was regenerated by continuation of the flow with 100% buffer B for 3 min followed by buffer A for 5 min. Throughout the run of 28 min, the absorbance at 280 nm and the percentage of buffer B were recorded and fractions of 1 ml were collected. In this way, hybridoma supernatant B, the Na_2SO_4 precipitate of the same supernatant and the ascites fluid from hybridoma cell line A were separated. The antibody containing fractions were selected with an immunoprecipitation assay, using 10 µl postheparin rat plasma and 90 µl of each fraction. The purification of the antibodies was followed by SDS-polyacrylamide gel electrophoresis according to Laemmli on a 3% stacking gel and a 10% resolving gel. Proteins were visualized by Coomassie Brilliant Blue staining.

Antibody preparations

Hybridomas were cultured in regular culture medium (RPMI medium supplemented with 15% (^{v}/v) fetal calf serum) in a humidified atmosphere at 37^{o} C, containing 95% air and 5% CO_2. Supernatants of the cultured hybridomas were kept at 4^{o} C for antibody precipitation or were frozen in liquid nitrogen and stored at -80^{o} C. Antibody precipitation was carried out by mixing an equal volume of 40% (^{w}/v) Na_2SO_4 with hydridoma supernatant at room temperature. The samples were kept for 2 h at room temperature and centrifuged (20 min, 3000 g). After discarding the supernatant, the pellet was washed with a 20% (^{w}/v) Na_2SO_4 solution twice and dissolved by dialysis against phosphate buffered saline.

Ascites fluid was obtained after the intraperitoneal injection of 10^6 cells from hybridoma clones "A" and "C" in BALB/c mice (10). The titre of both ascites preparations (that are called A or C after the hybridoma cell lines that were used for inoculation) was determined by immunoprecipitation of the salt-resistant triacylglycerol hydrolase activity in rat postheparin plasma. One ml of ascites fluid A or C turned out to be capable in the precipitation of respectively 222 or 130 U of enzyme activity (not shown). Based on these titres and assuming that all the extracellularly located enzyme is accessable to the antibodies, 27 µl of ascites fluid A or 46 µl of C should be sufficient to completely saturate all the enzyme found in a rat liver *in vivo*.

In vivo inhibition of enzyme activity

Normally fed, male Wistar rats were housed under controlled conditions (temperature 20-22° C with the lights on between 07.00 and 19.00 h). After anesthesia of 6 rats with ether, 100 µl of ascites fluid A or C or phosphate buffered saline (2 control rats) were intravenously administered. The animals were kept under narcosis for 10 min and then decapitated. Salt-resistant triacylglycerol hydrolase activity was tested in 50 µl of a 2.5% (^{w}/v) liver homogenate in phosphate buffered saline (the mean wet weight of the six livers is 10.3±0.6 g). Adrenal lipase activity was tested on 50 µl of a homogenate of the two adrenals of each animal in 1 ml phosphate buffered saline.

Immunoprecipitation

A mixture of postheparin rat plasma or a purified sample of liver lipase was incubated with monoclonal antibodies against liver lipase for 1 h on ice. Whole rabbit anti-mouse immunoglobulins (Nordic Immunology, Tilburg, The Netherlands) were used as a second antibody (10 µl from a stock solution of 1 ml of lyophylized antibody (10 mg/ml) dissolved in phospha-

te buffered saline was incubated for 30 min on ice). Fifty µl of a 1:1 suspension of protein A-Sepharose 4B (Pharmacia) in phosphate buffered saline was used as the precipitant. Incubation was performed at 4° C for 30 min with gentle rotation. Samples were centrifuged and liver lipase activity was determined in 100 µl of the supernatants.

Biochemical measurements

Lipase activity was measured as salt-resistant triacylglycerol hydrolase activity using an artificial tri[^{3}H]oleoylglycerol emulsion in the presence of 1.0 M NaCl (assay was for 30 min at 30° C) (2). Lipase activities are given in mU/ml (1mU represents 1 nmol free fatty acid released from the substrate in 1 min at 30° C).

RESULTS

Searching for inactive enzyme in normal rat plasma by immunoprecipitation

In Fig. 5.1, an increasing amount of a monoclonal antibody against rat liver lipase was used for immunotitration of a purified sample of rat liver lipase with or without 15 µl normal rat serum. The result obtained after immunotitration of 15 µl postheparin rat plasma (obtained 10 min after the intravenous injection of 100 I.U. heparin/kg body weight) is also presented in the same figure.

In vivo inhibition of salt-resistant lipase activity

For in vivo inhibition of liver lipase activity, 100 µl of ascites fluid of two different hybridoma cell lines (A + C) was used. In comparison to two control rats, the injection of monoclonal antibodies A and C resulted in the inhibition of both the liver and the adrenal salt-resistant triacylglycerol hydrolase activity for 85% and 42% respectively in the liver and 81% and 36% in the adrenal. The control value of lipase

activity in the liver and adrenal was 20.3±0.8 mU/ml and 13.6±1.0 mU/ml respectively.

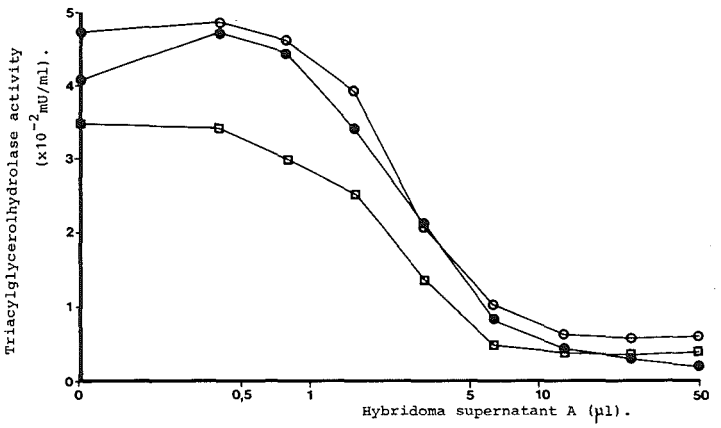

Figure 5.1
Immunotitration of a purified sample of rat liver lipase (●) and the same sample with 15 µl normal rat serum (O) with hybridoma supernatant A in an immunoprecipitation assay. Immunoprecipitation of 15 µl postheparin rat plasma (□) is also presented.

Separation of monoclonal antibodies by Fast Protein Liquid Chromatography

The results of anion exchange-fast protein liquid chromatography of hybridoma supernatant B, the Na_2SO_4 precipitate of the same sample and ascites fluid A are presented in Figs. 5.2, 5.3 and 5.4, respectively. In all three separations, the antibodies eluted from the mono Q column at about 0.1 M NaCl (10% (^{v}/v) of Buffer B) determined by immunoprecipitation. (The presence of antibody is indicated in the figures.) The

relative amount of antibodies compared to the total amount of A_{280} detectable proteins that eluted from the column greatly differed between the several runs. A 20% Na_2SO_4 precipitation of hybridoma supernatant B resulted in the precipitation of about 10% of the total amount of protein (as was determined according to Lowry) without any loss of antibody activity. (The supernatant was dialysed against phosphate buffered saline and tested for antibodies in an immunoprecipitation assay using 10 µl postheparin rat plasma.) In Fig. 5.4 it is shown that the major part of the protein content in ascites fluid that eluted from the column consists of antibodies.

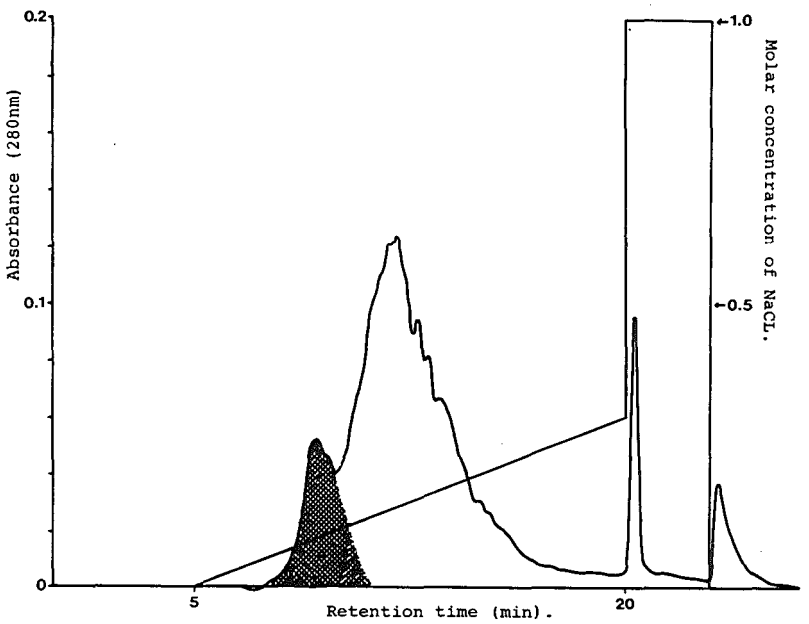

Figure 5.2
Anion-exchange-fast protein liquid chromatography of 0.5 ml hybridoma supernatant B. The presence of antibodies in the eluted fractions is indicated in black.

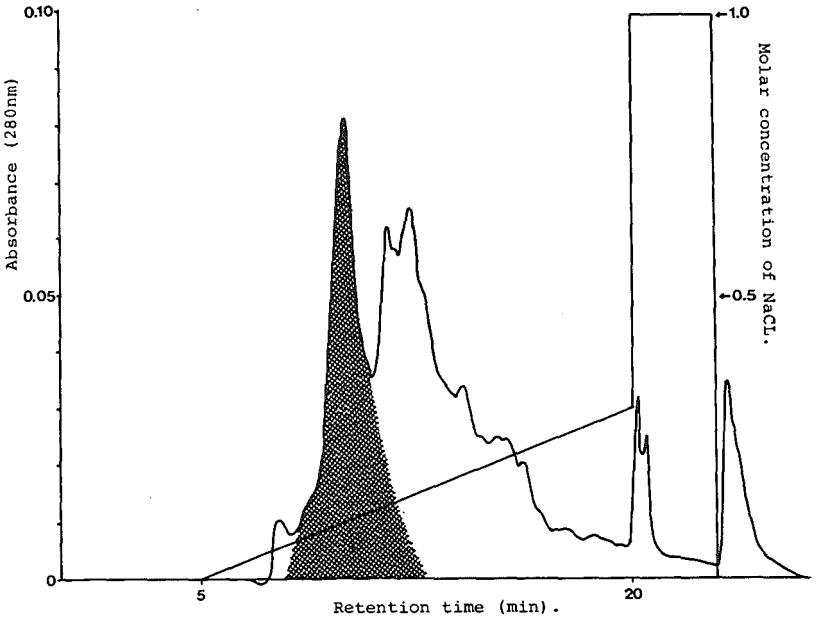

Figure 5.3
Anion-exchange-fast protein liquid chromatography of the Na_2SO_4 precipitate of hybridoma supernatant B after dialysis against start buffer (see Materials and Methods). The antibodies are indicated in black.

Polyacrylamide gel electrophoresis (Fig. 5.5) of the hybridoma supernatant and the Na_2SO_4 precipitate of the same sample (compare lanes 1 and 2 respectively) reveals a loss of a 81 kD protein band and serum albumin (68 kD) by the Na_2SO_4 precipitation (lane 2). In lane 3 it is shown that the antibody top fraction of a Na_2SO_4 precipitate of hybridoma superna-

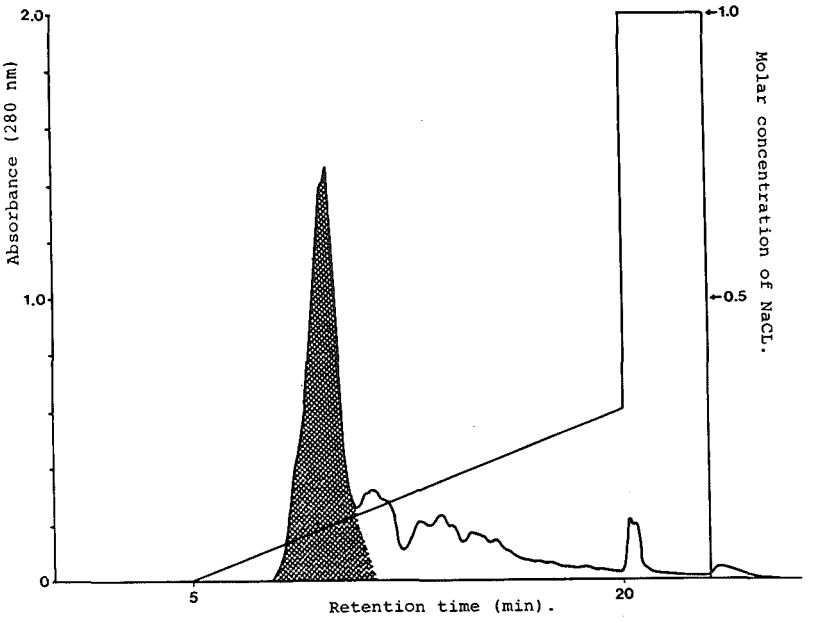

Figure 5.4
Anion-exchange-fast protein liquid chromatography of ascites fluid A. The antibodies are indicated in black.

tant after separation by fast protein liquid chromatography consists of pure antibody (the large and small subunits of IgG_1 are only present). Lane 4 shows the protein content in the first peak that eluted after the antibodies in the same run. The protein pattern of a part of the fractions that had been obtained after separation of ascites fluid on mono Q is

shown in lanes 5-9.

Lanes 5, 6, 7 and 8 represent the four fractions that had been collected in the antibody peak and lane 9 shows the protein composition in the first peak that eluted after the antibodies. It is clearly shown that the antibody peak contains the molecular forms of the two subunits of the antibodies but that it is also contaminated with serum albumin and the 81 kD protein band.

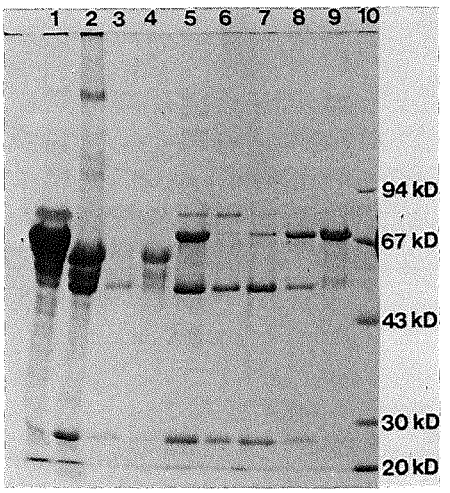

Figure 5.5
Polyacrylamide gel electrophoresis of lane 1: hybridoma supernatant, lane 2: the Na_2SO_4 precipitate of hybrydoma supernatant, lane 3: the antibody top fraction of Fig. 5.3, lane 4: the first peak that eluted after the antibodies in the same run (Fig. 5.3), lanes 5-8: the antibody peak fractions of Fig. 5.4, lane 9: the first peak that eluted after the antibodies in the same run (Fig. 5.4) and lane 10: marker proteins (phosphorylase B (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD) and soybean trypsin inhibitor (20.1 kD)). Electrophoresis was carried out on a 3% stacking gel and a 10% resolving gel.

DISCUSSION

In this study, monoclonal antibodies have been applied for the detection of inactive enzyme that might possibly be present in normal rat serum and for the *in vivo* inhibition of the enzyme activity in the liver and in the adrenal gland. The different antibody preparations that were used, have been analysed on anion-exchange - fast protein liquid chromatography and polyacrylamide gel electrophoresis. The effect of Na_2SO_4 precipitation in antibody purification was also studied with these techniques.

In Fig. 5.1, it is shown that the addition of rat serum does not change the pattern of immunotitration of a purified sample of rat liver lipase when using monoclonal antibody A in an immunoprecipitation assay. As the figure was not shifted to the right upon the addition of serum to the purified enzyme, it is concluded that normal rat plasma does not contain enzymatically inactive antibody precipitable forms of the lipases. In Fig. 5.1, the result of immunotitration of 15 µl postheparin rat plasma (with monoclonal antibody A) is also shown which illustrates the great difference that is observed before and after the injection of heparin into a rat with respect to the serum detectable amount of enzyme activity and enzyme precipitation with antibodies. The results obtained after *in vivo* inhibition of liver lipase activity with the different monoclonal antibodies A and C are identical with those of the *in vitro* inhibition of the enzyme as was published earlier (2). This could mean that *in vivo* inhibition with monoclonal antibody A will cause just a minor decrease in phospholipase A_1 activity as was also observed after the *in vitro* inhibition of both triacylglycerol hydrolase and phospholipase A_1 activity by the monoclonal antibody. The liver-type lipase that is located in the adrenal gland was also inhibited in a similar manner. Ascites fluids A and C therefore, turned out to be potent antibody preparations for the 80% or 40% inhibition of the triacylglycerol hydrolase activity in the liver and the

adrenal gland. The different epitopes that are located on the enzyme seem therefore to be accessable to antibodies that are present in the blood. It is noted that based on the excess amount of antibody that had been injected, all the enzyme will be associated with antibody. A differential decrease of enzyme activity will then be the result of the binding of antibodies A and C with different epitopes.

Separation of the different antibody preparations by Fast Protein Liquid Chromatography and analysis of fractions that had been collected by polyacrylamide gel electrophoresis clearly showed an enrichment of the antibody containing fractions by a 20% ($^{w}/v$) Na_2SO_4 precipitation. After pretreatment of hybridoma supernatant by Na_2SO_4, the sample is freed from albumin and a 81 kD protein band. The protein profile of antibody containing fractions obtained after the separation of ascites fluid by Fast Protein Liquid Chromatography shows the antibody's large and small subunits together with a 81 kD and a 68 kD protein band. Purification of ascites fluid by Na_2SO_4 precipitation alone will therefore result in an almost pure sample of antibody without the need of a column procedure. When using the monoclonal antibodies for <u>in vivo</u> inhibition of lipase activity, a purified sample with a high titre is recommended. After Na_2SO_4 precipitation of ascites fluid, a sample will most probably be obtained that meets such requirements.

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CHAPTER VI

LOCALIZATION OF THE SALT-RESISTANT HEPARIN RELEASABLE LIPASE IN THE RAT LIVER, ADRENAL AND OVARY

N.L.M. Persoon, W.C. Hülsmann and H. Jansen (1986)
Eur. J. Cell Biol. 41:134-137

ABSTRACT

The localization of the salt-resistant heparin-releasable lipase in rat liver, adrenal and ovary was studied. For this purpose, female rats were injected with a polyclonal antibody, monospecific for rat liver lipase, that crossreacts with the adrenal and ovarian enzymes. Immunofluorescence on thin frozen sections of these tissues and rat heart, kidney, spleen and striated muscle, using a labeled secondary antibody, only revealed the lipase in the liver, adrenal gland and ovary. In the liver, label was detectable along the endothelial cells, facing the sinusoidal cavities. The adrenal gland exclusively showed the label on endothelial cells in the zona fasciculata. In the ovary, label was exclusively found in the corpora lutea.

INTRODUCTION

In 1981, a distinct lipase activity in rat adrenals and ovaries, similar to the well defined lipase that is found in the liver (liver lipase or hepatic lipase) (1), was reported (2). These lipases can be released rapidly and almost completely from the organs by the in vivo administration of heparin which suggests an extracellular localization of the enzymes. In the case of the liver lipase, Virtanen and coworkers indeed showed the enzyme on the rat liver endothelium (7). In vitro, however, the rat liver lipase is synthesized and secreted by liver parenchymal cells only (10). It is therefore suggested that in vivo, after the secretion of the lipase by the liver parenchymal cells, the enzyme is bound onto the liver endothelium from where it could exert its physiological function in lipoprotein metabolism (4). With respect to the function of the lipase in the rat adrenal gland and ovary, a strong relationship between lipase activity and the level of steroidogenesis was suggested (2). In the adrenal gland, a drastic

increase in lipase activity was observed after stimulation of corticosteroid production in the rat by the prolonged treatment with Synacthen (3). Inhibition of steroidogenesis on the other hand by the administration of cortisol shows the opposite (2). Induction of steroidogenesis in rat ovary by (pseudo) pregnancy and lactation was positively related to ovarian lipase activity and the serum concentration of progesterone and 20α-hydroxyprogesterone (2,5). This chapter presents a study on the cellular localization of the lipase in the rat liver, adrenal gland and ovary. For this purpose a polyclonal antibody, monospecific for rat liver lipase which was shown to crossreact with the heparin-releasable lipases from rat adrenal and ovarian origin (2) and a fluorescein conjugated second antibody on thin frozen sections of the three organs from rat before and after heparin administration were used. Several other tissues served as controls.

MATERIALS AND METHODS

Preparation of the tissues

After anaesthesis of female rats (strain Wistar, 200-250 g) by the intraperitoneal injection of Nembutal (20-35 mg/ kg body weight, CEVA, Avenue George-V, Paris, France) 600 µl of a polyclonal antibody directed against rat liver lipase was intravenously administered. In the control rat, the injection of antibody was preceded by the intravenous administration of heparin (360 IU/kg body weight, Tromboliquine, Organon, Oss, The Netherlands) for 15 min. Eight min after antibody circulation, rats were perfused in vitro through the portal vein with 250 ml Tyrode buffer (4°C, 22.5 ml/min), gassed with 95% O_2 - 5% CO_2, to remove all serum components. Rats were fixed by continuation of the perfusion with 150-175 ml 2% (g/v) paraformaldehyde, 0.5% glutaraldehyde in 0.2 M phosphate buffer, pH = 7.4. After the fixation, the adrenal gland, ovary, liver, kidney, spleen, heart and striated muscle were excised, cut

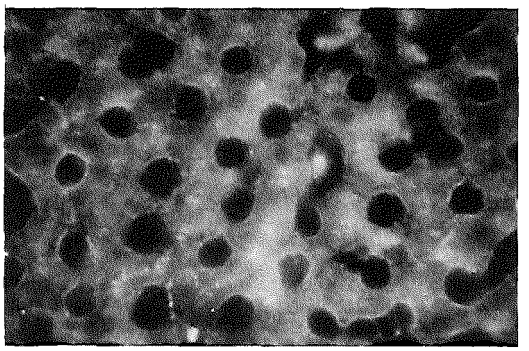
into small pieces and incubated in 2% paraformaldehyde, 1 M sucrose in the same buffer and frozen in liquid-N_2 and prepared for 5 µm thick sectioning on a cryostat (Bright, Instrument Company Ltd, Huntingdon, England) at -26° C.

Immunocytochemistry

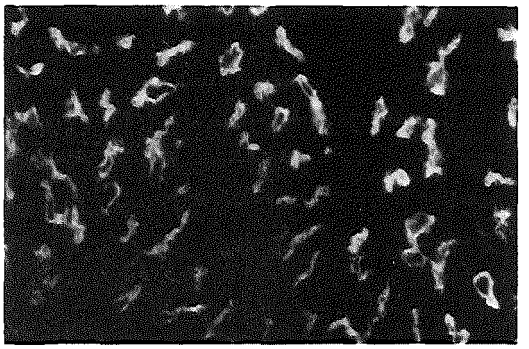
Cryosections were mounted on microscope glass slides, dried and incubated with glycin, 0.1 M in phosphate buffered saline (PBS-glycine). Sections were immunolabelled with fluorescein isothiocyanate conjugated anti-rabbit immunoglobulins from goat (Nordic, Immunological Laboratories, Tilburg, The Netherlands). After incubation for 30-60 min at 20°C, the sections were rinsed 3 times in PBS-glycine for 10 min, and 3 times in H_2O. After this washing procedure, the tissues were embedded in a drop of 10% (^{g}/v) 1,4-Diazobicyclo[2,2,2]-octan (Merck, West Germany) in 30% (^{v}/v) glycerol and a coverslip was placed and sealed. As soon as possible, the sections were observed and photographed.

Antibody-preparation

Antibodies were raised in rabbits against liver lipase purified as described earlier (6) from heparin containing rat liver perfusates. A purified γ-globulin fraction completely inactivates liver lipase activity and does not crossreact with lipoprotein lipase from extrahepatic tissues. 300 µl of the preparation is sufficient for a complete inactivation of lipase activities in the liver, adrenal glands and ovaries in vivo. Recently, by using the Western blotting technique, a monospecific interaction of the antibody with the liver enzyme was shown (8).



a



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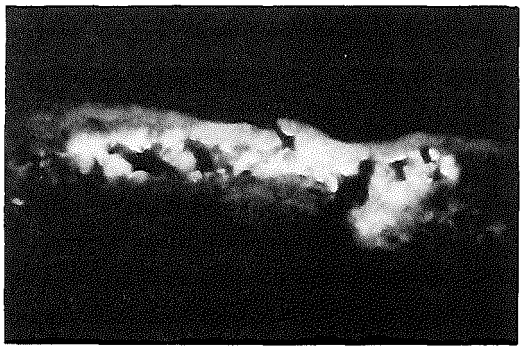
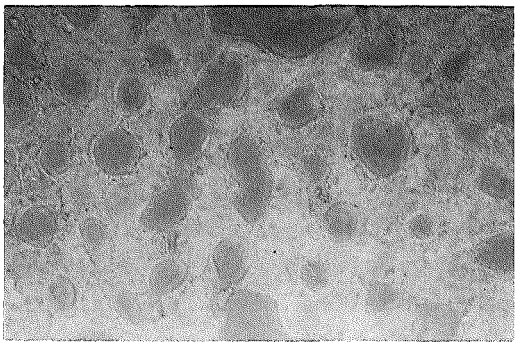


e

Figure 6.1
Cross-sections of the liver (1 a, 1 b), the zona fasciculata of the adrenal gland (1 c, 1 d) and part of the corpus luteum of the ovary (1 e, 1 f) after



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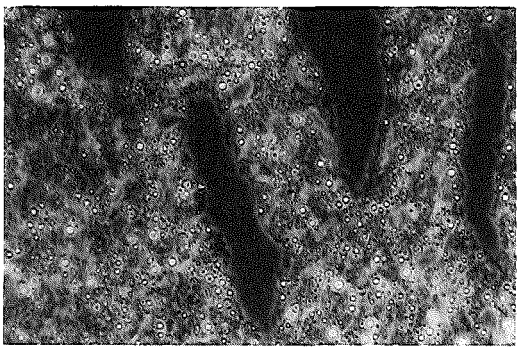

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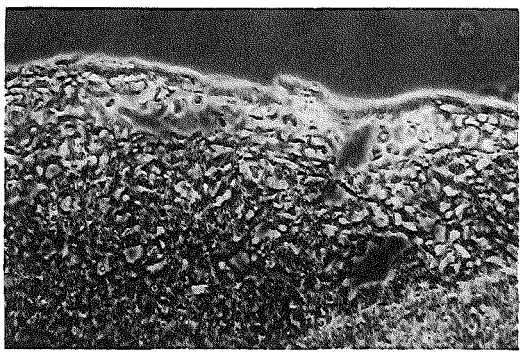

f

fluorescein exposure and the fluorescein and light combined result on the same part of the tissues (the adrenal gland and the ovary) or a different part of the tissue (liver) - Bar 10 µm.

RESULTS

After the administration of an anti-rat liver lipase antibody and immunofluorescence using a second antibody, label was detected in rat liver, adrenal and ovary. In none of the other tissues studied (kidney, heart, spleen and striated muscle) any fluorescence was visible (not shown). In the liver, fluorescein label was visible along the endothelial zone, facing the sinusoidal cavities (Fig. 6.1a) as was described earlier (7). A combined result of light and fluorescence microscopy on another part of the liver yields a better contrast of the location of this label (Fig. 6.1b). From these results it is not possible to say whether the label is also present in the subendothelium (space of Disse). In the liver, background fluorescence is rather high and is also present in unlabeled liver sections and in the control heparinized liver. In the adrenal gland, the label was exclusively located in the zona fasciculata; the major part of the adrenal cortex (9). Fig. 6.1c shows a clear location of the label on the endothelial cells of the straight capillaries in a section perpendicular to the sinusoids of the zona fasciculata. In this zone, the epithelial cells contain lipid droplets which is visualized in the combined results of light and fluorescein microscopy on the same part of the adrenal (Fig. 6.1 d). A longitudinal section of the cortex at a lower magnification showing the zonation of the label in the zona fasciculata is presented in Fig. 6.2. In the rat ovary, label is visible on endothelial cells, lining along the peripheral zone of the corpora lutea (Fig. 6.1e and 6.1f). In contrast to the results obtained in all the control heparinized tissues, the ovary is still labeled along the endothelial cells.

None of the tissues showed crossreactivity with the second fluorescein conjugated, anti-rabbit antibody.

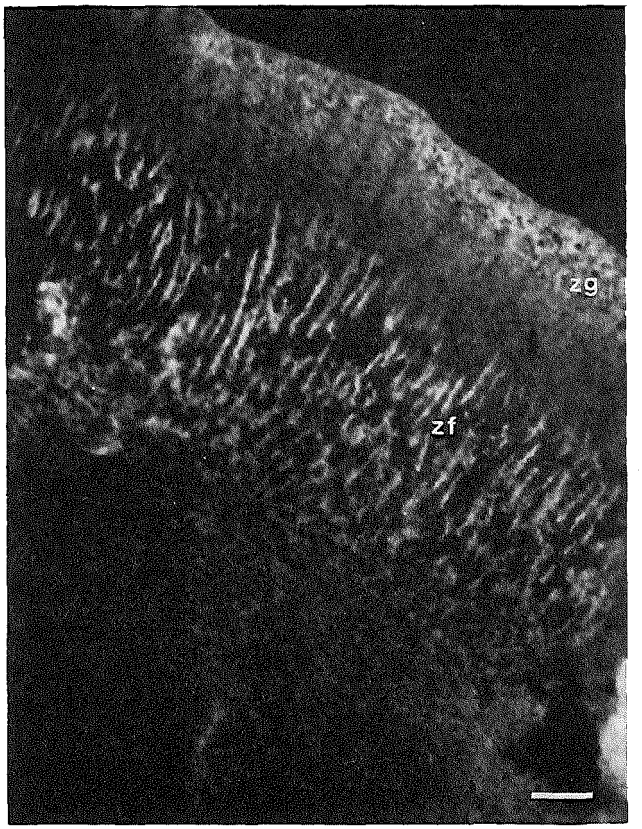

Figure 6.2
Fluorescein exposure of a radial section of the adrenal cortex. ZG = zona glomerulosa, ZF = zona fasciculata. - Bar 35 µm.

DISCUSSION

The exclusive localization of the anti-lipase antibody in the liver, adrenal and ovary is in accordance with the existance of the salt resistant lipase activities in the three organs mentioned (2).

After heparinization followed by antibody administration, label was no longer visible in cryosections of the liver and the adrenal gland. It was shown before that the lipase activity is removed from these tissues by this treatment (2). The present results indicate that also no enzymatically inactive material remains bound to these tissues. It also demonstrates the specificity of the labeling exclusively for "liver" lipase. In contrast, after heparinization label was still detectable in the rat ovary, which may be due to the relatively low blood flow in this organ. Compared with the adrenal and the liver, a 3-4 fold increase of heparin is needed for a complete release of the ovarian lipase (2). In the adrenal cortex, zonal differentiation and function are highly associated, mineralocorticoids are synthesized in the zona glomerulosa, glucocorticoids in the zona fasciculata and androgenic corticoids in the zona reticularis. As discussed in the Introduction, the lipase has been suggested to play a role in steroidogenesis. The present finding that the lipase is exclusively located in the zona fasciculata further supports a role in glucocorticoid production. The extracellular localization of the enzyme on the luminal surface of capillaries enables the enzyme to interact with plasma lipoproteins. By this way lipoproteins or components of lipoproteins may be taken up in the tissues. In a similar way, the localization of the ovarian enzyme in the corpus luteum demonstrates its proposed role in progesterone synthesis. In conclusion the results in the present study on the localization of the lipase in the liver, adrenal gland and ovary, give further support for a role(s) of the salt-resistant heparin-releasable lipases in steroidogene-

sis in the rat.

ACKNOWLEDGEMENTS

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CHAPTER VII

HUMAN HEPATOMA (Hep G2) CULTURES CONTAIN SALT-RESISTANT TRIGLYCERIDASE ("LIVER LIPASE")

N.L.M. Persoon, H.J. Sips and H. Jansen (1986)
Life Sci. 38:1029-1033

ABSTRACT

The culture fluid of Hep G2 human hepatoma cells contains triacylglycerol hydrolase activity resistant to high-salt concentrations. The lipase binds to Sepharose-heparin columns from which it can be eluted by 0.8 to 0.9 M NaCl. The nature of this lipase was studied using antibodies raised against "liver" lipases from human and rat origin. The anti-rat liver lipase inhibits both the postheparin human and rat plasma enzyme while the anti-human liver lipase has no effect on the rat enzyme. The lipase of the Hep G2 cultures showed affinity to the antibodies raised against rat as well as human "liver" lipase as shown by inhibition experiments. These results show that Hep G2 cells secrete "liver" lipase and that there seems to exist a structural homology between the lipases from rat and human origin.

INTRODUCTION

The liver of many, if not all, species contains an extracellularly located lipase. This enzyme is commonly indicated as hepatic triacylglycerol hydrolase or liver lipase. The enzyme can be released from the tissue by heparin and seems to play an important role in lipoprotein metabolism (for recent reviews see refs. 1,2). Its activity can be measured in the blood plasma after intravenous injection of heparin and, in laboratory animals, directly in the liver. Regulation and synthesis of the rat enzyme can be studied on freshly isolated (3) or maintenance-cultured parenchymal cells (4). Studies on the synthesis of the human enzyme are obviously more difficult to perform in view of the difficulty to obtain freshly isolated human liver tissue. Knowles and coworkers (5) established human hepatoma cell lines (Hep G2 and Hep 3B) which synthesize and secrete a variety of the major plasma proteins and therefore, in biosynthetic capacity, resemble normal parenchymal

liver cells. In this paper we demonstrate that one of these lines, Hep G2, secretes a triacylglycerol hydrolase with characteristics of liver lipase. This cell line is therefore potentially useful for studying the biosynthesis of human "liver" lipase.

MATERIALS AND METHODS

Cell culture

Hep G2 cells were grown to confluency in glass bottles (225 cm2, 14.5 mg total cell protein) in 50 ml of DMEM medium (Flow Laboratories, Irvine, Scotland) supplemented with 5% fetal calf serum, antibiotics and 10 IU heparin/ml (Tromboliquine, Organon, Oss, The Netherlands).

Sepharose-heparin affinity chromatography

70 ml of cell medium was kept on ice and loaded on a column of Sepharose-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) to which heparin was covalently bound (Sepharose-heparin). Loading of the column with supernatants was followed by a washing procedure with 50 ml of 30% (v/v) glycerol in 10 mM Na phosphate buffer, pH=7.0. The column was subsequently washed with 50 ml of 0.2 M NaCl, 30% (v/v) glycerol in 10 mM Na phosphate buffer, pH=7.0, followed by another washing procedure with the same buffer as described above. To the column a linear salt gradient from 0.0-2.0 M NaCl, in the same buffer as used before with a total volume of 80 ml was applied. Fractions (1.1 ml) were collected on ice and triacylglycerol hydrolase activity was measured on 100 µl of the fractions immediately.

Biochemical measurements

Triacylglycerol hydrolase activity was measured using an artificial tri[^{3}H]-oleoylglycerol emulsion in the presence of 1.0 M NaCl (6). Lipase activities are given in mU/ml (1 mU

represents 1 nmol free fatty acid released from the substrate in 1 min at 30°C). Protein content of Hep G2 cells was estimated by a standard method (7).

Anti-liver lipase polyclonal antibodies

Antibodies against human and rat liver lipase were raised in goats. The human enzyme was purified from postheparin plasma by 2 cycles of Sepharose-heparin affinity chromatography (8). The rat enzyme was purified from heparin-containing liver perfusates as described by Kuusi et al. (9). From both antisera a partly purified immunoglobulin fraction (50% ammoniumsulphate precipitate) was used (anti-human liver lipase, A-HLL, 53 mg/ml and anti-rat liver lipase, A-RLL, 48 mg/ml). As control served a similar fraction of the plasma of a non-immunized goat (control-γ-globulins, 52 mg/ml).

Inhibition of enzyme activity by anti-liver lipase antibodies

Aliquots (100 µl) fractions from Sepharose-heparin chromatography were incubated for 1.75 h with anti-human or anti-rat liver lipase antibodies or control-γ-globulins on ice and tested for triacylglycerol hydrolase activity.

RESULTS

In the media of confluent Hep G2 cultures a distinct triacylglycerol hydrolase activity was detectable (0.18±0.04 mU/ml). The lipase activity per flask amounted to about 12 mU (0.84 mU/mg cell protein). After removal of the media, cells were harvested by trypsination. The total lipase activity associated with the cells of 1 flask was 1.87 mU (0.13 mU/mg protein). In the absence of heparin a 2-3 times lower lipase activity in the medium of the cells was detected. Liver lipase exhibits a characteristic elution pattern during Sepharose-heparin affinity chromatography (8-10). Therefore the cell medium was applied to such a column and eluted with a linear

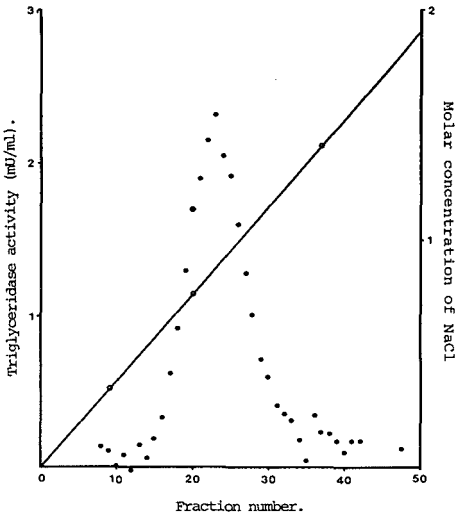

Figure 7.1
Sepharose heparin affinity chromatography of medium from confluent Hep G2 cells. Proteins were eluted with a linear salt gradient from 0-2 M NaCl (O) and salt resistant triacylglycerol hydrolase (●) activity was measured in the fractions collected. The shape of the gradient was determined by conductivity measurements.

salt gradient. Fig. 7.1 shows a typical example out of 4 separate experiments. Lipase activity eluted from the column as a symmetric peak. All enzyme activity in the medium was bound to and recovered from the column. The fractions with the highest lipase activity were eluted at 0.8 to 0.9 M NaCl, which is slightly higher than reported for liver lipase from postheparin plasma of different species. Therefore the identity with liver lipase was further studied using antibodies. Fig. 7.2 shows that anti-human liver lipase (A-HLL) does not cross-react with the rat enzyme, while the anti-rat liver lipase (A-RLL) partly inhibits the enzyme in postheparin human plasma (Fig. 7.3). The interaction between liver lipase and antibody was consistently better in the homologous systems.

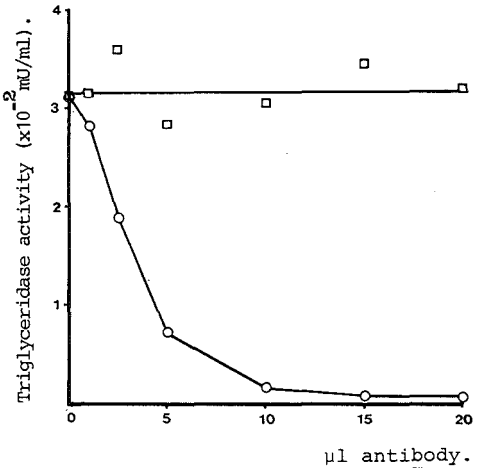

Figure 7.2
Immunotitration of liver lipase from rat postheparin plasma with goat anti-rat liver lipase (A-RLL, O) and goat anti-human liver lipase (A-HLL, □). Rat postheparin plasma was incubated on ice for 1.75 h with different amounts of antibodies. 0.9% NaCl pH 7.0 was added to obtain a total volume of 40 µl. Control-γ-globulins were added in the O µl antibody sample.

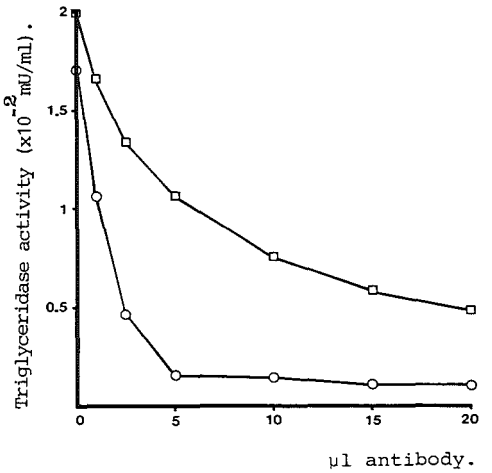

Figure 7.3
Immunotitration of liver lipase from human postheparin plasma by A-RLL (□) and A-HLL (O). The experiment was carried out exactly as described in Fig. 7.2.

The partially purified enzyme from Hep G2 cultures was inhibited by the anti-human as well as the anti-rat antibodies (Table 7.1). In separate experiments 51 to 60% inhibition was found with the anti-human liver lipase while with the anti-rat liver lipase more variable results were obtained.

Table 7.1
Effect of anti-liver lipase on triacylglycerol hydrolase activity from Hep G2 cells.

Experiments I and II were performed with different enzyme preparations. To 100 µl of the enzyme preparations 20 µl of antibody or control-γ-globulins (comp. legend of Fig. 7.2 and 7.3) was added. The incubations of enzyme with control-γ-globulins or antibodies were carried out in triplicate. Given are mean values ± standard deviation.

	Enzyme activity (mU/ml)	Inhibition (% of control)
Experiment I:		
Control-γ-globulins	0.88±0.06	-
Anti-human liver lipase	0.43±0.04	51
Anti-rat liver lipase	0.26±0.05	70
Experiment II:		
Control-γ-globulins	0.75±0.06	-
Anti-human liver lipase	0.30±0.03	60
Anti-rat liver lipase	0.47±0.05	37

DISCUSSION

In this chapter we describe a lipase in the medium of Hep G2 cells that binds to Sepharose-heparin columns from which it can be eluted by 0.8-0.9 M NaCl. It is resistant to a high NaCl concentration *in vitro* and is inhibited by antibodies against liver lipases. Therefore we conclude that the lipase is identical with or at least very similar to "liver" lipase.

The Hep G2 liver lipase differs in some aspects from the postheparin plasma enzymes. First of all, its binding affinity for the Sepharose-heparin column may be slightly higher than for the heparin eluted enzymes which have been described to be eluted at a lower (0.55 to 0.8 M NaCl (8-10)) salt concentration from the column. Secondly, the triacylglycerol hydrolase activity is, in contrast to the postheparin human enzyme, not completely inhibited by the anti-human liver lipase antibody. These differences may arise from intrinsic properties of the newly synthesized enzyme. As shown in this chapter, goat anti-rat liver lipase interacts with rat as well as human post-heparin plasma enzymes, while the anti-human lipase does not inhibit the rat enzyme. Since the Hep G2 lipase was inhibited by both antibody preparations, it seems that the lipase also resembles both the human and the rat enzyme, which is in accordance with the results that were obtained with the human enzyme (Fig. 7.3). The incomplete inhibition by the anti-human lipase preparation could suggest the presence of another, immunologically different, lipase activity. However, in preliminary experiments using monoclonal anti-rat liver lipase antibodies we observed that all the lipase activity of Hep G2 media binds to these antibodies (not shown), so that it is more likely that the incomplete inhibition of the activity arises from a structural difference with the postheparin plasma enzymes rather than from different enzymes. In contrast to the Hep G2 lipase, the enzyme in post-heparin human plasma does not show any significant immunoprecipitation with the monoclonal antibodies (see chapter III), which also indicates some structural heterology between both enzymes. This difference may be due to the fact that the enzyme is newly synthesized and not bound and released from the endothelium as are the postheparin plasma enzymes.

In conclusion: Hep G2 cells in culture are able to synthesize "liver" lipase and are therefore a useful tool to study the molecular basis of the synthesis and secretion of "human" liver lipase.

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CHAPTER VIII

GENERAL DISCUSSION

In this study on the salt-resistant heparin releasable lipases of the rat, monoclonal antibodies have been developed. Proliferating hybridomas in hundreds of wells were screened on antibody production, using a micro enzyme-linked immunosorbent assay (ELISA) which was adapted for the detection of anti-liver lipase antibodies. In this assay, the antigen was present as a partially purified preparation of rat liver lipase, immobilized on heparin pre-coated Terasaki-trays.

After immobilization of the lipase in this way, the enzyme is still active (capable in hydrolyzing palmitoyl-CoA), which indicates that the enzyme structure must be preserved. The *in vitro* presentation of the lipase on the trays might mimick the interaction of the enzyme with the vascular endothelium. The *in vivo* binding has also ionic (heparin-like?) binding characteristics. For the *in vivo* fulfilment of a function in lipoprotein metabolism the substrate binding site(s) of this enzyme will be pointing in the direction of the blood compartment to establish an enzyme substrate complex. Based on the comparison between *in vivo* and *in vitro* immobilization of the enzyme, the initial screening procedure with the micro-ELISA might be a highly suitable system for the detection of antibodies that could interfere with lipase activity. An enzyme activity immunoprecipitation with the ELISA positive supernatants was also carried out to avoid the disappointing selection for a different antigen that is present in the semi-purified sample on the trays and that might have escaped the purification procedures before immunization. Five hybridoma cell lines that produced antibodies, positive for both selection criteria after several times of subclonation, were isolated. Four of these antibodies (indicated as A, B, C and D) precipitated virtually all the triacylglycerol hydrolase and phospholipase A_1 activities (94% and 98% respectively) of the purified liver enzyme.

These results together with the fact that all the monoclonal antibodies recognize a 58 kD protein band on an immunoblot of the purified liver enzyme, suggest that both enzyme activities are performed by the same enzyme. However, the fifth of the monoclonal antibodies (indicated as E) precipitates 88% of the triacylglycerol hydrolase activity and only 59% of the phospholipase A_1 activity. Apparently, the liver contains two different forms of the enzyme which are both recognized by antibodies A, B, C and D. One of these forms cannot bind monoclonal antibody E and represents 6% of the triacylglycerol hydrolase and 39% of the phospholipase A_1 activity that is precipitated by the other antibodies (see Table 8.1). The difference between the two forms of the liver lipase which are indicated as I and I' (see Table 8.1) is not reflected in the molecular weight (58 kD) or the elution profile from a Sepharose-heparin column (0.75 M NaCl). These facts show that just a minor structural difference may account for the distinction of both forms with respect to the relative triacylglycerol hydrolase and phospholipase A_1 activities (see the ratios in Table 8.1). It is suggested that differences in glycosylation are involved. In vivo, different forms of the enzyme might arise from a differential post translational processing required, after secretion of the lipase from the liver parenchymal cell, for binding to its "receptor" on the vascular endothelium. The relative amount of the two forms of liver lipase (one with a relative high triacylglycerol hydrolase activity that is called Form I and one with a relative high phospholipase A_1 activity that is called Form I') in different species or even under different physiological conditions could determine whether the enzyme acts predominantly on triacylglycerol rich particles (such as intermediate density lipoproteins) or phospholipid rich particles (such as high density lipoproteins). With respect to the hydrolysis of triacylglycerol, the initial breakdown of the surface phospholipids of the lipoprotein by form I' could be an essential

Table 8.1
Immunoprecipitation of rat liver lipase activity.

			E		ABCD
x Triacylglycerol hydrolase activity	94%	=	88%	+	6%
y Phospholipase A_1 activity	98%	=	59%	+	39%
Ratio x/y*	20		31		3
	liver lipase	=	Form I	+	Form I'

* Ratio is based on the triacylglycerol hydrolase and phospholipase A_1 activity of the purified enzyme at pH=8.5 (350 mU/ml versus 17.5 mU/ml, see chapter IV, Materials and Methods section "purification of rat liver lipase").

step prior to the hydrolysis of core constituents by form I. The existence of two forms of liver lipase could explain the different results that have been published with respect to the function of the enzyme in vivo (see refs. 77,79,81,87,90, 101, chapter I).

All the monoclonal antibodies are able to inhibit both the phospholipase A_1 and triacylglycerol hydrolase activity to a certain degree. In case of monoclonal antibodies A and C, a similar degree of inhibition of the liver (and the adrenal) enzyme was observed after the in vivo administration of ascites preparations. This means that the epitopes for antibodies A and C that are located on the enzyme, are accessible to antibodies transported by the blood. As all the antibodies seem to interfere with the lipase activities, interactions with the active centre(s) of the enzyme (those parts of the enzyme, including the active sites, which are involved in substrate binding and catalysis) may be the case.

In Table 8.2, an overview of immunoinhibition and immunoprecipitation of enzyme activity by the different monoclonal antibodies is presented. From the different results in immunoprecipitation and immunoinhibition assays and the fact that only antibodies B and C gave a positive reaction in immunolocalization on acetone-fixed Hep G2 cells (not shown), it is concluded that all five monoclonal antibodies are different.

In Table 8.2 it is shown that whereas antibodies C and D show a similar degree of inhibition of the triacylglycerol hydrolase and phospholipase A_1 activity, binding of antibodies A, B en E result in a more drastic decrease of triacylglycerol hydrolase activity alone. As a similar result was also obtained with an emulsified mixture of phosphatidylcholine and triacylglycerol, the selective inhibition of enzyme activity by these antibodies cannot be related to the difference in size of micellar substrates that has been used. This result, together with the differential decrease of triacylglycerol hydrolase and phospholipase A_1 activity after a mild tryptic

Table 8.2
Percentage of liver lipase activity

	Triacylglycerol hydrolase		Phospholipase A_1	
Antibody	Precipitation	Inhibition	Precipitation	Inhibition
(in vitro)				
A	94	85	98	29
B	94	76	98	24
C	94	41	98	43
D	94	35	98	29
E	88	73	59	34
(in vivo)				
A		85		
C		42		

digestion of the enzyme and the result that was obtained after modification of the enzyme with phenylisothiocyanate, favour the existence of different structural domains on the molecule that are involved in the hydrolysis of phosphatidylcholine and trioleoylglycerol. The interaction of the enzyme with a polar lipid as phosphatidylcholine or a nonpolar lipid such as triacylglycerol will be different (at pH 7, phosphatidylcholine has no net electric charge with a positive charge at the choline headgroup ($-N^{+}(CH_3)_3$) and a negative charge at the phosphorus group). Shirai and others (ref. 19, chapter I), clearly showed the presence of a binding site for dipalmitoylphosphatidylcholine vesicles on the enzyme.

Based on the characteristics discussed above and the different polarity of triacylglycerol and phosphatidylcholine, it is postulated that two different substrate-interacting domains on the enzyme may be present.

As the hydrolytic action on the ester bond of both phospholipids and triacylglycerols most probably will support the same reaction mechanism, the presence of two different active sites seems less likely. The existence of two different substrate binding sites may then be necessary for the appropriate presentation of polar or nonpolar lipids before the hydrolysis by a common active site can take place. Phenylmethylsulfonylfluoride (PMSF) and N-tosyl-L-phenylethyl-chloromethyl ketone (TPCK) did not influence the phospholipase A_1 or triacylglycerol hydrolase activity of the enzyme, which would suggest that liver lipase may not be a serine-histidine hydrolase as has been suggested for lipoprotein lipase (ref. 26, chapter I) and pancreatic lipase. Although, it remains possible that such amino acids on liver lipase are not accessible to modification.

In vitro, all monoclonal antibodies cross-react with the salt-resistant heparin releasable lipases from the rat adrenal gland and ovary. For the rat adrenal gland, this was also shown ten minutes after the injection of monoclonal antibodies

A and C *in vivo* (both the liver and the adrenal lipase were similarly inhibited by the antibodies). All monoclonal antibodies also precipitated a part of triacylglycerol hydrolase activity from the rat testis (not shown). These results indicate further the large homology that seems to exist between the heparin releasable lipases of the steroid-hormone producing tissues.

The lipases from the liver, adrenal gland and ovary were studied, using a polyclonal antibody by immunofluorescence microscopy. The endothelial localization of the adrenal lipase in the *zona fasciculata* of the adrenal cortex and of the ovarian lipase in the corpus luteum is in good agreement with the heparin releasability of the enzymes and the *in vivo* inhibition of lipase activity by intravenously administered antibodies. The exclusive localization of the enzyme along the straight capillaries of the *zona fasciculata* supports the hypothesis of a role of the enzyme in glucocorticoid production. The hydrolysis of phospholipids of high density lipoproteins that arrive at the adrenal lipase located on the endothelium, could induce a flux of free cholesterol from the lipoproteins in favour of the underlying tissue (ref. 95, chapter I). Cholesterol entering the adrenal cells might be either esterified and stored in a cholesterol ester pool (indicated by the droplets that are found in these cells) or be directly converted to glucocorticoids.

In the ovary, the enzyme was localized along the sinusoids that surround the corpus luteum. Here the lipase could initiate a similar process as was discussed for the adrenal enzyme but now in favour of progesterone synthesis. As discussed earlier in this chapter, isolation of the rat liver enzyme (in the presence of protease inhibitor) reveals a 58 kD band visualized by the western blotting technique. It is shown in this thesis that a mild tryptic treatment of the purified enzyme degrades the protein, resulting in a 53 kD band and subsequently in a band of 48 kD. The phospholipase A_1 activity

was shown to be most sensitive to tryptic digestion.

In a number of studies, liver lipase was identified as a protein with a molecular weight of 53 kD. Apparently such preparations of liver lipase represent the proteolytic degraded form of the enzyme. The use of the degraded enzyme in the research on the function of liver lipase may result in an underestimation of its phospholipase A_1 activity. Therefore the application of protease inhibitors during the isolation procedures of the enzyme is highly recommended.

In this thesis, a triacylglycerol hydrolase is presented that was identified in the medium of a culture of a hepatoma cell line of human origin (called Hep G2). This enzyme, isolated by Sepharose-heparin affinity chromatography cross-reacted with antibodies that were raised against human and rat liver lipase. The cross-reactivity of the anti-rat liver lipase antibodies with the enzyme in postheparin human plasma and in the medium of Hep G2 cells shows that the rat and human enzymes are very much alike. This hepatoma cell line offers a new tool as an *in vitro* model system for the research on the production and the regulation of the human enzyme.

SUMMARY

Blood lipids derived from the dietary intake or produced by the liver, circulate as organized structures which are called lipoproteins. Based on the density of lipoproteins, several different classes can be recognized. Certain disorders in lipoprotein profiles have been shown to be associated with a higher chance to develop atherosclerosis. In this disease, the formation of cholesterol-rich plaques in the wall of bloodvessels may increase the incidence of a heart attack. From recent medical research on atherosclerosis, it was learned that a number of patients that mostly showed benefit of a lipid lowering intervention, had a higher activity of a certain enzyme that is located in the liver and that is called "liver lipase", than patients who showed a further progession of the disease. From earlier research on this enzyme in a rat model it was already postulated that liver lipase might be involved in the metabolism of cholesterol in the liver and, as similar liver-type lipases are found in the adrenal gland, in the ovary and in the testis, in steroid hormone producing tissues. Fundamental research on the rat enzyme will be helpful in the elucidation of the function(s) of the rat (and probably also the human) enzyme in lipoprotein metabolism. This thesis presents new information about the structure, the function and the localization of the liver (and liver-type) lipases that are present in the rat. In this research, five different monoclonal antibodies against rat liver lipase have been developed that all cross-react with the lipases that are found in the adrenal gland, the ovary and the testis. By using these antibodies in immunoblotting, the liver enzyme (purified in the presence of a protease inhibitor) was identified on the blot as a protein with a molecular weight of 58,000 Dalton. This protein turned out to be degraded by mild proteolysis with trypsin into a 53,000 molecule and subsequently into a 48,000 Dalton one.

It was already known that liver lipase is capable of hydrolysis of both triacylglycerol and phospholipid. From the characteristics of the five monoclonal antibodies in immunoprecipitation of phospholipase A_1 and triacylglycerol hydrolase activity, the purified liver enzyme was shown to exist of two different forms. Both forms of the enzyme do obtain both enzyme activities but differ in the ratio of triacylglycerol hydrolase activity versus phospholipase A_1 activity. The relative amount of the two forms of liver lipase in different species or even under different physiological conditions could determine whether the enzyme acts predominantly on triacylglycerol-rich particles (such as IDL) or phospholipid-rich particles (such as HDL). The fact that these enzyme activities are not inhibited to the same degree by some monoclonal antibodies and dissimilar results were also observed after trypsin degradation of the purified enzyme, shows that different domains on the protein are involved with the phospholipase A_1 and with the triacylglycerol hydrolase activity. This phenomenon was further studied after chemically changing the structural properties of the purified enzyme.

Modification of the lysine(s) residues with phenylisothiocyanate completely inhibited the triacylglycerol hydrolase activity, whereas the phospholipase A_1 activity was stimulated. These results favour the possibility that liver lipase contains two different substrate-binding sites, one for polar lipids such as phosphatidylcholine and one for non-polar lipids such as triacylglycerol. In an immunofluorescence microscopy study, the localization of the lipase in the adrenal gland and in the ovary of the rat was studied. In restricted areas of these organs, the lipases are extracellularly located along the vascular endothelium which explains the heparin releasability of the enzymes. The exclusive localization of the enzyme along the straight capillaries that are found in the zona fasciculata of the adrenal cortex strongly favours a function of the enzyme in glucocorticoid production. In the

rat ovary, the enzyme is located on endothelial cells lining the peripheral zone of the corpora lutea, which is highly suggestive for a role of the enzyme in progesterone synthesis. The proposed function of the enzyme in steroidogenesis in these organs is further supported by these results.

Different kinds of preparations of the monoclonal antibodies were separated and studied by Fast Protein Liquid Chromatography. Ascites preparations (with different monoclonal antibodies) turned out to be highly effective tools in the *in vivo* inhibition of triacylglycerol hydrolase activity in the rat liver and adrenal gland. Finally, cross-reactivity of a polyclonal antibody (directed against the rat enzyme) with the enzyme in postheparin human plasma, shows that the human and rat enzymes are very much alike. A salt-resistant triacylglycerol hydrolase that has been identified in the medium of human Hep G2 cells also cross-reacted with the same antibody. As this hepatoma cell line of human origin secretes the salt-resistant lipase, this cell line can be a useful *in vitro* model to study the production and processing of the human enzyme.

SAMENVATTING

De vetten die in ons lichaam in het bloed voorkomen (enerzijds afkomstig uit ons voedsel en anderzijds geproduceerd door de lever) circuleren als georganiseerde deeltjes die "lipoproteïnen" worden genoemd. Deze lipoproteïnen worden (gebaseerd op de dichtheid van de deeltjes) onderverdeeld in verschillende klassen. Het blijkt dat sommige afwijkingen die voorkomen in deze klassen de kans vergroten op het verkrijgen van atherosclerose. Bij deze ziekte worden cholesterolrijke afzettingen in de vaatwand gevormd hetgeen (in het geval van het dichtslibben van een hartkransslagader) kan leiden tot een verhoogde kans op het krijgen van een hartaanval. Uit recent medisch onderzoek aan atherosclerose is gebleken dat patienten die gunstig reageren op een langdurig cholesterol verlagend dieet een hogere activiteit hebben van lever lipase dan patienten waarbij een dergelijk dieet niet had geholpen. Uit eerder werk aan dit enzym (van de rat) kon geconcludeerd worden dat lever lipase waarschijnlijk betrokken is bij het cholesterolmetabolisme in de lever en (aangezien het enzym ook gevonden wordt in de bijnier en het ovarium en in de testis) in steroïd hormoon synthese. In dit onderzoek zijn 5 verschillende monoclonale antilichamen tegen het lever enzym geïsoleerd die allemaal tevens binden aan de lipasen die voorkomen in de bijnier, het ovarium en de testis. Door gebruik te maken van deze antilichamen in een eiwit "blotting"-techniek kon het molecuulgewicht van 58.000 dalton van het lever enzym worden bepaald. Lever lipase bleek door het eiwitafbrekende "trypsine" heel gemakkelijk te worden afgebroken tot een kleiner deeltje met molecuulgewicht 53.000 dalton en verder tot 48.000 dalton. Het was reeds bekend dat lever lipase in staat is om vetten zoals fosfolipiden en triacylglycerol af te breken. Precipitatie van de fosfolipase A_1 activiteit en de triacylglycerol hydrolase activiteit van het enzym met behulp van de monoclonale antilichamen bracht

aan het licht dat gezuiverd lipase bestaat uit 2 verschillende vormen die beide enzymactiviteiten bezitten maar verschillen in de verhouding van de triacylglycerol hydrolase activiteit tot de fosfolipase A_1 activiteit. De relatieve hoeveelheid van beide vormen in verschillende diersoorten of bij verschillende fysiologische omstandigheden zou kunnen verklaren of lever lipase vooral op triacylglycerol rijke deeltjes (zoals IDL) of op fosfolipide rijke deeltjes (zoals HDL) zal werken. Het feit dat deze enzymactiviteiten door sommige monoclonale antilichamen op een ongelijke manier worden geremd en verschillende resultaten ook werden waargenomen na afbraak van het enzym door trypsine, suggereert dat verschillende plaatsen op het enzym betrokken zijn bij de fosfolipase activiteit en bij de triacylglycerol hydrolase activiteit. Dit verschijnsel werd verder onderzocht door de structuur van het gezuiverde enzym enigszins te veranderen. Verandering van het aminozuur lysine met de stof fenylisothiocyanaat remde volledig de triacylglycerol hydrolase activiteit terwijl de fosfolipase A_1 activiteit werd gestimuleerd. Vanwege deze resultaten is de mogelijkheid aanwezig dat lever lipase twee aparte gedeelten bevat die kunnen binden met een polair of een apolair lipide zodat het enzym een interactie kan aangaan met of een fosfatidylcholine (polair) of een triacylglycerol (apolair). Door gebruik te maken van immunofluorescentie-microscopie werd de localisatie van het enzym in de bijnier en in het ovarium van de rat bestudeerd. Het blijkt dat de enzymen op zeer bepaalde gebieden in deze organen (aan de buitenkant van de cellen aan/bij de vaatwand van een bloedvat) aanwezig zijn (hetgeen verklaart waarom deze enzymen door heparine zijn vrij te maken in het bloed). De aanwezigheid van het bijnier enzym bij de straalsgewijs lopende haarvaten in de zona fasciculata van de bijnierschors is een sterke aanwijzing dat het enzym mogelijk betrokken is bij de productie van glucocorticoïden. In het ovarium wordt het enzym aangetroffen op het endotheel dat de bloedvaten bekleedt die aan de buitenkant en om het

gele lichaam (corpus luteum) heen liggen. Dit laatste duidt hoogstwaarschijnlijk op een functie van het enzym in progesteron synthese. De reeds eerder voorgestelde hypothese van de functie van de lipase in hormoon synthese in deze organen wordt door deze resultaten verder versterkt. Verschillende antilichaampreparaten zijn tijdens dit onderzoek gescheiden en werden bestudeerd met behulp van "Fast Protein Liquid Chromatography". Ascites-preparaten met verschillende monoclonale antilichamen bleken zeer effectief te zijn om de lipase activiteit in de lever en in de bijnier van het levende dier te remmen. Tenslotte blijkt uit de kruisreactiviteit van een antilichaam (dat gericht was tegen het ratte enzym) met het menselijke enzym dat het lever lipase van de mens en van de rat veel op elkaar lijken. Een zoutresistente lipase activiteit aanwezig in het medium van Hep G2 cellen bleek ook te binden met hetzelfde antilichaam. Deze leverkankercellijn (afkomstig van de mens) scheidt lever lipase uit en kan daarom geschikt zijn om als model te dienen in het onderzoek van de productie en de afwerking van het menselijke enzym in vitro.

LIST OF PUBLICATIONS

1. **Persoon, N.L.M., H.J. Sips, W.C. Hülsmann and H. Jansen** (1985) Monoclonal antibodies to rat liver-, adrenal- and ovarian salt-resistant lipase. Federation Proceedings **44**, 1456.
2. **Persoon, N.L.M., H.J. Sips, W.C. Hülsmann and H. Jansen** (1986) Monoclonal antibodies against salt-resistant rat liver lipase. Cross-reactivity with lipases from rat adrenals and ovaries. Biochimica et Biophysica Acta **875**,286-292.
3. **Persoon, N.L.M., H.J. Sips and H. Jansen** (1986) Human hepatoma (Hep G2) cultures contain salt-resistant triglyceridase ("Liver lipase"). Life Sciences **38**, 1029-1033.
4. **Persoon, N.L.M., W.C. Hülsmann and H. Jansen** (1986) Localization of the salt-resistant heparin-releasable lipase in the rat liver, adrenal and ovary. European Journal of Cell Biology **41**, 134-137.
5. **Persoon, N.L.M., W.C. Hülsmann and H. Jansen** (1987) Structural modulation of salt resistant rat liver lipase alters the relative phospholipase and triacylglycerol hydrolase activities. Biochimica et Biophysica Acta **917**, 186-193.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 10 mei 1959 geboren te 's-Gravenhage. Na het behalen van zijn atheneum B diploma in 1978 aan het Thomas More College te 's-Gravenhage begon hij in hetzelfde jaar aan de studie biologie aan de Rijksuniversiteit te Leiden.

Op 20 oktober 1981 behaalde hij het kandidaatsexamen met als specialisatierichting scheikunde (B_4). Het doctoraalexamen biologie legde hij op 20 december 1983 af met als hoofdvak celbiologie (Dr. W. de Priester, vakgroep Celbiologie en Genetica) en als bijvakken biochemie (Dr. B. Kraal, vakgroep Biochemie) en immunocytochemie (Dr. L.A. Ginsel, Laboratorium voor Electronenmicroscopie).

1 Januari 1984 trad hij in dienst bij de Nederlandse Organisatie voor Zuiver-Wetenschappelijk Onderzoek (Z.W.O.) als onderzoekmedewerker bij de Stichting Scheikundig Onderzoek in Nederland (S.O.N.) binnen het Instituut Biochemie I van de Erasmus Universiteit Rotterdam (hoofd Prof.dr. W.C. Hülsmann). De resultaten van drie jaar onderzoek zijn in dit proefschrift weergegeven.

Vanaf 1 juni 1987 is hij werkzaam als biochemicus binnen de afdeling Toegepaste Enzymologie behorende tot de centrale hoofdafdeling Research & Development van Gist-Brocades N.V. te Delft.

NAWOORD

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