ASPECTS OF THE REGULATION OF LIVER LIPASE

ASPECTEN VAN DE REGULATIE VAN LEVER LIPASE

PROEFSCHRIFT

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CONTENTS

APPENDIX PAPERS

V. EFFECTS OF HORMONES, FASTING AND DIABETES ON TRIGLYCERIDE LIPASE ACTIVITIES IN RAT HEART AND LIVER

H. Stam, K. Schoonderwoerd, W.A.P. Breeman and W.C. Hülsmann (1984) Horm. Metab. Res. **16**, 293-297

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ABBREVIATIONS

EFA	essential fatty acids
LPL	lipoprotein lipase
HL	hepatic lipase
LLA	liver lipase activity
A.A.	amino acids
Funct.	functional
ConA	concanavalin A
HDL	high density lipoprotein
LDL	low density lipoprotein
IDL	intermediate density lipoprotein
VLDL	very low density lipoprotein
Apo	apoprotein
HMG-CoA	3-hydroxy-3-methyl-glutaryl-Coenzyme-A
SD	standard deviation
SEM	standard error of the mean

CHAPTER I. INTRODUCTION

I.1. History

In 1943 it was shown that serum of hyperlipemic dogs was cleared by heparin injection (1). The factor released by heparin was called "Clearing Factor". Twelve years later Korn (2) postulated that a lipase from rat hearts, that was activated by heparin and serumproteins, and therefore called lipoprotein lipase (LPL), was identical with the "Clearing Factor". For many years it was assumed that the postheparin lipase activity consists only of lipoprotein lipase, although Spitzer had already shown in 1956 that also from liver a lipase was released by heparin (3). It was not until 1972 that it was recognized that postheparin plasma lipase activity is exerted by at least two different enzymes. La Rosa and coworkers (4) demonstrated that the lipase activity from the liver was resistant to the inhibitors of LPL, a high salt concentration and protamine sulphate. Greten et al (1972) isolated a similar enzyme from postheparin human plasma using Sepharose-heparin chromatography (5). However, the criteria used to distinguish the lipase from lipoprotein lipase were criticized (6,7). It only became clear in 1973, after it was found that both lipase activities were immunologically unrelated (8-11)that different enzymes exist in liver and in extrahepatic tissues . Since then the knowledge of the liver enzyme has rapidly expanded. In this thesis the enzyme is referred to as

liver lipase. In the literature synonyms such as hepatic lipase, hepatic endothelial lipase, heparin-releasable hepatic lipase, salt-resistant postheparin lipase, postheparin plasma protamine resistant lipase and liver plasma membrane phospholipase A_1 also appear. The enzyme has been purified (5,12-17) and characterized. Antibodies were raised to learn more about the function and localization of liver lipase (18,19-22). A few years ago a lipase activity similar to liver lipase was found in the adrenal gland of rats and humans and ovaries of the rat and was indicated as "liver lipase-like" or "livertype lipase" activity (24).

I.2. Characterization of liver lipase

In Table 1 some characteristics of liver lipase are summarized. The enzyme can be purified by affinity chromatography on heparin-Sepharose from which it can be eluted by 0.75 M NaCl (12,16,17). The pH-optimum of the enzyme is 8.0-8.5 as measured by triglyceride hydrolysis (25-27) and the lipase is, in contrast to lipoprotein lipase, not inhibited by 1 M NaCl (13,29,30). This property is frequently used to measure plasma liver lipase activity specifically (31). The molecular weight is 53,000-58,000 (77), determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and immunoblotting with monoclonal antibodies. The enzyme appears to be a glycoprotein as it binds to ConA. The amino acid composition of liver lipase has been published,

although doubts on the reliability of the data exist (26).

In vitro the enzyme exhibits a broad substrate specificity. It not only catalyzes the hydrolysis of triglycerides but also of di- and monoacylglycerols, acyl-CoA thiolesters and phospholipids. Also an acyl transfer activity of the enzyme has been described (13,25,28-30,78). The nature of the preferred substrate in vivo may be phospholipid as can be seen from the low K_m.

TABLE I
CHARACTERIZATION OF LIVER LIPASE

	Liver lipase	References
pH optimum	8.0-8.5	25-27
Effect of molar NaCl	Slight stimulation	13,29,30
Eluted from heparin Sepharose	0.75 M NaCl	12,16,17
Molecular weight	53,000-58,000	77
Substrates	Triglycerides (Km 6.2 mM), Diglycerides, Monoglycerides (Km 0.6 mM), Phospholipids (Km 0.1 mM), Acyl-CoA thioesters	13,25,28-30
Stereospecificity	Prefers Sn-1 over Sn-3	32

I.3. Physiological role of liver lipase

It has already been mentioned that in vitro liver lipase has a broad substrate specificity. The nature of its substrate

in vivo has been investigated by injection of antibodies to liver lipase *in situ* and the subsequent analysis of the different lipoprotein fractions in the rat (18-21) and cynomolgus monkey (22). A common finding in most of these studies was an increase in HDL-phospholipid contents, strongly suggesting a role of liver lipase in HDL-phospholipid metabolism. Indeed HDL-phospholipids are good substrates as has been shown in vitro (54). Because of the close correlation between the phospholipid and cholesterol content, a role of liver lipase in HDL cholesterol uptake has been proposed (18,33). In line with this proposal is the inverse relationship between liver lipase activity and HDL cholesterol levels found in a number of conditions (55-57), increase in HDL cholesterol during hypercortisolism (appendix paper II) and after norepinephrine treatment (appendix paper IV), conditions which lower liver lipase activity. An effect of liver lipase on the uptake of HDL cholesterol has been demonstrated by Bamberger et al. (58), who showed that preincubation of HDL with liver lipase leads, after incubation with liver hepatoma cells, to an increased uptake of HDL free cholesterol by the cells . A role of liver lipase in the metabolism of IDL has also been suggested (21), which was concluded from the increase in IDL observed after prolonged treatment of rats with anti-liver lipase. Because of the rapid turnover of IDL it may be questioned whether this increase is a direct consequence of the inhibition of liver lipase activity, or reflects the close relationship between the metabolism of all lipoproteins. Moreover, the

secretion of lipoproteins may be affected after inhibition of liver lipase activity since an increase of cholesterol synthesis has been found after inhibition of liver lipase activity (59).

Liver lipase activity has also been found in the adrenal glands and ovaries (24). A common aspect of these organs is that they are cholesterol demanding: the liver for the synthesis of bile acids and lipoproteins, the adrenal gland for the synthesis of corticosteroids and the ovaries for the synthesis of progesteron and estrogens. As shown by several investigators, the cholesterol needed for the synthesis of corticosteroids in the adrenal gland is mainly derived from extracellular sources rather than from de novo synthesis (60,61). The major cholesterol carrying lipoprotein in the rat is HDL. Indeed, in the rat, HDL cholesterol is the major source of cholesterol for the adrenal gland (62) and for the ovaries (63). Schwarz et al. (65) showed that in liver HDL free cholesterol is the precursor for the synthesis of bile acids. Given the demand for HDL cholesterol by the above mentioned organs and the proposed role of liver lipase in the uptake of HDL cholesterol, it may be that liver lipase activity in the adrenal gland and ovaries is involved in the regulation of steroidogenesis. Some factors that contributed to this idea were the increased liver lipase activity in the adrenals after prolonged ACTH administration (appendix paper II) and the increased liver lipase activity in the ovaries of pseudopregnant (24,64) rats. For the liver the physiological role is

less clear; the enzyme may be involved in hepatic metabolism as well as in the maintenance of serum lipoprotein levels. It is interesting to note that in the liver HDL free cholesterol is preferentially used for the synthesis of chenodeoxycholic acid and the amount of chenodeoxycholic acid is lowered after thyreoidectomy, in diabetes, after castration of males and in females, all situations associated with a lowered liver lipase activity (67,68). Chenodeoxycholic acid inhibits HMG-CoA reductase in the liver (69). Therefore, it could be that the observed increase in HMG-CoA reductase after inhibition of liver lipase activity may be mediated by a decrease in the amount of chenodeoxycholic acid. All these findings are suggestive for an important role of liver lipase in HDL cholesterol metabolism, as well as an explanation of its being a negative risk factor for the development of atherosclerosis (76).

I.4. Aim of the study

It is evident that factors that influence the activity of liver lipase could be important because of the role of liver lipase in HDL-cholesterol metabolism. At the start of this study not much was known about the regulation of liver lipase. The activity had been found to be decreased after fasting (10,34), during hypercortisolism (12), hypothyreoidism (35), insulin deficiency in diabetes (35,12) and by estrogen treatment (36) suggesting a role for respectively insulin, corti-

costeroids, thyroxine and estrogens in the regulation of the activity of the enzyme, respectively.

This thesis describes the effects of several hormones (corticosteroids, catecholamines, insulin, thyroxin) on the liver lipase activity in vivo and in vitro. In chapter II and appendix paper I the model used to study the regulation of liver lipase in vitro is shown in addition to several factors influencing the liver lipase activity in vitro. Aspects of the regulation of liver lipase in vivo are described in chapter III including the turnover of liver lipase and the influence of essential fatty acid (EFA) deficiency. In chapter IV factors influencing the enzyme activity are discussed by an integration of literature data with our own experiments.

CHAPTER II. FACTORS AFFECTING LIVER LIPASE ACTIVITY IN VITRO

II.1. In vitro model

The liver consists of parenchymal-, fat-storing, Kupffer and endothelial cells. The latter two are often taken together as non-parenchymal cells. The bulk of the liver mass consists of parenchymal cells.

After incubation of parenchymal cells liver lipase activity is found in the medium (39,40), indicating that liver lipase is synthesized by these cells. On the other hand, if non-parenchymal cells are incubated no liver lipase activity is found; neither in cells nor incubation medium. Incubation of non-parenchymal liver cells with purified liver lipase however, causes the rapid disappearance of the lipase activity from the medium. The enzyme is then bound to the non-parenchymal cells (38) in a specific manner (41). It has been found previously that the lipase is mainly extracellularly located. An indication for this localization of liver lipase was the rapid release of liver lipase activity after heparin injection and by the loss of liver lipase activity upon cell isolation with collagenase (37,38). In addition Kuusi and coworkers (81) and Persoon et al. (82) have demonstrated an extracellular localization, presumably at the vascular endothelium, by immunological methods.

The regulation of the synthesis and release was studied by following the secretion of liver lipase from the isolated

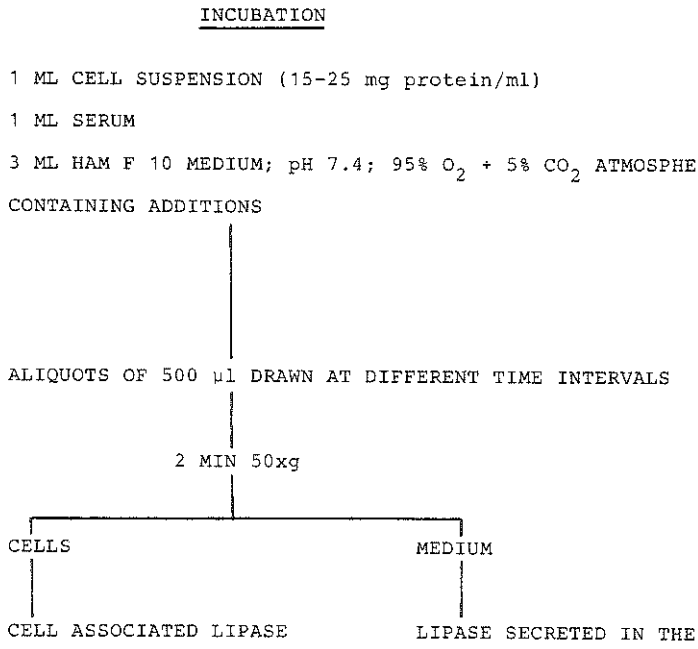

Fig. 1. Model used to study the synthesis and secretion of liver lipase activity in vitro. Isolated parenchymal cells were incubated as indicated.

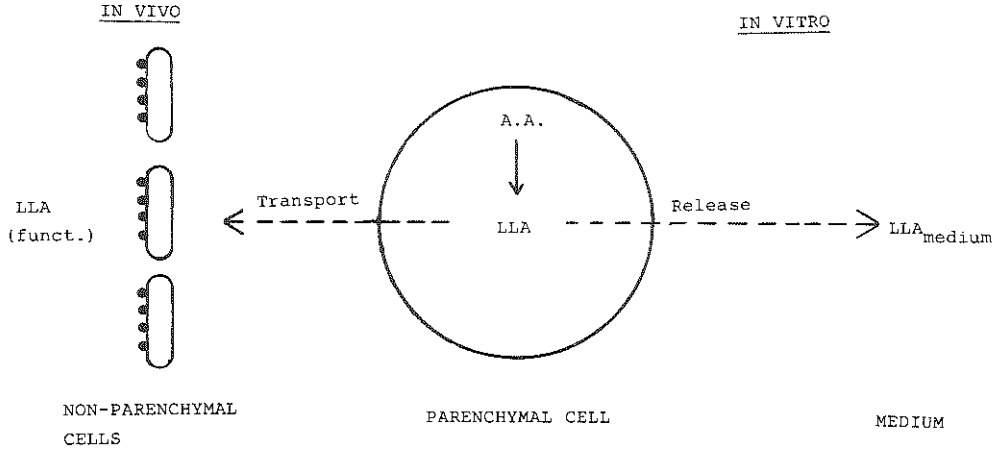

Fig. 2. Model used to study the synthesis and release as well as the binding of liverlipase activity in vitro with isolated parenchymal and non-parenchymal liver cells. The cells were isolated as described in appendix paper I.

parenchymal cells into the medium (Fig. 1). The binding of the enzyme to non-parenchymal cells was studied with purified enzyme and isolated parenchymal cells (Fig. 2).

II.2. Effects of a heat-stable serum factor on the secretion of liver lipase activity

Parenchymal liver cells require the presence of serum for an optimal release of liver lipase activity (48) (Fig. 3). Omission of serum resulted in a much lower release of liver lipase activity into the medium. The activity in the medium without serum reached a plateau level after 2 hrs. To learn

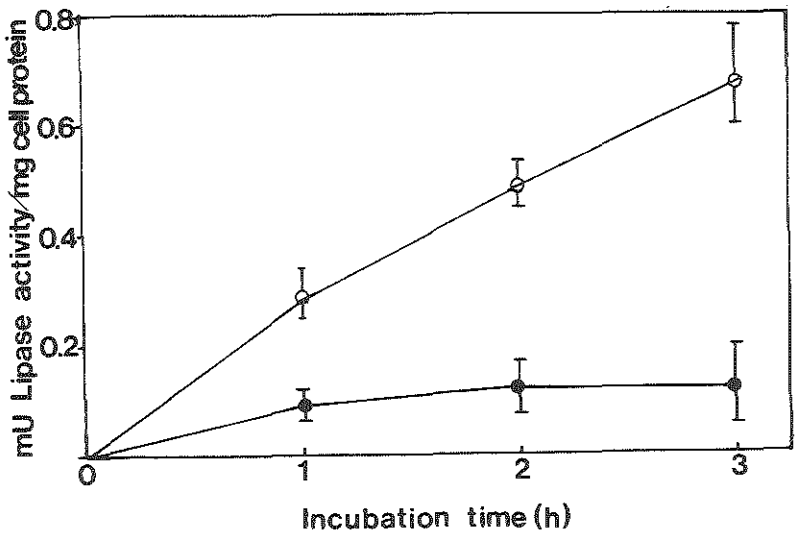

Fig. 3. The secretion of liverlipase activity from isolated parenchymal liver cells with (o) or without (●) 20% serum present in the incubation medium.

TABLE II
EFFECT OF DIFFERENT SERA ON THE SECRETION OF LIVER LIPASE ACTIVITY BY PARENCHYMAL LIVER CELLS

Isolated parenchymal liver cells were incubated in the presence of 20 percent serum from different species during 3 hr. The secretion of lipase activity is given as the percentage of enzyme activity secreted in the presence of 20% male rat serum (0.68 mU/mg cell protein).

Species serum	Sex	n	Liver lipase activity (%)
Rat	Male	12	100
Rat	Female	6	95±10
Bovine	Female	12	102±12
Human	Male	6	98± 5
Rat (lipoprotein free)	Male	6	90±15
Rat (1 hr 56°C)	Male	12	105± 8

more about the factor responsible for the secretion of liver lipase sera of different species have been used. No difference was found in the effect of sera of rat, cow or man, nor between sera from female rats or male rats (Table II). This table also shows that the stimulatory activity of the serum was not inactivated by prior heating of the serum at 56° C for 1 hr and that it is also present in lipoprotein-free serum. So it can be concluded that the factor is probably not an enzyme and lipoproteins are not involved. The stimulatory activity of the serum proved to be highly resistant to high temperatures as it was still active after heating the serum for 5 min at

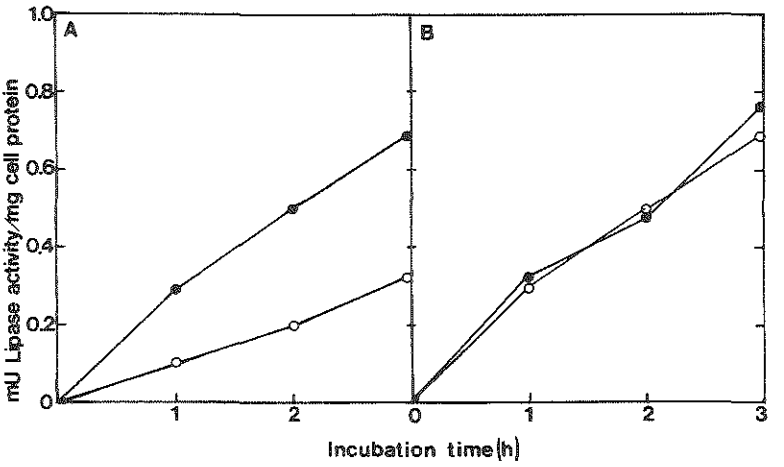

Fig. 4. The secretion of liverlipase activity from isolated hepatocytes. Parenchymal liver cells were suspended in 5 ml incubation medium A. containing 20% control serum (●) or 20% heated serum (5 min 100° C) (○); B. containing 20% control serum (○) or 80 mg albumin in addition to the 20% heated serum (●).

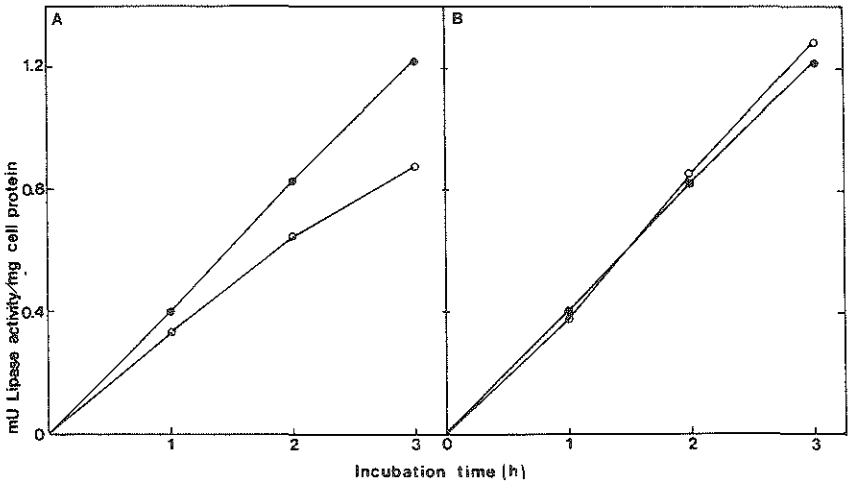

Fig. 5. Liverlipase activity secreted by isolated parenchymal cells. A. In the presence of 20% serum + heparin (15 U/ml)(●) or 20% heated serum (5 min, 100°C) + heparin (15 U/ml)(○). B. in the presence of 20% serum + heparin (15 U/ml)(●) or 20% heated serum (5 min, 100° C), heparin (15 U/ml) and albumin (20 mg/ml)(○).

100° C. The secretion in the presence of this heated serum was 50 percent of the control (Fig. 4 A) while the protein content was decreased after heating to 3 mg/ml. Since during secretion a loss of enzyme activity occurred, which could be prevented by the addition of heparin (appendix paper I), heparin was added to the incubation media to study whether this loss in liver lipase activity was due to an increased inactivation of the secreted liver lipase activity or to a loss of the stimulating serum factor. It can be seen that addition of heparin resulted in an almost complete maintenance of liver lipase activity in the presence of heated serum (Fig. 5 A). Addition of albumin to correct for the protein content resulted in a complete recovery of liver lipase activity in the absence (Fig. 4 B) as well in the presence of heparin (Fig. 5 B). Neither dialysis nor chromatography on Sepharose-heparin influenced the stimulating factor in this heated serum indicating that a positively charged polypeptide, like apo E is not the factor (results not shown). In Fig. 6 it can be seen that just like control serum the secretion was optimal in the presence of 20% heated serum. Thefore the factor is heat-stable and of high molecular weight because heating at 100° C nor dialysis affected the stimulating properties. Only the stabilizing effect, probably due to the high protein content of control serum disappeared after heating. A heat-stable factor has been found by Fujiwara (42) to stimulate DNA and protein synthesis in hepatocytes and by others in rat heart cell cultures (43,44). However, this factor was dialyzable,

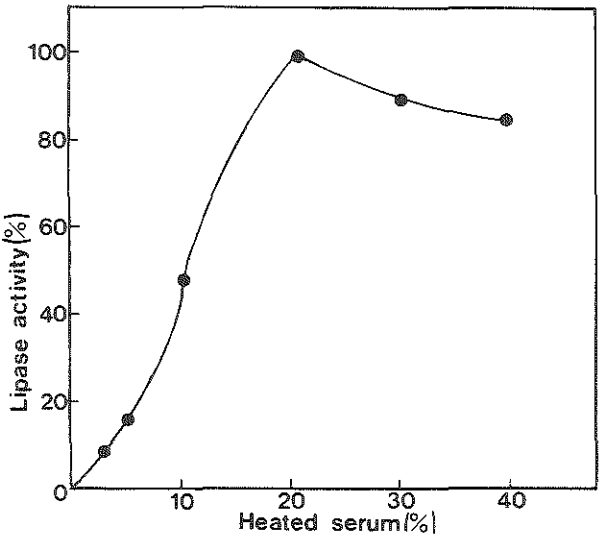

Fig. 6. Effects of different amounts of heated serum (5 min, 100° C) on the lipase activity secreted by parenchymal cells in vitro. The lipase activity secreted in the presence of 20% control serum was taken as 100%.

only active in the presence of serum and required for the stabilization of the protein, which is at variance with the factor studied by us where the stabilizing effect was lost after heating. Our serum factor could be required for the de novo synthesis of the enzyme, although the secretion of liver lipase in the absence of serum was not inhibited by puromycin (39) and reached a plateau level after 1 or 2 hrs, which would resemble the heat-stable factor found by Fujiwara (42).

III.3. Factors affecting the secretion of liver lipase activity

Not much is known about the short-term regulation of liver lipase. Most of the effects, such as the lowering of lipase activity after corticotrophin administration (appendix paper II), estrogen treatment (36) and hypothyreoidism (35) are observed after several hours or even days. Therefore the mechanisms and hormone sensitivity of the liver lipase activity in the short term were studied. To study the dependency of the de novo synthesis on the secretion of liver lipase we performed the secretion experiments in the presence of cyclo-

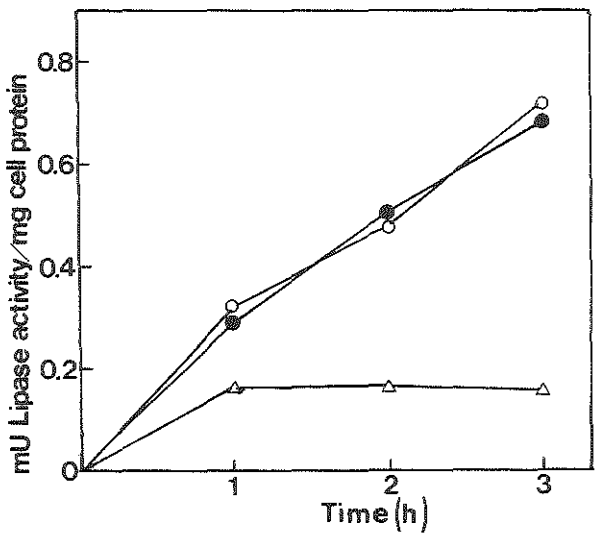

Fig. 7. The effect of cycloheximide or actinomycin D on the secretion of liverlipase activity. The cells were incubated as described in Fig. 1 (●) with the addition of cycloheximide (Δ) or actinomycin D (o).

heximide or actinomycin D. As shown previously (40), secretion of liver lipase activity was completely inhibited after 1 hr in the presence of cycloheximide (Fig. 7). During the first hour, however, the secretion was about 60% of the control. The secretion of liver lipase activity was not affected by actinomycin D over the studied time period of 3 hrs, indicating that the transcription was not involved during this short time period. Since liver lipase is apparently a glycoprotein the possible involvement of the glycosylation on the secretion of liver lipase activity was also studied (Figs. 8 A,B). In the presence of tunicamycin, secretion is completely blocked after 1 hr. During the first hour tunicamycin hardly affects the secretion of lipase activity. In the insert it is also shown that cellular lipase activity is inhibited indicating the necessity of the glycosylation for the activation of liver lipase. Proteins destinated for secretion have to be transported from the Golgi apparatus to the plasma membrane where they are secreted. The involvement of the microtubuli and or microfilaments in this transport process was investigated. To differentiate between the microfilaments and microtubuli experiments were performed in the presence of colchicin or cytochalasin B (an inhibitor of the microfilaments). It can be seen that the secretion is not influenced by cytochalasin B while it was reduced by colchicin (Fig. 8 A), indicating an involvement of the microtubular system. The cellular lipase activity was not affected by both drugs.

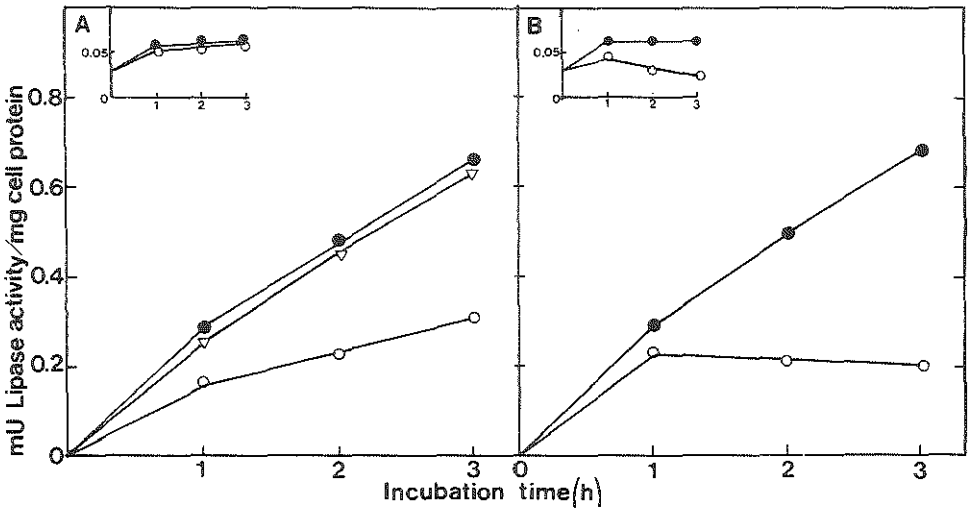

Fig. 8. The effect of colchicin, cytochalasin B (A) or tunicamycin (B) on the secretion of liverlipase activity by isolated hepatocytes. The cells were incubated as described in Fig. 1. Control (●) A; with colchicin (o) or cytochalasin B (▽), B with tunicamycin (o). In the insert the lipase activity in the cells are measured under the same conditions.

The effects of several hormones, known or at least suggested to affect the lipase in vivo on liver lipase activity in the short term, are shown in Table III. The secretion of liver lipase was only affected by insulin and epinephrine, while corticosterone, 17 β-estradiol, thyroxin and glucagon had no effect. The inhibition of secretion of lipase activity by the catecholamines is further investigated in appendix paper IV.

TABLE III
THE EFFECT OF DIFFERENT HORMONES ON THE SECRETION OF LIVER LIPASE ACTIVITY

Parenchymal liver cells were incubated in the absence or presence of different hormones for 3 hr. After the incubation the lipase activity was measured in the medium. Lipase activity is expressed as the percentage of liver lipase activity secreted in the absence of hormones.

Additions	n	Liver lipase activity (mU/mg cell protein)
None	6	0.82±0.08
Epinephrine (10^{-7}M)	4	0.55±0.06[a]
Glucagon (50 ug/ml)	3	0.80±0.09
Insulin (10 mU/ml)	6	1.02±0.06[b]
Thyroxin (34 ug/ml)	3	0.78±0.07
Corticosteron (20 ug/ml)	6	0.74±0.10
17 β-Estradiol (4 ug/ml)	4	0.71±0.09

[a] $p < 0.005$
[b] $p < 0.01$

II.4. Binding of liver lipase to non-parenchymal cells in vitro

After secretion of liver lipase by the parenchymal liver cells, liver lipase is probably transported to the non-parenchymal cells where it is bound to specific binding sites (41). These binding sites are also involved in the regulation of liver lipase (appendix paper III). Besides secretion and syn-

thesis of liver lipase there is a continuous degradation of the enzyme. Therefore factors influencing the turnover of the receptor are also involved in the regulation of the lipase activity. Since the functional lipase activity is bound to the non-parenchymals liver cells, the turnover of the enzyme was investigated with isolated non-parenchymal liver cells. As liver lipase bound to the non-parenchymal cells can be released by heparin (41) we estimated the amount of the enzyme that was released from the non-parenchymal liver cells by heparin at different time periods. Because HDL is believed to be the major substrate of liver lipase we studied the effect of HDL on the releasability of liver lipase. No difference was found between the amount of liver lipase activity that could be released by heparin with or without HDL (Fig. 9 A). The amount of liver lipase activity remaining on the cells, however, decreased at a faster rate in the presence of HDL (Fig. 9 B) which was not due to an effect of HDL on the assay of liver lipase activity. So it seems that the turnover of the enzyme is affected by HDL; an effect which would be analogous to the enhanced turnover of the LDL receptor after binding of LDL in coated pits. This analogy, also proposed for liver lipase (46), is suggestive for the internalization or degradation of the liver lipase through receptor-mediated endocytosis. After interaction with its substrate HDL the lipase may be internalized via coated pits.

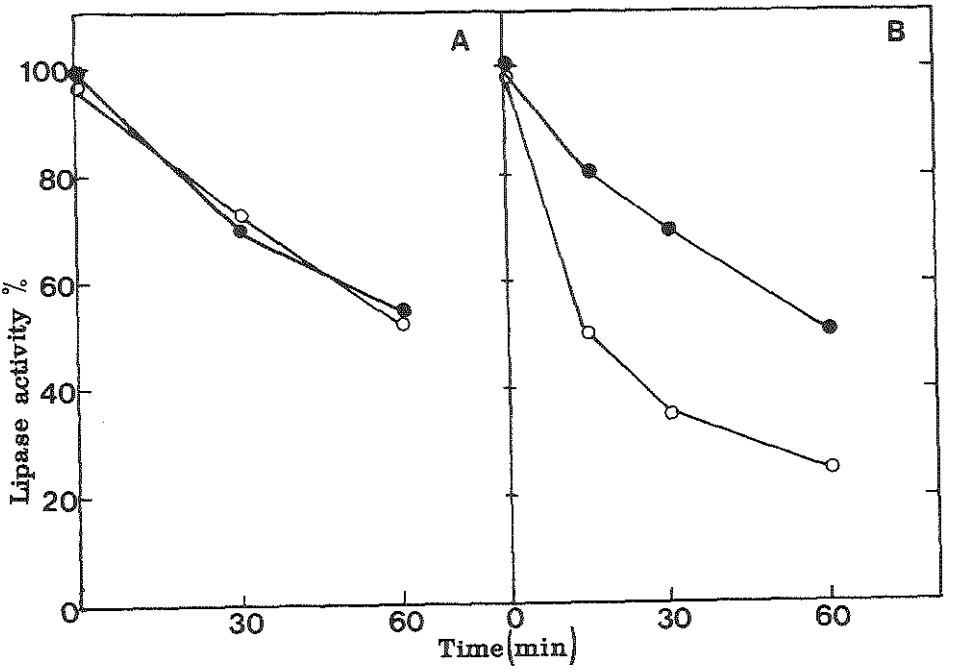

Fig. 9. The effect of HDL on the liver lipase activity bound to the non-parenchymal liver cells in vitro. Liver lipase was bound as described in appendix paper III, and incubated with HDL for different time periods. A, the relative amount of liver lipase activity released with heparin (5 U/ml), 100% is the amount releasable at t=o with (o) or without (●) HDL. B, The relative amount of liver lipase activity remaining on the cells, as percentage of the amount remaining on the cells at t=0 with (o) or without (●) HDL after heparin release.

TABLE IV

INHIBITION OF LIVER LIPASE ACTIVITY BY CYCLOHEXIMIDE AND NOT BY ACTINOMYCIN D IN INSULIN TREATED FASTED RATS

Glucose was administered to fasted, male rats, 15 min before the injection of insulin (0.5 I.U. s.c.), by a stomach tube (3 ml 10% glucose). 50 I.U. of heparin were injected (i.v.) 1 hr after the administration of insulin. A number of rats had received cycloheximide (1 mg/kg body weight, i.p.), 2 hr prior to the injection of heparin or Actinomycine D (500 ug/kg body weight, s.c.) 5 hr prior to the injection of heparin. 6 min after the heparin injection blood was withdrawn and liver lipase activity was measured in the serum.

Condition	n	Treatment	Liver lipase activity (mU/ml posthep. serum)	
Normally fed	6	None	450±28	P 0.01
Fasted	6	None	350±15	P 0.005
Fasted	4	Glucose + insulin	438± 8	
Fasted	4	Glucose + insulin + cycloheximide	263±25	P 0.001
Fasted	4	Glucose + insulin actinomycin D	435±17	

CHAPTER III. REGULATION OF LIVER LIPASE IN VIVO

III.1. Short term regulation by insulin

Liver lipase activity is lowered after fasting and in diabetes suggesting a role of insulin in the regulation of liver lipase (Table III). Indeed, insulin has been found to enhance the secretion of liver lipase activity in vitro. Therefore we investigated whether insulin could enhance liver lipase activity in the short term in vivo. In these experi-

TABLE V
EFFECT OF ACTINOMYCIN D ON LIVER LIPASE ACTIVITY

Rats were divided in 3 groups. Group 1 was injected with saline, group 2 received Actinomycin D 4.5 hrs prior to killing and group 3 was injected just as group 2 with Actinomycin D (150 g/100 g body weight, s.c.) but 20 hrs prior to killing.

Group	n	Liver lipase activity (mU/total liver)
Control	4	6930±69
Actinomycin 4.5 hrs	4	6007±50
Actinomycin 20 hrs	4	4720±588*

* $P < 0.005$

ments rats were fasted overnight which led to a lowering of the lipase activity. The lipase activity was normalized when rats were given glucose plus insulin (Table IV) (80). This normalization of the lipase activity was abolished by prior treatment of the animals with cycloheximide but not with actinomycin D. Insulin probably enhances the liver lipase activity by stimulation of protein synthesis at the level of translation because the insertion of ^{3}H leucine and ^{3}H-glucosamine was raised in the secretory proteins in vitro and the effect of insulin was abolished by cycloheximide. The transcription of liver lipase is probably not involved in the short term regulation of liver lipase activity since actinomycine D affects liver lipase activity only after 20 hrs. Therefore, the half-life time for the related mRNA is more than 20 hrs (Table V).

III.2. <u>In vivo</u> studies on the turnover of liver lipase in fed, fasted and hypercorticistic rats

Insulin and corticosteroids are involved in the regulation of liver lipase activity. Besides the synthesis or binding (appendix paper III) the turnover of the enzyme may also be involved. Therefore, the turnover of the enzyme was studied <u>in vivo</u> in normally fed rats (Fed-rats), rats fasted for 24 hrs (fasted rats) and rats that had been treated with corticotrophin for 10 days (ACTH-rats). In addition rats were treated

TABLE VI

EFFECT OF CYCLOHEXIMIDE TREATMENT ON LIVER LIPASE ACTIVITIES (LLA) IN NORMALLY FED, FASTED AND ACTH-TREATED RATS

Normally fed, fasted (overnight) and ACTH-rats were divided into 2 groups, one group was injected with cycloheximide (1 mg/kg body weight, i.p.) and the other with 0.9% NaCl. After 4.5 hrs the rats were killed and lipase activity was measured in the liver. The relative lipase activity was expressed as the percentage of the NaCl-treated animals. The results are given as the means ± S.E.M.

Conditions	Liver lipase activity		
	-cycloheximide	+cycloheximide	
	(mU/total liver)		(% of control)
Control rats (5)	5686±159	3158± 10	55
Fasted rats (3)	4405±240	2177± 53	49
ACTH-rats (3)	3314±126	1749±256	53

with either cycloheximide (experimental group) or 0.9% NaCl (control) and the liver lipase activity was determined in the liver 4.5 hrs later. In the experimental groups the lipase activity was 50% lower, compared to the controls (Table VI). The decrease in the absolute amount of liver lipase activity, however, is highest in the normally fed rats (2500 ± 150) followed by the fasted and hypercorticistic rats (2200 ± 200 and 1600 ± 210, respectively). It is known that the lipase is synthesized and secreted by the parenchymal liver cells and bound to the endothelial cells where the enzyme exerts its function. Therefore the turnover of the heparin releasable enzyme was investigated in the following manner. Rats, pre-

treated with either saline (control) or cycloheximide (1 mg/kg body weight, experimental group), were injected with heparin, 60 min after the cycloheximide injection. Blood was withdrawn 6 and 60 min after the first heparin injection and the heparin-releasable liver lipase activity was measured in the serum. Sixty min after the first heparin injection rats were injected with heparin for the second time, blood was withdrawn 6 min thereafter and liver lipase was measured in the serum. The results of Table VII and Fig. 10 show that liver lipase activity decreased 1 hr after heparin injection but full activity may be obtained by reinjecting heparin in all groups of rats. Pretreatment with cycloheximide, however, causes a failure of heparin to elicit a full effect after the second injection. This effect was more pronounced in the fed and fasted rats compared to the ACTH rats. From these experiments, it is concluded that the turnover of liver lipase is decreased in the ACTH and fasted rats because less lipase activity is formed in 4.5 hr. It can be calculated that the synthesis of liver lipase in the fasted and ACTH rats is 80% and 60% of the control rats, respectively which is in accordance with the in vitro experiments (appendix paper III). From Table VII it can be seen that after heparin injection, the liver mobilizes heparin releasable lipase in a short time period. Within 1 hr almost 40-50% of the activity is removed from the plasma but full activity is obtained by reinjecting heparin. Part of this effect must be due to replenishment of the liver plasma-membranes with liver lipase synthesized de novo in 1 hr. It

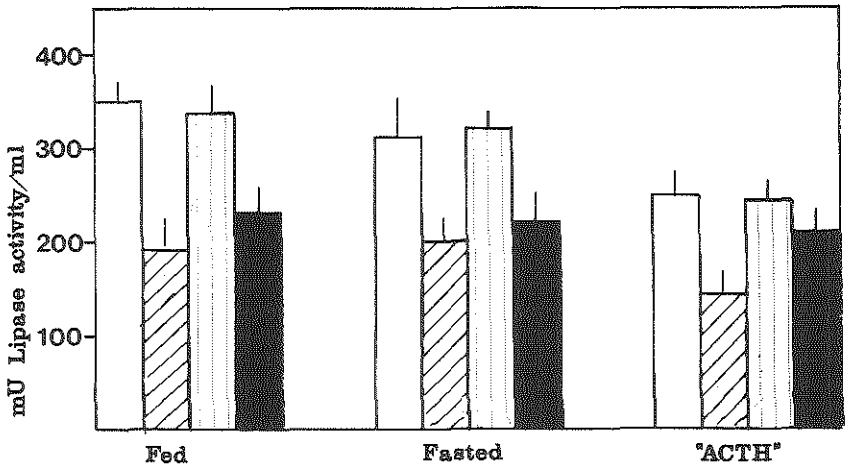

Fig. 10. Release of liver lipase activity into the blood after one or two heparin injections. Rats were treated as described in the legends to Table VII. Liver lipase activity in the blood 6 min after the first heparin injecton □. Liver lipase activity in the blood 60 min after the first heparin injection ▨. Liver lipase activity in the blood after the second heparin injection ⁞⁞. Liver lipase activity in the blood of rats, treated with cycloheximide, after the second heparin injection ■.

can be calculated from this Table that within 1 hr 1900 mU, 1500 mU and 700 mU liver lipase activity is synthesized in the livers of the control, fasted and ACTH rats, respectively. This indicates an enhanced synthesis of liver lipase after heparin injection. Of interest is the observation that in spite of the stimulated synthesis, the liver lipase activity did not exceed the lipase activity released by the first

TABLE VII

RELEASE OF LIVER LIPASE ACTIVITY (LLA) INTO THE BLOOD AFTER ONE OR TWO HEPARIN INJECTIONS

Control, fasted and ACTH-rats were injected with heparin (50 I.U., i.v.). After 6 min and 60 min blood was withdrawn. At that time heparin was injected again and blood was sampled 6 min later (66 min sample). A number of rats had received cycloheximide (1 mg/kg body weight) 1 hr before the first heparin injection. As indicated LLA was measured in the serum of the samples. Results are expressed as the percentage (± S.D.) of the LLA measured in the first blood sample (6 min).

Conditions	Time of blood sampling	Number of heparin injections	LLA +cyclo-heximide (%)	LLA -cyclo-heximide (%)
Control rats (3)	6	1	100	100
	60	1	56±4	55± 7
	66	2	68±5	94± 6
Fasted rats (3)	6	1	100	100
	60	1	61±4	57± 6
	66	2	67±5	102± 4
ACTH-rats (5)	6	1	100	100
	60	1	56±8	51±14
	66	2	79±8	98± 6

heparin injection. In the control, fasted and ACTH rats, the difference in lipase activity between these groups was maintained (Fig. 10).

III.3. Effect of an essential fatty acid (EFA)-deficient diet on liver lipase activity

III.3.1. Introduction

Animals, fed a diet without essential fatty acids (EFA), develop a syndrome known as EFA deficiency (47,48). Biochemically, alterations in the fatty acid composition of tissue lipids (49), development of a fatty liver (50) and changes in the levels of the serum lipoproteins (51) are found. The concentrations of circulating triglycerides, unesterified cholesterol and phospholipids are greatly decreased (51). De Pury and Collins suggested that the increase in lipase activity, released into the bloodstream after injection of heparin, which was considered to represent lipoprotein lipase activity, is responsible for the reduction in the level of very low density lipoprotein (VLDL)-triglycerides (52). As it became clear later on that besides lipoprotein lipase, liver lipase is also released into the bloodstream after injection of heparin, it may well be that at least part of the effects found in EFA-deficient rats on postheparin lipase activity is due to changes in the liver enzyme, since liver lipase contributes largely to the overall postheparin lipolytic activity in the rat (4,10). Therefore the effect of EFA-deficient diets on the liver lipase activity in the liver as well as in the blood plasma after injection of heparin was studied. Since in EFA-deficient animals the prostaglandin synthesis is impaired, the

TABLE VIII

EFFECT OF EFA-DEFICIENCY ON SERUM TRIGLYCERIDES, LIVER WEIGHT, LIVER PROTEIN CONTENT AND RELATIVE LIVER WEIGHT IN FED AND FASTED RATS

Animals	n	Feeding condition	Serum triglycerides (mM)	Body weight (g)	Liver weight (g)	Liver protein content (mg/g wet weight)	Relative liver weight (g/100 g body weight)
Control	4	fed	1.96±0.035	288±37	11.7±0.8	181± 2	4.08±0.33
EFA-deficient	4	fed	1.50±0.28*	278±39	14.2±1.7*	159± 5*	5.14±0.30*
Control	4	fasted	0.77±0.02	247±10	6.9±0.1	210±11	2.78±0.07
EFA-deficient	4	fasted	0.78±0.30	294±57	8.8±1.1*	200±10	3.04±0.25

Values statistically different from controls are marked with an asterisk ($P < 0.01$); n=number of rats.

involvement of prostaglandins in the regulation of liver lipase activity was studied as well.

III.3.2. Experimental design

EFA-deficient male Wistar rats of about 200 g were obtained from Unilever Research, Vlaardingen, The Netherlands. They showed all the signs of EFA-deficiency such as a scaly, dry skin, loss of hair and scaly tail. The rats had free access to food and water, unless noted otherwise. They were housed under controlled conditions; temperature 20-22^{o}C, lights on between 7.00 and 19.00 h.

The rats were divided in two groups; one group was fed an EFA-deficient diet (Unilever Research, Vlaardingen, The Netherlands), composition by percentage of weight: casein 23, saccharose 70, cellulose 5.5, dried yeast 1.0, calciumcarbonate 1.0, calciumbiphosphate 1.6, potassiumchloride 1.0, sodiumchloride 0.5, magnesiumoxyde 0.3, dl-methionin 0.2 and vitamins 1.4; the other group was fed laboratory chow for two weeks. Normal male Wistar rats of about the same weight were also divided in two groups, one group was fed laboratory chow and the other group received the EFA-deficient diet for two weeks.

III.3.3. Results

Table VIII shows the effect of an EFA-deficient diet on

TABLE IX

EFFECT OF EFA DEFICIENCY ON THE TOTAL AND THE HEPARIN RELEASABLE LIVER LIPASE ACTIVITY.

Liver lipase activity is measured in the liver and in posthep. serum of fed and fasted EFA-deficient and control rats.

Animals	n	Feeding condition	Liver lipase activity mU/total liver	mU/ml posthep. serum
Control	4	fed	6955± 930	465±37
EFA-deficient	4	fed	4036±1770*	800±98*
Control	4	fasted	4950± 780	410±37
EFA-deficient	4	fasted	6960±1240*	530±17*

*P < 0.01

TABLE X

INFLUENCE OF EFA DEFICIENCY ON THE TOTAL AND NON-RELEASABLE LIVER LIPASE ACTIVITY.

Liver lipase activity was measured in the livers of rats before or after injection with heparin.

Animals	n	Feeding condition	Liver lipase activity Total activity (mU/mg protein)	Non-releasable activity (mU/mg protein)
Control	4	fed	3.39±0.08	0.21±0.02
EFA deficient	4	fed	6.13±0.55*	0.45±0.14*
Control	4	fasted	2.53±0.23	0.24±0.03
EFA deficient	4	fasted	4.01±0.73*	0.88±0.60*

*P < 0.05

serum triglycerides, relative liver weight (expressed as g liver/100 g body weight) and protein content of the liver, in normally fed and overnight fasted rats. In fed EFA-deficient rats the serum triglycerides were lower than in control animals. After an overnight fast, no difference in serum triglycerides between controls or EFA-deficient animals was found. The absolute and relative liver weight was higher during EFA-deficiency and this was more pronounced in the fed than in the fasted state. In contrast to the higher liver weight, there was a lower protein content in the liver of EFA-deficient rats (181 mg/g weight in control rats, 159 mg/g wet weight in EFA-deficient rats).

The effect of the EFA-deficient diet on the liver lipase activity is shown in Table IX. To obtain a measure of the total amount of the enzyme activity and the heparin-releasable part of this activity, liver lipase was measured in the liver and in the serum after heparin injection. The lipase activity in the liver was doubled in the EFA-deficient rats. In the postheparin plasma the liverlipase activity was increased by 60%. The difference in liver lipase activity was most pronounced in the fed EFA-deficient rats. The lipase activity which remained in the liver after heparin administration was considered as the non-(easily)-releasable lipase activity. This non-releasable lipase activity was much higher in the fasted EFA-deficient rats than in control animals (Table X).

Short term effects of dietary EFA's were studied by feeding control rats an EFA-deficient diet for 14 days and by

feeding EFA-deficient animals a diet of normal laboratory chow for the same period. The results of these experiments are shown in Table XI. An EFA-deficient diet led to a significant

TABLE XI
INFLUENCE OF THE DIET ON THE LIVER LIPASE ACTIVITY IN EFA-DEFICIENT AND CONTROL RATS.

EFA-deficient and control rats were fed with an EFA-deficient diet or with laboratory chow for 14 days. Liver lipase activity was measured in the liver and in the serum after injection of heparin in fed rats.

Animals	n	Diet	Liver lipase activity mU/total liver	mU/ml posthep. serum
Control	4	chow	6955± 930	465±37
Control	4	EFA-deficient	8500± 807*	585±79*
EFA-deficient	4	EFA-deficient	14036±1770	800±98
EFA-deficient	4	chow	7400± 800*	502±53*

*P < 0.01

increase in lipase activity in control rats, while liver lipase activity was normalized in EFA-deficient rats on laboratory chow for 14 days.

To investigate the possible involvement of prostaglandins in this process liver lipase activity was measured in rats treated with indomethacin for two days. In these rats the total and non-releasable liver lipase activity was significantly increased (Table XII).

TABLE XII

EFFECT OF INDOMETHACIN ON LIVER LIPASE ACTIVITY

Rats were treated for two days with indomethacin (5 mg/kg body weight, i.p.) and liver lipase activity was measured in the liver of fed rats before or after injection with heparin (200 U/kg body weight) on the second day. To determine the lipase activity that is not easily released from the liver by heparin, the liver of heparin injected animals were rinsed by perfusion through the portal vein with a modified Tyrode buffer, pH 7.4, during 5 min (12 ml/min). Then tissue extracts were prepared and lipase activity was estimated as described elsewhere.

Treatment	n	Liver lipase activity (mU/liver)	
		before heparin	after heparin
None	4	6940±694	398±40
Indomethacin	4	8350±577*	549±35*

*P < 0.05

Experiments with isolated liver cells

The apparent involvement of some prostaglandins in the regulation of liver lipase acitivity was also studied in vitro on the synthesis or secretion of liver lipase activity by isolated parenchymal liver cells. Prostaglandin E_2 was found to inhibit the secretion of liver lipase activity by 25 percent (Table XIII), while prostaglandin $F_{2\alpha}$ did not influence the secretion of lipase activity.

TABLE XIII

EFFECT OF PROSTAGLANDINS ON THE SECRETION OF LIVER LIPASE ACTIVITY

Parenchymal liver cells were incubated in the presence of solvent (ethanol) or prostaglandin E_2 or prostaglandin $F_{2\alpha}$, during 4 hrs. After the incubation liverlipase activity was measured in the medium. n denotes number of experiments.

Additions	n	Liver lipase activity (mU/mg cell protein)
Control (1%o ethanol)	4	2.21±0.12
Prostaglandin $F_{2\alpha}$ (2 µg/ml)	3	2.09±0.14
Prostaglandin E_2 (0.4 µg/ml)	4	1.74±0.05*

*P < 0.01

III.3.4. Conclusions

We have investigated the effect of an EFA-deficient diet on liver lipase activity. It was found that this activity in postheparin serum as well as in the liver is remarkably higher after feeding an EFA-deficient diet. Relatively short periods of EFA deprivation were sufficient to cause a significant increase in liver lipase activity. The activity did not reach the values found in EFA-deficient animals presumably because a complete EFA-deficient state is not reached in a relatively short period, as EFA's may still be mobilized from the fat stores. On the other hand, the feeding of a normal laboratory chow to EFA-deficient animals leads rapidly to normalization

of the lipase activity. This strongly suggests that the EFA-deficiency is the primary cause of the high liver lipase activity and that this is not due to other factors, such as breeding conditions or the strain of the rats or the composition of the diet, in particular the high carbohydrate content, since feeding of a high saccharose diet to control rats leads only to a slight enhancement of the liver lipase activity. The increase in liver lipase activity in EFA-deficient rats may be responsible for the decrease of HDL-(free)-cholesterol and phospholipids, and the increase in liver cholesterol, observed in these rats (51).

One consequence of EFA-deficiency is impairment of prostaglandin synthesis (53). An acute inhibition of prostaglandin synthesis cannot be obtained by feeding this EFA-deficient diet. Therefore prostaglandin synthesis was inhibited by the administration of indomethacin. Within two days the liver lipase activity was significantly increased, indicating that the lack of prostaglandins may be responsible for the enhancement of the enzyme activity in the EFA-deficient animals. The apparent involvement of prostaglandins is also evident from the inhibition of the secretion of liverlipase activity by prostaglandin E_2. However, to elucidate the precise role of the prostaglandins in the regulation of liver lipase, more studies are necessary.

CHAPTER IV. GENERAL DISCUSSION

IV.1. Regulation of liver lipase

Because of the separation of the sites of synthesis and function of liver lipase, the regulation of its activity may be exerted at various levels: 1) enzyme structure; 2) synthesis and secretion by parenchymal cells; 3) binding of the enzyme to the non-parenchymal cells; 4) degradation of the enzyme (Fig. 11). The enzyme structure was investigated in hypercorticistic rats by means of stability and immunotitration experiments. It was found (appendix paper IV) that the enzyme structure was not altered under this condition, because heat stability, immunotitration and affinity of the enzyme for non-parenchymal cells was identical in control and hypercorticistic rats. Secretion and synthesis have been investigated with freshly isolated parenchymal cells. The secretion was inhibited by cycloheximide, tunicamycin, colchicin, prostaglandin E_2, epinephrine, vasopressin and the calcium ionophore A23187 (Table III, XIII, appendix paper IV) and stimulated by insulin (Table III). The hormones corticosteron, glucagon, thyroxin and 17β-estradiol did not affect the secretion of lipase activity from the isolated parenchymal cells (Table III). Inhibition of de novo synthesis by cycloheximide confirmed earlier findings (40). Sundaram et al. (39) found no inhibition of the secretion of liver lipase activity by puromycin. This, however, could have been due to the absence of

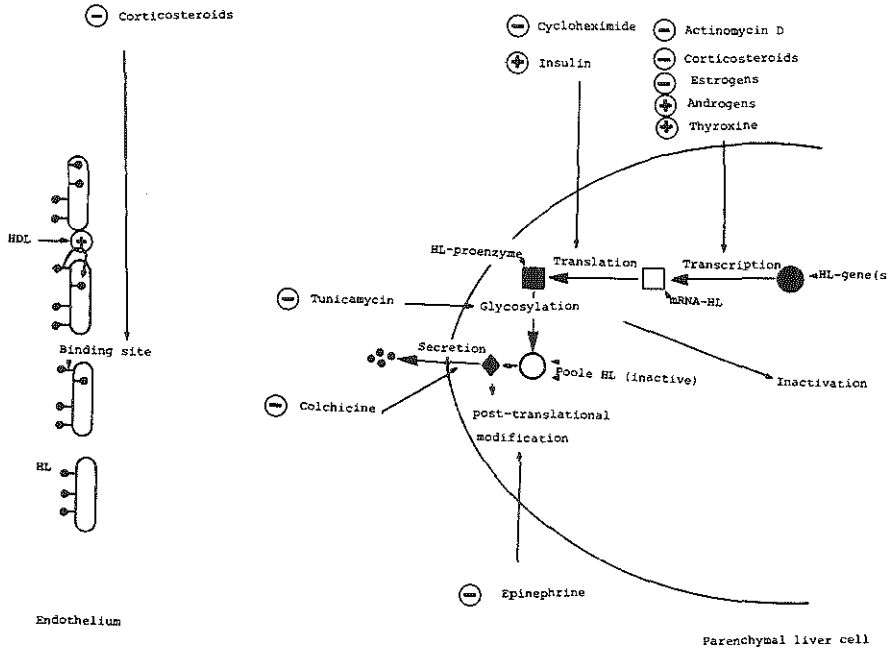

Fig. 11. Possible sites of the regulation of liver lipase by different hormones and drugs.

serum in their secretion experiments, which we have found to be indispensible for optimal secretion. Sundaram et al. (39) probably studied the secretion of enzyme coming from an intracellular pool of liver lipase. The existence of such a pool is apparent from the experiments described in this thesis. The secretion of lipase activity was inhibited by only 40% during the first hour (Fig. 7). The enzyme activity secreted into the medium under our conditions exceeded the activity originally

present in the hepatocytes to a considerable extent (Fig. 8), which suggests the intracellular conversion of the enzyme from an inactive proenzyme to the active enzyme. The activation may be coupled to the release of the enzyme from the parenchymal cells (39). Therefore the situation may be analogous to the release of LPL from the adipocytes (70). The nature of this activation is not known but it could be due to the limited proteolysis of the N-terminal sequence, a process frequently coupled to protein secretion (71). Glycosylation of the enzyme is also involved in the activation and secretion of the enzyme since tunicamycin (an inhibitor of the N-glycosylation) not only inhibited the secretion of lipase activity but also the intracellular lipase activity which is in accordance with the findings of Leitersdorf et al. (79) in cultured hepatocytes. Of the hormones tested only epinephrine and insulin affect the secretion of liver lipase activity by the isolated parenchymal cells in vitro. Neither corticosteron, thyroxin or β-estradiol influence the secretion of lipase activity during short exposure (3 h). They probably exert their effects at the transcription level although also in cultured hepatocytes they did not affect the secretion of liver lipase activity during 12 hrs (79). This is possibly due to the long half-life time, of more than 20 hrs, of the mRNA of liver lipase. Actinomycin D only lowered the lipase activity by 30% 20 hrs after administration of the drug (Table V). Whether insulin specifically enhances the secretion of liver lipase activity or whether this is a consequence of the stimu-

lated protein synthesis in general is not known. Insulin exerts its effect on translation level, since it was already apparent after 1 hr (*in vivo*) and could not be inhibited by actinomycin D. Epinephrine inhibited the secretion of liver lipase *in vitro* by 30% in accordance with Sundaram. This effect could not be blocked by the β-adrenergic -antagonist propranolol, while the lowering of lipase activity was prevented in the presence of the α_1-adrenergic blocker prazosin. The extracellular free calcium concentration may be involved in this process since the secretion of liver lipase activity was also inhibited by the Ca-ionophore A23187 and vasopressin while glucagon did not affect the enzyme activity which makes it unlikely that cAMP is involved as the second messenger. It is interesting to note that Sundaram et al. (39) did not find an inhibition of the secretion of phospholipase A_1 activity from the isolated parenchymal cells by epinephrine as liver lipase has besides triglyceridase activity, phospholipase activity. Perhaps epinephrine is involved in the post-translational modification of the enzyme, which might result in the loss of triglyceridase activity of the enzyme and a gain in phospholipase activity. The mode of conversion of the enzyme, however, is unknown so that only speculations can be made. Epinephrine raises the cytosolic free calcium concentration which may lead to activation of a calcium-dependent protease, which then might promote the cleavage of a hydrophobic peptide of the enzyme necessary for the binding to triglycerides leaving the phospholipase activity unchanged. This suggestion

is supported by the fact that treatment of hepatic lipase with collagenase selectively abolishes the lipase's ability to act on triglycerides, leaving the activity upon more hydrophilic monoacylglycerol unaltered (72). A hydrophobic peptide is necessary for the binding of triacylglycerol, as can be inferred from the alteration of collagenase inhibition of triglyceridase activity when the enzyme is bound to a triacylglycerol substrate. Maintenance of phospholipase A_1 and monoacylglycerol hydrolase activity of hepatic lipase isolated from liver membranes (lacking triglyceridase activity), supports the suggestion that proteolytic digestion of the lipase selectively alters its ability to act on triacylglycerol (74,75,84).

Corticosteroids also lowered the liver lipase activity, probably at the transcription level. This may also be the case for the other steroid hormones, estrogens and androgens which inhibit and increase the lipase activity respectively (57) (appendix paper V). All these hormones, as well as thyroxin, exert their effects at the transcription level, which explains why we did not find an effect of these hormones on the secretion of lipase activity from the isolated parenchymal cells, because of the long t 1/2 of the mRNA for liver lipase (Table V). Corticosteroids not only lower the secretion of liver lipase, but also the number of binding sites. The decrease in secretion of liver lipase activity from the parenchymal cells as well as the decrease of the number of specific binding sites of the non-parenchymal cells indicates that both processes are synchronized. This is supported in vivo in the

"ACTH" rats (Chapter III, Fig. 10), where in spite of the enhanced synthesis of liver lipase after heparin injection, the liver lipase activity did not exceed the original lipase activity after the first heparin injection. Although heparin cannot be expected to affect the synthesis directly, it may be that it enhances synthesis indirectly because of the enzyme release from its binding sites. How this intercellular communication proceeds is unknown. In Chapter III the inhibition of the secretion of liver lipase activity by prostaglandin E_2 is described. Liver lipase is involved in the hydrolysis of phospholipids from HDL. HDL may be involved in the regulation of liverlipase binding to non-parenchymal cells as this decreased in its presence (Chapter II). This situation is analogous to the LDL receptor which is internalized after binding of LDL in coated pits. This analogy has been mentioned earlier (46).

This thesis shows a prominent role of the stress-hormones, corticosteroids and epinephrine in the regulation of liverlipase. Prolonged stress leads to an increase in atherogenic risk. Whether liver lipase is the link between atherogenesis and stress remains to be elucidated. Very interesting is the relationship found between liver lipase activity and coronary atherosclerosis (76) and the degree of regression of atherosclerosis and liver lipase activity (83). Therefore factors that influence liverlipase activity may be worthwhile to consider in the study of atherosclerosis.

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SUMMARY

The enzyme liver lipase plays an important role in the metabolism of HDL cholesterol. HDL cholesterol is considered as a prominent negative risk factor in the development of atherosclerosis. Therefore factors influencing the HDL cholesterol are of great importance. In this thesis aspects of the regulation of liver lipase have been described. The regulation was investigated *in vivo* and *in vitro*.

In chapter II a model was described to study the regulation of liver lipase *in vitro*. The secretion and synthesis of the lipase was investigated with isolated parenchymal liver cells and the binding of the enzyme with isolated non-parenchymal liver cells. Because the place of synthesis and function of liver lipase differs, regulation may occur at several sites: 1. enzyme structure; 2. secretion and synthesis; 3. binding and 4. degradation of the enzyme.

The enzyme structure does not seem to play a role in the regulation of the lipase activity because the heat-stability, binding-affinity and immunotitration of the enzyme are not changed in animals treated with synacthen (appendix paper III). Aspects of the regulation of the secretion and synthesis were studied with isolated parenchymal liver cells. The synthesis of liver lipase by these cells is about the same as *in vivo* (appendix paper I). The secretion of the enzyme is optimal in the presence of a heat-stable serum factor present in the sera of several species. Besides this factor a stabili-

zing factor is necessary because of the inactivation of the lipase secreted into the medium. An important stabilizing factor is heparin or binding to non-parenchymal liver cells (appendix paper I). The secretion of liver lipase *in vitro* is inhibited by cycloheximide, tunicamycin, colchicin, prostaglandin E_2, epinephrine, vasopressin and the calcium ionophore A 23187. Inhibition of the secretion of liver lipase by tunicamycin indicates that glycosylation of the enzyme is necessary for the expression of its activity. After glycosylation in the Golgi apparatus the lipase is transported to the cell membrane and subsequently secreted. In this transport the microtubuli are involved. The secretion *in vitro* is regulated by the hormones insulin, epinephrine and possibly prostaglandin E_2 in a short term and by the corticosteroids in the long term.

The regulation of liver lipase by insulin, epinephrine, thyroxin and the corticosteroids is also investigated *in vivo*. Insulin enhances the secretion of liver lipase probably by a stimulation of the protein synthesis at the translation level because normalisation of liver lipase activity *in vivo* by administration of insulin and glucose, in fasted rats, was inhibited by cycloheximide and not by actinomycin D. From the treatment with actinomycin D it appears that the half-life time of the liver lipase mRNA is more than 20 hours.

Besides insulin, epinephrine plays an important role in the short term regulation of liver lipase (appendix paper IV). Administration of epinephrine lowers the liver lipase activi-

ty. Moreover, endogenous enhancement of the catecholamine level also reduces the lipase activity. The lowering of the lipase activity is probably a consequence of the inhibition of the secretion of liver lipase by the parenchymal liver cells after interaction of epinephrine with the α_1-receptor, because as well *in vivo* as *in vitro* the inhibition is abolished by the α_1-antagonist prazosin while the β-antagonists, atenolol, metoprolol and propranolol have no effect. The inhibition is probably mediated by a change in the intracellular free calcium concentration because the secretion is also inhibited by vasopressin and the calcium ionophore A 23187.

The involvement of the corticosteroids in the regulation of liver lipase was investigated in hypercortisistic rats, treated with synacthen. The lowering of the lipase activity is 25%, 16 hours after treatment with synacthen and 50% after treatment for 20 days. The reduction in lipase activity is smaller in fasted animals, probably because of the rapid lowering of the lipase activity in fasted animals. The change in lipase activity in these hypercortisistic rats may be due to a lowering of the synthesis next to a reduction in the number of binding sites on the non-parenchymal liver cells (appendix paper III). An indication of a limited number of binding sites has been found also *in vivo* (chapter III).

The close relationship that seems to exist between the secretion and binding suggests a possible intercellular communication between both types of liver cells. The prostaglandins may be involved in this communication process, because they

are synthesized in the non-parenchymal, endothelial cells and they inhibit the secretion of liver lipase by the parenchymal liver cells (chapter III).

SAMENVATTING

Het enzym lever lipase speelt een belangrijke rol in het HDL cholesterol metabolisme. HDL cholesterol wordt gezien als een van de belangrijkste negatieve risico factoren in het proces van atherosclerose. Inzicht in de regulatie van het enzym lever lipase is dan ook van groot belang. In dit proefschrift worden verschillende aspecten van de regulatie van het lever lipase beschreven. De regulatie werd zowel in vivo als in vitro onderzocht.

Eerst werd er een in vitro model opgesteld aan de hand waarvan de regulatie van het enzym lever lipase is bestudeerd (hoofdstuk II). De secretie en synthese van het lipase is onderzocht met behulp van geisoleerde parenchymale lever cellen en de binding van het enzym met geisoleerde niet-parenchymale lever cellen. Daar de plaats van synthese en functie van het lever lipase verschilt, kan de regulatie op verschillende niveau's plaatsvinden: 1. structuur van het enzym; 2. secretie en synthese; 3. binding en 4. afbraak van het enzym.

Een verandering in de structuur van het enzym blijkt geen rol te spelen bij de regulatie van de enzym activiteit, daar de hitte stabiliteit, bindingsaffiniteit en immunotitratie van het enzym niet veranderen (appendix paper III). Aspecten van de regulatie van de secretie en synthese van lever lipase werden onderzocht met behulp van geisoleerde parenchymale lever cellen. De synthese van lever lipase door deze cellen in vitro is ongeveer gelijk aan de synthese in vivo (appendix

paper I). Voor een optimale secretie van het enzym is een hitte stabiele, niet dialyseerbare factor vereist, die wordt aangetroffen in de sera van diverse species. Naast deze factor is er een stabiliserende factor nodig daar er in het medium een inactivering plaats vindt van het gesecreteerde enzym. Een belangrijke stabiliserende factor is heparine. Naast heparine blijkt ook binding van het enzym aan de niet-parenchymale lever cellen het enzym te stabiliseren. De secretie van lever lipase in vitro wordt geremd door cycloheximide, tunicamycine, colchicine, prostaglandine E_2, adrenaline, vasopressine en de calcium ionofoor A 23187. De remming van de secretie van lever lipase door tunicamycine duidt er op dat de glycosylering van het enzym nodig is voor de expressie van de enzym activiteit. Na glycosylering in het Golgi apparaat wordt het lipase getransporteerd naar het celmembraan, waarbij de microtubuli betrokken zijn en vervolgens gesecreteerd. Hormonaal wordt de secretie op korte termijn in vitro gereguleerd door insuline, adrenaline en mogelijk prostaglandine E_2 en op lange termijn eveneens door de corticosteroiden.

De regulatie van lever lipase door insuline, adrenaline, thyroxine en de corticosteroiden werd tevens in vivo onderzocht. Insuline stimuleert de secretie waarschijnlijk door een verhoging van de eiwitsynthese op translatie niveau, daar in vivo de normalisering van de lever lipase activiteit in gevaste ratten door toediening van insuline en glucose wordt geremd door cycloheximide en niet door actinomycine D. Uit de behandeling met actinomycine D blijkt dat de halfwaarde tijd

van het mRNA van lever lipase langer is dan 20 uur.

Adrenaline is ook van belang voor een snelle regulatie van lever lipase (appendix paper IV). Niet alleen toediening van adrenaline maar ook een endogene verhoging van de catecholamine spiegel verlagen de lipase activiteit. Deze verlaging is waarschijnlijk het gevolg van een remming van de secretie van lever lipase door de parenchymale lever cellen, na interactie van adrenaline met de α_1-receptor, daar zowel in vivo als in vitro de remming kan worden opgeheven door de α_1-antagonist prazosin en niet door de β-antagonisten atenolol, propranolol en metoprolol. De remming is waarschijnlijk gemedieerd door een verandering in de vrije intracellulaire calcium concentratie, daar de secretie van lever lipase eveneens wordt geremd door vasopressine en de calcium ionofoor A 23187.

De rol van de corticosteroiden in de regulatie van lever lipase werd onderzocht in proefdieren die behandeld waren met synacthen. De verlaging van de lever lipase activiteit bedraagt 25%, 16 uur na behandeling en 50% na een behandeling van 20 dagen met synacthen. In gevaste proefdieren is deze verlaging minder, waarschijnlijk als gevolg van de snelle verlaging van de lever lipase activiteit na vasten. In vitro is vastgesteld dat de verandering in lipase activiteit, in proefdieren behandeld met synacthen gedurende 10 dagen, het gevolg is van zowel een verlaging van de novo synthese door de parenchymale lever cellen als van een vermindering van het aantal bindingsplaatsen op de niet-parenchymale lever cellen

(appendix paper III). Een aanwijzing voor een beperkt aantal bindingsplaatsen is tevens gevonden in vivo (hoofdstuk III).

De nauwe samenhang welke er lijkt te bestaan tussen de secretie en de binding heeft geleid tot de gedachte van een mogelijke intercellulaire communicatie tussen de beide typen lever cellen. Mogelijk kunnen de prostaglandines hier een rol in spelen, daar zij worden gesynthetiseerd in het ene celtype, de endotheel cel en de secretie van lever lipase remmen in de parenchymale lever cellen.

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* In this thesis.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 27 oktober 1952 geboren te Gouda. Na het behalen van het HBS-b diploma aan het St. Antonius College te Gouda heeft hij gewerkt als analist op het research laboratorium van Nutricia te Zoetermeer. Gedurende deze tijd volgde hij de opleiding HBO-b aan het Van 't Hoff Instituut te Rotterdam.

In 1971 is hij begonnen met de studie chemie aan de Vrije Universiteit te Amsterdam. Het kandidaatsexamen (S2'), legde hij af in april 1975 en het doctoraalexamen, met als hoofdvak moleculaire farmacologie en als bijvakken chemische fysiologie en organische synthese, in augustus 1979.

Het in dit proefschrift beschreven onderzoek werd gestart in september 1979 op de afdeling Biochemie I van de Erasmus Universiteit te Rotterdam. In de periode 1984-1985 werd scheikunde les gegeven aan het Instituut voor Hoger Beroeps Onderwijs West Brabant te Breda en aan de Zeeuwse Academie voor Chemie en Gezondheidszorg te Goes. Vanaf oktober 1985 is hij werkzaam op de afdeling Biochemie I van de Erasmus Universiteit te Rotterdam.

APPENDIX PAPER I

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STABILIZATION OF LIVER LIPASE IN VITRO BY HEPARIN OR BY BINDING TO NON-PARENCHYMAL LIVER CELLS

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Key words: Lipase stability; Heparin; Cell binding; (Liver cell)

The effect of heparin on the secretion of acylglycerol hydrolase activity by isolated parenchymal liver cells was studied. In the presence of heparin, the lipase activity, secreted in 3 h, was almost doubled. Heparin did not influence the activity of the enzyme, but affected the stability of the enzyme. In the absence of heparin, the triacylglycerol hydrolase activity declined to 50% of the initial value during 1 h incubation at 37°C. The addition of heparin prevented this loss of activity almost completely. The optimal stabilization of enzyme activity was reached at 15 U heparin/ml NaCl (1 M) and protamine sulphate (120 μg/ml) abolished this effect of heparin. Instead of heparin, liver lipase activity could also be stabilized by binding to non-parenchymal liver cells. The results are discussed in connection with the binding of the enzyme in vivo.

Introduction

Several lipolytic activities are found in the blood after heparin injection [1–3]. The acylglycerol hydrolase activity of postheparin plasma can be attributed to at least two immunologically different enzymes. One of the enzymes is lipoprotein lipase (EC 3.1.1.34) derived from extrahepatic tissues, the other enzyme is of hepatic origin (liver lipase) [4–6]. Isolation of parenchymal or non-parenchymal liver cells with collagenase results in the loss of more than 90% of the total neutral lipase activity present in the liver before collagenase perfusion [7,8]. Isolated parenchymal rat liver cells synthesize and secrete liver lipase in vitro [9,10]. The enzyme can be bound to non-parenchymal cells both in vitro [8] and in vivo [11]. This situation may be analogous to adipose tissue where one cell type synthesizes and secretes a lipolytic enzyme (lipoprotein lipase) which is subsequently bound to other cells where the enzyme exerts its metabolic function [8,12,13]. The release of lipoprotein lipase from heart cell cultures and from fat pads in vitro is stimulated by heparin [14,15]. Also, the release of liver lipase activity from isolated hepatocytes is enhanced by heparin [9]. The present study was undertaken to characterize the nature of the effect of heparin on liver lipase.

Methods and Materials

Animals. Normally fed male Wistar rats were used (200–250 g).

Cell preparation. Parenchymal cells and a preparation enriched in non-parenchymal cells were isolated from rat liver by collagenase treatment followed by differential centrifugation, exactly as described elsewhere (Ref. 16, method 2). The intactness of the cells was routinely checked before and after incubation by the trypan blue exclusion test and by measuring the leakage of lactate dehydrogenase from the cells. In the experiments presented, more than 90% of the cells excluded trypan blue, while less than 20% of the total lactate dehydrogenase was found in the media before or after the incubation studies.

Rat liver perfusion. Rat liver was perfused in vitro through the portal vein, as described earlier [4]. After preperfusion during 10 min with Krebs-Ringer bicarbonate buffer (pH 7.2), the liver was perfused for 5 min with the incubation medium described below, containing in addition 25 U heparin/ml.

Secretion of lipase by isolated liver cells. Experiments to study the secretion of lipase activity by the parenchymal cells were carried out as described before [10] with some modifications. The cells were incubated in Ham F 10 medium supplemented with 10 mM piperazine-*N*,*N*'-bis(2-ethanesulphonic acid), 11.2 mM *N*,*N*'-bis(2-hydroxylethyl)-2-aminoethanesulphonic acid, 8.9 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, 2.3 mM $CaCl_2$ and 20% cow serum instead of rat serum (pH 7.4). This incubation medium, hereafter referred to as the medium, was used either in the presence or absence of varying amounts of heparin. At different times, cells were separated from the medium by centrifugation (2 min at $50 \times g_m$) and in the cell-free media lipase activity was measured.

Studies on the stability of liver lipase. Studies on the stability of liver lipase were carried out at 37°C. Liver lipase containing media were incubated in the presence or absence of heparin and lipase activity was subsequently measured. In some experiments, the medium, isolated after incubation with the parenchymal cells (see above), was incubated with non-parenchymal liver cells, essentially as described before [16]. The medium (1.2 ml, containing about 3.6 mU lipase activity) was mixed with 0.75 mg non-parenchymal cells and incubated for 5 min at 25°C. The non-parenchymal cells were then separated from the medium by centrifugation (2 min at $600 \times g_m$). The supernatant (2.0 mU lipase/ml) was incubated at 37°C. The sedimented cells were washed and taken up in 1 ml fresh incubation medium and also kept at 37°C. Samples of 250 µl were drawn and lipase activity was measured. Non-parenchymal cells, preincubated with incubation medium, without lipase activity were treated in the same way. No lipase activity was measurable in these samples.

Biochemical measurements. Liver lipase activity was measured using an artificial trioleoylglycerol emulsion, exactly as described before [8] with the exception that the final NaCl concentration was 0.6 M during the assay. Lipase activities are expressed in mU (nmol free fatty acids released/min). Protein contents and lactate dehydrogenase activities were estimated by standard methods.

Materials. Heparin (Thromboliquine) was purchased from Organon (Oss, The Netherlands), collagenase from Sigma (St. Louis, MO, U.S.A.) and Ham F 10 medium from Gibco Europe (Glasgow, U.K.).

Results are given as means ±S.D.

Results

Incubation of freshly isolated parenchymal rat liver cells at 37°C in a medium containing 20% cow serum (v/v) resulted in an increase of the lipase activity in the medium. The increase was almost linear with the incubation time (Fig. 1). In the presence of heparin, the lipase activity in the medium was enhanced at all time points studied. The effect was more pronounced after a longer incubation period. The increase of secreted lipase activity was dependent

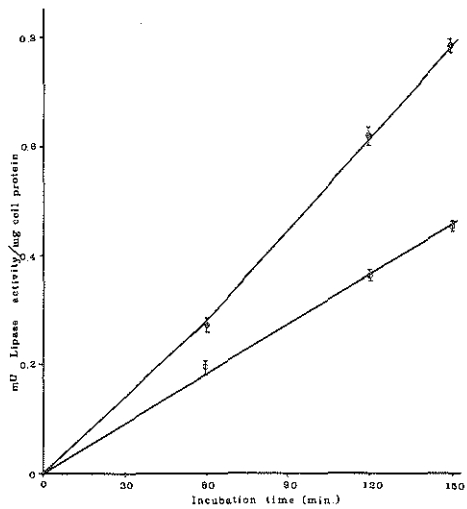

Fig. 1. Lipase activity in vitro secreted by isolated hepatocytes. Parenchymal liver cells (20–25 mg cell protein) were suspended in 5 ml incubation medium with (●——●) or without (○——○) heparin and incubated at 37°C under an atmosphere of 95% O_2, 5% CO_2 [10]. Samples were drawn at various times and lipase activities were measured. The figure shows the mean ±S.D. of three separate experiments.

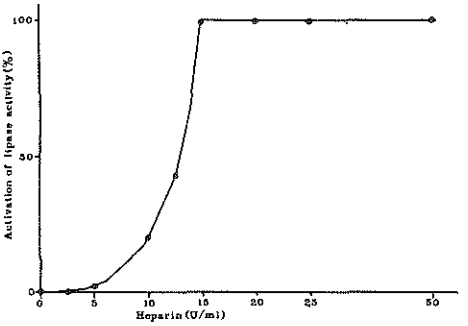

Fig. 2. Effect of different amounts of heparin on the lipase activity secreted by parenchymal cells in vitro. Incubations were carried out as described in the legend to Fig. 1. Different amounts of heparin were added at the start of the incubations to final concentrations as indicated. The effect of the additions was compared with the activation of the lipase activity with 50 U heparin/ml (100%). The results are means ±S.D. from three experiments.

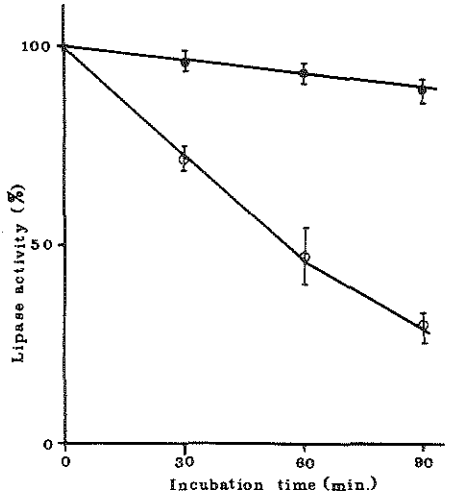

Fig. 3. Effect of heparin on the stability of liver lipase at 37°C. Parenchymal liver cells were incubated for 3 h, as described in the legend to Fig. 1. After the incubation, the medium was separated from the hepatocytes by centrifugation and incubated during the times indicated with (●——●) or without (○——○) heparin (50 U/ml). The lipase activities are given as the percentage of the activity before the incubation (2.1 ± 0.4 mU/ml). The figure shows the mean ±S.D. of six separate experiments.

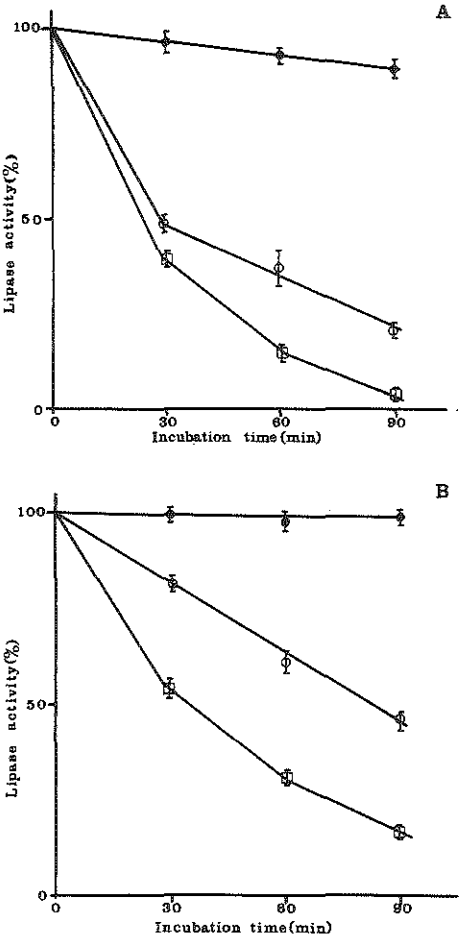

Fig. 4. Effects of NaCl and protamine sulphate on the stability of liver lipase secreted by isolated parenchymal cells or released from liver. (A) Incubations were carried out as described in the legend to Fig. 3 in the presence of heparin. NaCl (1 M) (○——○) or protamine sulphate (120 μg/ml) (□——□) was added in addition to heparin. The lipase activities measured are given as a percentage of the activity before the incubation. The figure shows the mean ±S.D. of three experiments. (B) Rat liver was perfused with a heparin-containing medium, as described under Methods and Materials. The perfusate was collected in ice and incubated, as described under (A) without further addition of heparin. The mean ±S.D. of three separate experiments is given.

on the amount of heparin added to the incubation medium. An optimal effect was reached at a heparin concentration of 15 U heparin/ml (Fig. 2).

The elevation of secreted lipase activity may be explained by activation of the lipase activity by heparin in the assay, by prevention from inactivation of the enzyme, normally occurring at 37°C (cf. Ref. 10) or by an increased secretion rate of the enzyme. Addition of heparin to the assay did not alter the enzyme activity (result not shown). To study the inactivation of the enzyme secreted by hepatocytes at 37°C, media were separated from the cells after 2 h incubation and kept at 37°C for another 2 h. The lipase activity in the media without heparin declined by 95% (1.85 ± 0.05 to 0.10 ± 0.05 mU/ml, $n = 6$). In the media with heparin, a loss of only 9% of the initial activity was observed (3.15 ± 0.20 to 2.91 ± 0.15 mU/ml, $n = 6$). Addition of heparin to the medium, after separation from the cells, was also found to prevent inactivation of secreted lipase activity (Fig. 3). In the presence of NaCl (1 M) or protamine sulphate (120 μg/ml), the stabilization of secreted lipase activity by heparin was completely abolished (Fig. 4A). The stabilization of the enzyme by heparin and the effect of NaCl and protamine sulphate was also studied with enzyme released from the liver by perfusion with a heparin-containing medium in vitro. During incubation of the liver perfusate at 37°C, 3% of the activity was lost in 90 min. The addition of NaCl or protamine sulphate caused a loss of enzyme activity of 54 and 84%, respectively, in 90 min (Fig. 4B).

We have shown before that liver lipase secreted by parenchymal cells in vitro can bind to non-parenchymal liver cells [16]. The effect of this binding on the stability of the enzyme was studied. From Fig. 5 it can be seen that enzyme once bound to the non-parenchymal cells is stabilized to a similar extent as by heparin.

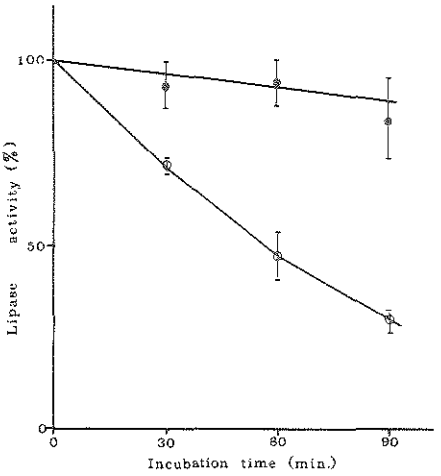

Fig. 5. Stability of liver lipase after binding to non-parenchymal liver cells. Incubation conditions and assays are described under Methods and Materials. Liver lipase bound to non-parenchymal liver cells (●——●); liver lipase remaining in the supernatant (○——○). Lipase activities are expressed as a percentage of the initial value. The figure shows the mean ±S.D. of three experiments.

Discussion

Isolated parenchymal rat liver cells can secrete triacylglycerol hydrolase activity in vitro [9,10]. The activity of the secreted enzyme was found to decline after removal of the cells from the medium [10]. Therefore, during the secretion, a continuous inactivation of the lipase seems to occur, leading to underestimation of the total activity secreted. The inactivation seems not to be caused by proteolytic degradation of the enzyme since the proteolytic inhibitors Trasylol and phenylmethylsulphonyl fluoride had no effect on the lipase activity secreted (data not shown). In the presence of heparin, an increase in secreted lipase activity has been found [9]. This can be explained by a stimulation of the secretion of enzyme or by protection of the enzyme activity against inactivation. Heparin was found to stabilize enzyme secreted and released from the liver. During 2 h incubation at 37°C, about 90% of the lipase activity was inactivated when no heparin was present, whereas with heparin a loss of only 10% of the lipase activity was found. Liver lipase binds to heparin, as is indicated by its retention on a column of Sepharose-bound heparin [17]. Binding to heparin may be essential for the stabilization of the enzyme. To study the effect of cleavage of the binding on the stability of the enzyme, NaCl was added to the incubation mixtures. A complete loss of the stabilizing effect of

heparin was found. Protamine sulphate, a heparin antagonist, was found to be even more effective. A similar result has been obtained before by Assmann et al. [18], with liver lipase partially purified from liver plasma membranes and liver perfusates. Interestingly, liver lipase can be measured in the presence of 1 M NaCl [19] or protamine sulphate, so that its substrate seems to stabilize the enzyme, presumably by binding to the enzyme, as is found for many other enzymes. The binding of substrate is clearly not affected by NaCl or protamine sulphate. It was proposed that also in vivo, the enzyme is secreted by the parenchymal cells and then bound to the non-parenchymal cells [10]. As shown in Fig. 5, the enzyme is also stabilized by binding to non-parenchymal cells. This suggests that in vivo the enzyme may be inactivated rapidly after being secreted by the hepatocytes, unless it is bound to the non-parenchymal cells. From the data of Fig. 1, it can be seen that in the presence of heparin, about 0.32 mU lipase activity/mg cell protein can be secreted in 1 h. So that with 1 800 mg cell protein (corresponding to the parenchymal cell protein of 10 g of liver) in 4.6 h (the half-life time of liver lipase in vivo), 2 600 mU lipase activity is formed in vitro. Since 10 g of whole liver contains about 5 300 mU lipase activity [10], it can be calculated that in 4.6 h, 2 650 mU lipase activity is synthesized in vivo. Therefore, the activity formed in vitro is in agreement with that formed in vivo.

The binding of liver lipase to heparin or to non-parenchymal cells in vitro displays a number of similarities with the binding of the enzyme in vivo. In vitro or in vivo bound enzyme is relatively stable (cf. Ref. 16 and Fig. 3). Furthermore, the enzyme bound in vitro [16] or in vivo [4] can be released by heparin. It is tempting to speculate that binding of lipase in vivo occurs at heparin-like binding sites, as has been proposed before for lipoprotein lipase binding at non-hepatic (endothelial) cells [22]. Whether these binding sites are specific for each of these enzymes remains to be established.

Acknowledgements

We thank Dr. H. Stam for stimulating discussions and Dr. Th.J.C. Van Berkel for advice concerning the isolation of the liver cells. Miss A.C. Hanson is thanked for preparing the manuscript. This work was carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from the Netherlands Organization for the Advancement of Pure Scientific Research (Z.W.O.).

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APPENDIX PAPER II

Biochimica et Biophysica Acta, 753 (1983) 205–212
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THE EFFECT OF CORTICOTROPHIN ON LIVER-TYPE LIPASE ACTIVITY IN ADRENALS, LIVER AND HIGH-DENSITY LIPOPROTEIN SUBFRACTIONS IN THE RAT

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Hypercortisolism was induced in rats by the administration of a corticotrophin analogue (Synacthen depot). The effect of this treatment during different periods was studied in normally fed and overnight-fasted rats. The activity of liver-type lipases, i.e., of lipases similar to the heparin-releasable lipase of rat liver (liver lipase), was determined in the adrenal gland and in the liver. Short-term (16 h) treatment had no effect on the lipase activity in the adrenal gland. During prolonged treatment, however, the lipase activity rose to 600–700% of control values in 10 days and from then on remained constant. The effect was similar in fed and overnight-fasted rats. The lipase activity in the liver decreased upon Synacthen administration. In the fed rats a decrease of 25% of the initial value was found after 16 h, 40% after 3 days and 50% after 20 days of treatment. In overnight-fasted rats the lowering of the lipase activity was less marked than in fasted controls. Serum lipid levels and high-density lipoprotein (HDL) subclass concentrations were also measured. The cholesterol concentration in the lipoproteins with a density > 1.050 g/ml (HDL) was elevated in rats treated for 3–20 days. If the rats were treated for longer than 10 days, overnight fasting led to a normalization of the HDL-cholesterol levels. After separation of the HDL into two subfractions, a relatively 'light' apolipoprotein E-rich fraction and a more 'heavy' apolipoprotein A-I-rich fraction, in fed and fasted animals treated with Synacthen for 3 days both HDL subfractions were elevated. After 10 days treatment only the apolipoprotein A-I-rich HDL fraction was still enhanced in both fed and fasted rats.

Introduction

Recently it was shown that rat ovaries and rat and human adrenals contain a lipase, similar to an enzyme previously demonstrated in the liver of different species (liver lipase or hepatic triglyceridase) [1–4]. In the liver the lipase is bound to the endothelial cells [5,6], where it may play a role in the uptake of serum lipoproteins or components thereof [7–10]. The enzyme of ovaries and adrenals is, as in the liver, presumably extracellularly located and varies along with the steroidogenic activity of the tissues, as was shown in a number of conditions [2]. For example, the adrenal lipase activity was increased in rats bearing a transplantable corticotrophin-secreting tumor [2] and in hyperplastic human adrenal tissue [3], while its activity is suppressed after corticosteroid administration [2]. Earlier it was shown that corticosteroid administration lowers the liver lipase activity of postheparin plasma [11] and that the activity in the livers of the corticotrophin-secreting tumor-bearing rats is decreased [1]. Therefore, it seems that adrenal stimulation leads to opposite effects

Abbreviations: HDL, high-density lipoprotein(s); LDL, low-density lipoprotein(s); VLDL, very-low-density lipoprotein(s).

on liver-type lipase activities in adrenals and liver. This then may affect metabolism in the lipase-containing tissues and serum lipoprotein levels as well. To study the relation between lipase activities in different organs and serum (high-density) lipoprotein levels, we treated rats with a corticotrophin analogue during different time periods and determined liver-type lipase activity in the adrenal gland and the liver and high-density lipoprotein subclass concentrations in the serum.

Materials and Methods

Animals

Male rats of the Wistar strain were used. They were 2.5–4 months old and housed under controlled conditions (temperature, 20–23°C; lights on, 7.00–19.00 h). The rats had free access to standard chow and tap water, unless noted otherwise.

Animal preparations

Hypercortisolism was induced by subcutaneous injection with 50 μg (= 5 I units) of a synthetic corticotrophin analogue (tetracosactide hexaacetate, zinc complex; Synacthen depot (1 mg/ml), Ciba-Geigy N.V., Belgium) in the suprascapular region. The drug was administered daily between 16.30 and 17.30 h. Previously this treatment had been found to give a distinct stimulation of the corticosterone production (Vreeburg, De Greef, Ooms and Weber, unpublished data). In our experiments the serum corticosteroid concentrations were similar in normally fed and overnight-fasted rats treated with Synacthen for 3 or 10 days (610 ± 137 ng/ml, $n = 24$, controls 258 ± 120 ng/ml, $n = 24$). For each experimental condition 12 rats with body weights differing less than 5% were selected and randomly placed in a control ($n = 6$, injected with 0.15 M NaCl) or experimental group ($n = 6$). At the initiation of the treatments the body weights of the rats varied between 228 and 251 g.

Tissue preparations

The rats were killed by decapitation between 9.00 and 10.00 h. The blood was collected in ice-cooled tubes containing 10 mg Na_2EDTA. From the tissues extracts were made in 0.15 M NaCl containing 5 I units heparin/ml (Thromboliquine, Organon, Oss, The Netherlands) exactly as described previously [2].

Assay of liver (-type) lipase

Lipase activities were measured in the tissue extracts by determination of the free fatty acids released from a glycerol [9,10(n)-^{3}H]trioleyl emulsion stabilized with gum arabic at pH 8.5 in the presence of 0.6 M NaCl, and are expressed as munits (munits = nmol fatty acid released from the substrate in 1 min) [3]. The lipase activity in the adrenals was measured after preincubation of the tissue extracts with antibodies against heparin-releasable liver lipase (anti-(liver)lipase) or control γ-globulins [2]. The activity of the liver-type lipase was calculated by subtracting the residual activity after incubation with anti-(liver)lipase, which was always less than 10% of the total activity, from the total lipase activity of the tissue extracts. The lipase activity measured in the extracts of the livers was always inhibited more than 95% by anti-(liver)lipase, and therefore represents heparin-releasable liver lipase.

Separation of serum lipoproteins

Serum lipoprotein with a density > 1.050 g/ml (high-density lipoproteins, HDL) were separated from $d < 1.050$ g/ml (very-low- and low-density lipoproteins, VLDL and LDL) by ultracentrifugation in an air-driven Beckman centrifuge in the same way as described for human lipoproteins by Bronzert and Brewer [12]. Subfractionation of HDL was obtained by ultracentrifugation, employing a shallow KBr density gradient between $d = 1.050$ and $d = 1.175$ g/ml. Rat plasma (1.5 ml) was adjusted to a density of 1.175 g/ml by the addition of solid KBr. The plasma was overlayered in a centrifuge tube with KBr solutions of density 1.150 1.125, 1.100, 1.075 (2.4 ml) and 1.050 g/ml (2.2 ml), respectively. The tubes were centrifuged in a SW 40 TI Beckman rotor for 22 h at 284000 $\times g_m$ at 15°C. Then the tubes were punctured 1 cm from the bottom and fractions of 0.4 ml were collected. In this way HDL was separated into two subfractions, one found at a density between 1.070 and 1.090 g/ml, the other between densities 1.108 and 1.119 g/ml. The cholesterol at the top of the tubes represents VLDL and LDL (Jansen, H.,

unpublished data). The recovery of total HDL-cholesterol in the gradient fractions 1–26 was 92 ± 4% ($n = 6$). Densities of gradient fractions were determined with a digital density meter (DMA 40, Paar, Graz, Austria) at 15°C.

Apolipoprotein electrophoresis

Electrophoresis was carried out on 12% polyacrylamide gels containing 0.1% (w/v) SDS for 7 h at 6 mA/tube, as described by Weber and Osborn [13]. The staining of the gels and the sample preparation was as described by Connelly and Kuksis [14].

Other biochemical methods

Corticosteroids were determined with a competitive protein-binding assay [15]. Free and esterified cholesterol were measured by an enzymatic method (Boehringer testkit combination, Cat. No. 124087), phospholipids according to the method of Bartlett [16] and protein following the procedure of Lowry et al. [17].

Chemicals

All chemicals were of analytical grade.

Statistical procedures

Results are expressed as mean ± S.D. Comparisons between groups were made with Student's *t*-test or one-way analysis of variance. If with this last procedure, significant overall effects were obtained the groups were compared using Duncan's multiple range tests.

Results

Body, liver and adrenal weights

Synacthen administration largely affected the body, liver and adrenal weight. While the body weight decreased to about 70% of control values, the liver weight showed a temporal increase (Table I). The increase was more marked after overnight fasting, mainly because of a lower weight loss in the hypercortisolistic rats than in the controls. The adrenal weight increased 4–5-fold during 10 days of treatment and from then on remained constant.

Adrenal and liver lipase activities

The adrenal liver-type lipase activity was not affected 16 h after the first injection of Synacthen but increased during 10 days treatment to 6–7 times control values (Fig. 1). Prolonged treatment had no further effect. The adrenal weights increased less than the total lipase activities in the adrenals, so that expressed on the weight base also the lipase activity was elevated in the hypercorti-

TABLE I

EFFECTS OF SYNACTHEN TREATMENT ON BODY, LIVER AND ADRENAL WEIGHT

The mean body, liver and adrenals weight of rats treated for different periods with Synacthen are given as the percentage of the mean values of control rats ± S.D. The range of mean values of the different control groups are given between brackets. Each group of rats consisted of six animals. The statistical significance of difference between the groups was calculated comparing each experimental group with its control group using Student's *t*-test. Values statistically significantly different ($P < 0.05$), from control values are marked with an asterisk.

Feeding condition	Duration of treatment (days)	Body weight (%)	Liver weight (%)	Adrenal weight (%)
Normally fed	0	100 (242–266 g)	100 (8.9–9.8 g)	100 (26–29 mg)
	1	101 ± 3	120 ± 5 *	127 ± 17 *
	3	93 ± 2 *	117 ± 6 *	233 ± 51 *
	10	77 ± 3 *	102 ± 5	503 ± 56 *
	20	67 ± 4 *	90 ± 4	449 ± 71 *
Overnight-fasted	0	100 (225–252 g)	100 (6.0–6.7 g)	100 (27–33 mg)
	1	101 ± 3	114 ± 6 *	127 ± 13 *
	3	95 ± 3	133 ± 3 *	224 ± 31 *
	10	71 ± 3 *	106 ± 3	414 ± 50 *
	20	74 ± 2 *	94 ± 5	380 ± 67 *

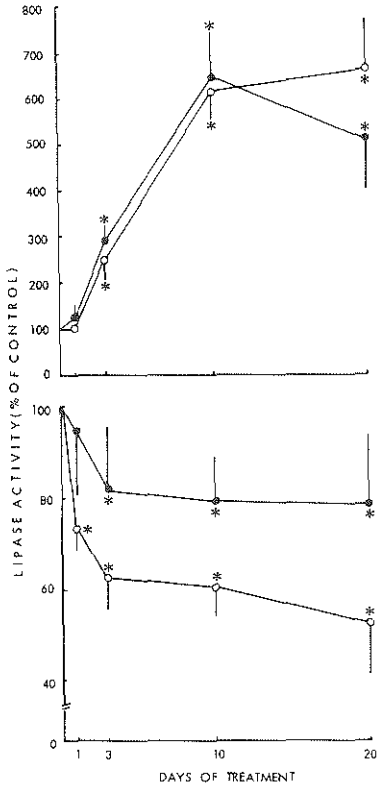

Fig. 1. Effect of Synacthen treatment on hepatic and adrenal liver-type lipase activity. The total liver-type lipase activities in adrenals (upper panel) and livers (lower panel) of rats treated during various periods with Synacthen (cf. Table I) are given as a percentage of the activities measured in control groups. Mean liver lipase activity of normally fed controls, 5525 ± 700 munits ($n = 24$), of fasted controls, 3218 ± 694 munits ($n = 24$). mean adrenal liver-type lipase activity of normally fed controls, 4.9 ± 1.1 munits ($n = 24$), and of fasted controls, 3.9 ± 0.6 munits ($n = 24$). Values of fed rats are represented by open symbols, fasted rats by closed symbols. The values that were significantly ($P < 0.05$) different from controls, using variance analysis followed by Duncan's multiple range tests are marked with an asterix.

solistic rats. The lipase activity of the liver decreased during hypercortisolism about 40% (Fig. 1). In the fed state the effect was much more marked than after overnight fasting. This was due to the fact that overnight fasting of control rats led to a lowering of the total liver lipase activity (5525 ± 700 munits/liver, $n = 24$, vs. 3218 ± 694 munits/liver, $n = 24$). Since in 1–3-day treated rats the liver weights were increased (cf. Table I) the decrease in lipase activity was even more pronounced when expressed per g tissue than per liver.

Serum lipid levels

The mean serum triacylglycerol concentration in the (very) low-density fraction increased gradually during 3 weeks of treatment (Table II). However, due to large differences in the individual responses, no statistically significant effect was found except for the 3-week treated rats after an overnight fast. The cholesterol content of the HDL fraction was increased after the first Synacthen injection (Table II) and remained, in the fed state, elevated during prolonged treatment. In rats treated for more than 3 days, fasting overnight resulted in a decrease of the HDL-cholesterol concentration to control values.

TABLE II

EFFECT OF SYNACTHEN TREATMENT ON VLDL PLUS LDL TRIACYLGLYCEROLS AND HDL-CHOLESTEROL LEVELS

The triacylglycerol content of VLDL plus LDL and the cholesterol content of the HDL fraction of the hypercortisolic rats of Table I were determined. The results were expressed as in Table I.

	VLDL + LDL triacylglycerol (%)	HDL-cholesterol (%)
Normally fed	100	100
	(1.68–1.74 mM)	(0.88–1.28 mM)
	83 ± 38	124 ± 22
	101 ± 13	144 ± 17 *
	126 ± 24	127 ± 10 *
	155 ± 73	139 ± 27 *
Overnight-fasted	100	100
	(0.43–0.50 mM)	(0.74–1.04 mM)
	103 ± 15	113 ± 14
	108 ± 26	127 ± 12 *
	121 ± 75	121 ± 25
	187 ± 74 *	88 ± 25

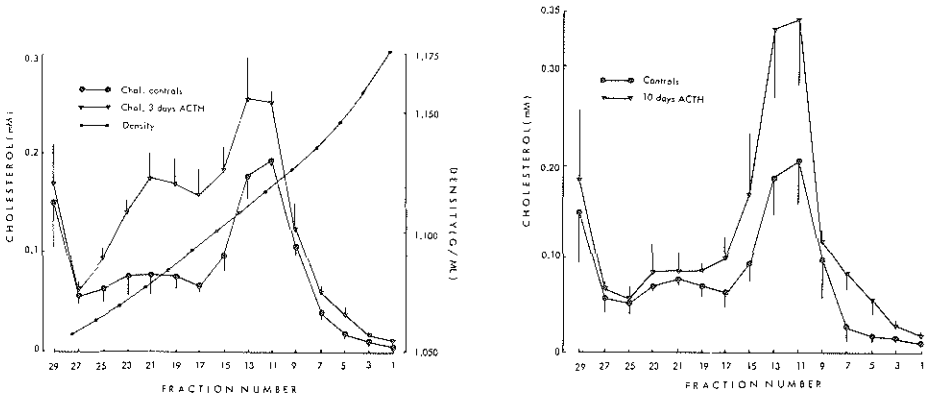

Fig. 2. Cholesterol distribution over HDL subfractions of control, 3-day or 10-day Synacthen-treated rats. Rats were treated for 3 or 10 days with Synacthen. The treated rats were killed by decapitation, together with a group of control rats. Equal volumes of the sera of two rats were mixed and subjected to ultracentrifugation as described in Materials and Methods. Always three samples from Synacthen-treated rats were processed simultaneously with three control samples. In the odd-numbered fractions obtained from each centrifuge tube cholesterol was measured. The mean cholesterol contents ± S.D. of corresponding fractions are plotted against the fraction number so that the fractions with the highest density, which were first recovered, are given at the right side of the figure (triangles, Synacthen-treated; circles, controls). Fig. 2a shows the results of 3-day treatment; Fig. 2b of 10-day treatment. Densities were measured in combined adjacent fractions. Given is the mean of four separate gradients (small dots, Fig. 2a). The differences between the values of different gradients were smaller than 5% and are not shown. The experiments were carried out with normally fed rats and after an overnight fast. The results with the fed rats are shown. With the fasted rats similar patterns were obtained (data not shown).

Distribution of cholesterol over, and composition of HDL, subfractions

The serum HDL fraction of 3- and 10-day treated rats was separated into two subfractions (Fig. 2). The lipoproteins found in the less dense region of the gradient (fractions 18–24) contained mainly apolipoprotein E (see also Fig. 3) and will be referred to as apolipoprotein E-rich HDL. In the lipoproteins found at higher densities (fractions 10–14) apolipoprotein A-I was the main component and are indicated as apolipoprotein A-I-rich HDL. The elevated total HDL cholesterol content of 3-day treated rats (Table II) was due to an increased cholesterol content of lipoproteins in the whole HDL density range (Fig. 2a). Similar patterns were found with fed and overnight-fasted rats (data not shown). Besides cholesterol, the phospholipid and protein content of both HDL subfractions were increased in the 3-day treated animals (Table III). The apolipoprotein composition remained the same (Fig. 3). After 10 days of

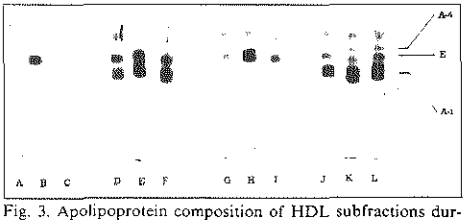

Fig. 3. Apolipoprotein composition of HDL subfractions during Synacthen treatment. The samples of apolipoprotein E-rich and apolipoprotein A-I-rich HDL remaining after determination of different components as described in Table II were dialyzed against 0.15 M NaCl. Then they were subjected to SDS-polyacrylamide electrophoresis. Of the apolipoprotein E-rich HDL 75 μl and of the apolipoprotein A-I-rich fractions 37.5 μl were used. A–C, gels of apolipoprotein E-rich HDL of fasted controls, 3- and 10-day treated rats, respectively; D–F, apolipoprotein A-I-rich HDL of the same rats; G–I, apolipoprotein E-rich HDL: of normally fed controls, 3- and 10-day treated rats; J–L, apolipoprotein A-I-rich HDL of the same rats. The identification of the apolipoproteins (right side of the figure; A-I, apolipoprotein A-I; A-4, apolipoprotein A-IV; E, apolipoprotein E) was based on experiments in which part of the fractions shown were subjected to electrophoresis simultaneously with purified apolipoproteins after delipidation.

TABLE III

LIPID COMPOSITION OF HDL SUBFRACTIONS DURING SYNACTHEN TREATMENT

Of the gradients shown in Fig. 2, fractions 10–14 and 18–24 were combined and referred to as apolipoprotein A-I-rich HDL and apolipoprotein E-rich HDL, respectively. In these combined fractions the phospholipid, unesterified cholesterol and cholesterol ester content was determined. The mean values ± S.D. are given. The values that are statistically different from the controls are indicated by [a]. If the values of 3 and 10 days adrenocorticotropin treatment are different this is indicated by [b]. (Student's t-test, $P < 0.05$ was considered as being significant). After measurement of the above-mentioned components the HDL subfractions of similarly treated animals were combined and protein was measured. All values are μg/ml.

	Fed rats					Overnight-fasted rats			
	Treatment	Protein	Phospholipid	Free cholesterol	Cholesterol ester	Protein	Phospholipid	Free cholesterol	Cholesterol ester
Apolipoprotein E-rich HDL	None ($n = 6$)	41	70 ± 10	12 ± 2	45 ± 10	36	45 ± 11	13 ± 2	39 ± 5
	3 days Synacthen ($n = 3$)	119	113 ± 10 [a]	29 ± 3 [a]	88 ± 9 [a]	81	137 ± 13 [a]	25 ± 2 [a]	65 ± 9 [a]
	10 days Synacthen ($n = 3$)	56	83 ± 8 [b]	13 ± 3 [b]	48 ± 11 [b]	26	43 ± 12 [b]	12 ± 2 [b]	20 ± 8 [a,b]
Apolipoprotein A-I-rich HDL	None ($n = 6$)	218	183 ± 17	18 ± 3	127 ± 13	204	151 ± 37	22 ± 2	101 ± 2
	3 days Synacthen ($n = 3$)	347	314 ± 18 [a]	33 ± 3 [a]	189 ± 22 [a]	267	248 ± 18 [a]	29 ± 1 [a]	137 ± 17 [a]
	10 days Synacthen ($n = 3$)	356	316 ± 118 [a]	31 ± 9 [a]	190 ± 32 [a]	278	192 ± 18 [b]	24 ± 5	133 ± 25 [a]

hypercortisolism the cholesterol, phospholipid and protein content of the apolipoprotein E-rich HDL in fed rats was similar to that in controls (Fig. 2b, Table III). During overnight fasting the cholesterol ester and the protein content of this HDL fraction decreased below control values (Table III). In these 10-day treated rats the apolipoprotein A-I-rich fraction remained elevated in the fed as well as in the fasted state. Also in these conditions no major changes in apolipoprotein composition of the HDL subfractions occurred (Fig. 3).

Discussion

The present paper shows that liver lipase activities in the liver and the adrenal gland change in opposing ways during prolonged corticotrophin administration to rats. The magnitude of the effect on the hepatic enzyme is dependent on the nutritional state of the animals, while the adrenal lipase was similar in fed and overnight-fasted rats. The most feasible explanation for the differential behaviour of both enzymes seems to be that the activity of the lipase in both tissues is under different (hormonal) control. In the adrenal gland the lipase activity seems to be related to the steroidogenic activity of the tissue [2]. This activity is determined by the corticotrophin administered, and is similar in the normally fed and overnight-fasted rats. In the liver a number of hormones affect liver lipase activity. The lipase changes during feeding and fasting, presumably under influence of insulin [11,18], while also steroid hormones affect the enzyme activity [11,19,20]. The relatively small effect of corticotrophin on the lipase in fasted rats suggests that both types of hormones act, at least partly, via the same mechanism.

The activity of the liver lipase seems to be an important determinant of the HDL concentration in the serum, as indicated by two lines of evidence. Firstly, inhibition of the lipase in situ by antibod-

ies leads to an increase in HDL levels [7–10]; secondly, in a number of conditions an inverse relationship between liver lipase activity and HDL-cholesterol levels have been shown to exist [19]. In line with these data, it was found that during hypercortisolism, when liver lipase activity was decreased, HDL levels were increased. In normally fasted rats, liver lipase is also decreased but no effect on HDL is found. However, in this situation also the generation of HDL components during the breakdown of triacylglycerol-rich lipoprotein will de diminished compared to the fed state. It seems likely that a lowering in liver lipase activity only leads to increased HDL levels if the formation of HDL is enhanced, or at least not affected. These situations seem to occur in the hypercortisolic rats. Corticosteroids profoundly affect intermediate metabolism (see, e.g., Refs. 21–23). During short-term hypercortisolism VLDL synthesis in the liver and the peripheral degradation is enhanced, which may lead to a high rate of formation of (apolipoprotein E-rich [24]) HDL. During long-term hypercortisolism the fat stores in the body become depleted and the degradation of muscle protein attenuated. This leads to a diminished supply of substrates for VLDL synthesis to the liver while VLDL degradation may become impaired [25]. These events, together with the lowered liver lipase activity, may explain the temporal increase and subsequent fall in apolipoprotein E-rich HDL. The administration of estrogens to male rats leads also to an increase of apolipoprotein E-rich HDL [26]. Estrogens lower liver lipase activity [20] and enhance the formation rate of HDL [26]. Therefore, the same mechanism as in the hypercortisolic rats (increased formation and decreased catabolism) may provoke the largely elevated apolipoprotein E-rich HDL levels in the estrogen-treated rats. What is not explained by these mechanisms is the continuously high level of apolipoprotein A-I-rich HDL in the hypercortisolic rats, and it is clear that more factors involved in lipoprotein metabolism may change during hypercortisolism and that also other mechanisms leading to the found results may be operative. An interesting question concerns the physiological importance of the effects of corticotrophin on the parameters studied. As discussed by Andersen and Dietschy [27], an increased cholesterol synthesis in the liver is needed to meet the demand of cholesterol by the adrenal gland during largely stimulated steroidogenesis. This suggests a cooperative relationship between different organs, the liver and the adrenal gland. The lowering in liver lipase activity in the liver fits in this idea because it may help to prevent the cholesterol being taken up again by the liver and may contribute to the increase in HDL levels. During long-term treatment with corticotrophin less cholesterol can be synthetized by the liver because of a lack of substrates, as already discussed. Then the liver-type lipase activity in the adrenals becomes maximal. If, as has been suggested, this enzyme plays a role in the uptake of HDL-cholesterol by the adrenals [28], then under these conditions the channelling of cholesterol to this tissue may become more efficient. These tentatively drawn interrelationships linking lipoprotein and hormone metabolism are clearly more complicated than presented here, and especially the role of lipoprotein receptors, which also changes during hypercortisolism [29], has to be taken into consideration. It may serve, however, as the basis for further research.

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APPENDIX PAPER III

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REGULATION OF LIVER LIPASE

I. EVIDENCE FOR SEVERAL REGULATORY SITES, STUDIED IN CORTICOTROPHIN-TREATED RATS

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The activity of liver lipase, an enzyme that can be released from the liver by heparin, varies under several hormonal conditions. The site(s) at which regulation of the enzyme activity may occur was investigated in vitro. As a model, rats were used which had been treated with a corticotrophin analogue, to induce hypercortisolism, a condition in which liver lipase activity is lowered. Lipases isolated from heparin-containing perfusates of livers from ACTH or control rats were identical with respect to heat stability and specific activity as determined by immunotitration and binding to isolated non-parenchymal liver cells, indicating that the enzyme structure was not affected by the treatment. The secretion of liver lipase by isolated parenchymal liver cells was studied. During incubation of parenchymal cells derived from ACTH rats, less enzyme activity was found to be secreted when compared with hepatocytes isolated from control rats (ACTH rats, 2.30 ± 0.2 mU/10^6 cells; control rats, 3.3 ± 0.3 mU/10^6 cells). Liver lipase partially purified from control rats could be bound specifically to saturation by non-parenchymal cells, isolated from ACTH or control rats. Non-parenchymal cells from ACTH rats bound less lipase activity (29 mU/mg cell protein) than cells from control rats (50 mU/mg cell protein). This reduction in binding capacity seems to be due to a diminished number of binding sites, since the affinity based on Scatchard analysis and half-maximal binding was not different. These results suggest that the lowered liver lipase activity found during hypercortisolism may be due to an impaired synthesis and/or secretion of the enzyme by the parenchymal cells and to a reduced binding capacity of the non-parenchymal cells for liver lipase.

Introduction

After heparin injection several lipolytic activities are released into the bloodstream [1–3] which can be attributed to at least two immunologically different enzymes; lipoprotein lipase and (heparin-releasable) liver lipase [4–6]. The latter enzyme may play an important role in high-density lipoprotein (HDL) metabolism [7,8].

The enzyme activity is lowered under several conditions, such as fasting [9,10], diabetes [10,11], hypercortisolism [11,12] and during estrogen treatment [13]. The mechanism underlying the regulation of liver lipase is not known. In vitro the enzyme is synthesized and secreted by the parenchymal liver cells [10,14,15] and can be bound specifically to non-parenchymal (endothelial) liver cells [16,17]. Immunofluorescence studies showed that also in vivo the liver lipase is localized on the endothelial liver cells [18]. From both in vitro binding studies [16] and immunofluorescence studies [18] it appears that almost all liver lipase is

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localized at the endothelium, where a limited number of binding sites seem to exist [17]. Therefore regulation of enzyme activity may take place at several sites: (1) enzyme structure; (2) synthesis and secretion by the parenchymal cells; (3) binding of the enzyme to the non-parenchymal cells.

The aim of the present study was to investigate the mechanism responsible for the reduced liver lipase activity during hypercortisolism.

Methods, Materials and Animal preparations

Animals. Normally fed, male Wistar rats were used. They were housed under controlled conditions; temperature 20–22°C, lights on between 7.00-19.00 h. Hypercortisolism was induced by daily subcutaneous injections of Synacthen depot (0.2 mg/kg body weight) during 10 days (ACTH rats). This treatment leads to corticosterone levels of about 600 ng/ml and to a lowering of liver lipase by 50% [19].

Cell preparations. Parenchymal cells were isolated from rat liver by collagenase treatment exactly as described before [20]. Nonparenchymal cells were isolated from rat liver as described elsewhere [20] with a slight modification (method in Ref. 21, omitting the centrifugation step using an elutriation rotor) to decrease parenchymal contamination. Protein contents of the nonparenchymal cell preparation were corrected for parenchymal cell contamination as described elsewhere [20].

Secretion experiments. Experiments to study the secretion of lipase activity were carried out as described before [15] with a slight modification: heparin (50 U/ml) was added to the incubation medium to minimize loss of lipase activity during the experiment [22]. At different times cells were separated from the medium by centrifugation, and in the cell-free media lipase activity was measured [15]. The amount of lipase secreted is expressed as $mU/10^6$ cells. Cells were counted before the incubation by a cell counter (control rats, $0.48 \pm 0.02.10^6$ cells/mg cell protein; ACTH-rats, $0.56 \pm 0.04.10^6$ cells/mg cell protein).

Binding experiments. Experiments to study the binding of liver lipase were carried out as described before [17] with the following modifications: liver lipase was partially purified from post heparin liver perfusates. The liver was perfused in situ as described earlier [4]. After perfusion during 10 min with a modified Tyrode buffer of pH 7.4 supplemented with glucose (11.1 mM) [23], the liver was perfused for 2 min with the buffer supplemented with 20% glycerol and 5 U heparin/ml. The perfusates of five or six rats were applied to a Sepharose-heparin column. The column was washed with 6 column volumes of 0.2 M NaCl/10 mM phosphate buffer (pH 7.0). The lipase was eluted with the same buffer containing 1.2 M NaCl and 1% albumin. Fractions of 1 ml were collected. Enzyme preparations of about 700 mU/ml were used for all the binding experiments.

Biochemical measurements. Liver lipase activity was measured using an artificial trioleoylglycerol emulsion in the presence of 0.6 M NaCl [16]. Lipase activities are given in mU (1 mU represents 1 nmol free fatty acid released from the substrate in 1 min). Protein contents and lactate dehydrogenase activities were estimated by standard methods.

Materials. Heparin (Thromboliquine) was purchased from Organon (Oss, The Netherlands), collagenase from Sigma (St. Louis, MO, U.S.A.), Ham F10 medium from Gibco-Europe (Glasgow, U.K.) and Synacthen depot (1 mg/ml) from Ciba-Geigy (Basel, Switzerland).

Results

'Structural' parameters of liver lipase from ACTH and control rats

To determine whether the enzyme isolated from ACTH rats is structurally different from 'control' enzyme, we performed stability and immunotitration experiments. Incubation of enzyme, purified from heparin-containing liver perfusates of control or ACTH rats at 37°C, resulted in 30 min in a decrease of enzyme activity to about 60% of the initial activity. During further incubation the activity decreased less rapidly. There was no difference in the inactivation rates of enzyme isolated from ACTH or control rats (Fig. 1).

The specific activities of lipase from ACTH and control rats were compared in immunotitration experiments. Incubation of enzyme with increasing amounts of anti-liver lipase resulted in a complete inhibition of lipase activity with enzyme from either

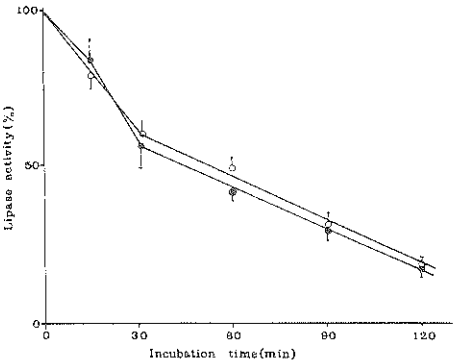

Fig. 1. Stability of purified liver lipase at 37°C. Liver lipase was purified from control (●) and ACTH-treated rats (○) as described under Methods and Materials except that the elution buffer contained 0.8 M instead of 1.2 M NaCl. The incubation of the enzyme preparation was at 37°C in the elution buffer. At different time intervals samples were drawn and the lipase activity was measured. Lipase activities are expressed as a percentage of the initial value. The figure shows the mean of three experiments.

source (Fig. 2). Each amount of antiserum inhibited the same lipase activity whether the enzyme was isolated from ACTH or control rats. This indicates that the enzymatic activity per enzyme protein moiety is not altered following ACTH-treatment.

In vitro secretion of liver lipase

The synthesis and secretion of liver lipase was studied employing parenchymal liver cells isolated from control or ACTH rats. In the presence of heparin (50 U/ml) secretion of lipase activity from control cells proceeded linearly for at least 3 h (Fig. 3). In this period 3.8 mU of lipase activity per 10^6 cells were secreted into the medium. Cells derived from rats treated for 10 days with ACTH secreted less enzyme activity. The difference between control and ACTH cells was, however, only statistically significant at incubation times of more than 1 h (in 3 h 2.2 ± 0.3 mU ($n = 3$) vs. 3.8 ± 0.3 ($n = 3$) $P < 0.01$). Also, if expressed per mg cell protein, less enzyme activity was secreted by the ACTH cells (1.24 ± 0.25 vs. 1.68 ± 0.16, $P < 0.05$) in 3 h.. In the absence of heparin less lipase activity is found in the incubation media because of lipase inactivation [22]. Nevertheless, the relative amount of lipase in the media of the ACTH cells is also lower than in the media of the control cells (1.0 ± 0.2 ($n = 3$) vs. 2.0 ± 0.2 ($n = 3$) per 10^6 cells, $P < 0.01$).

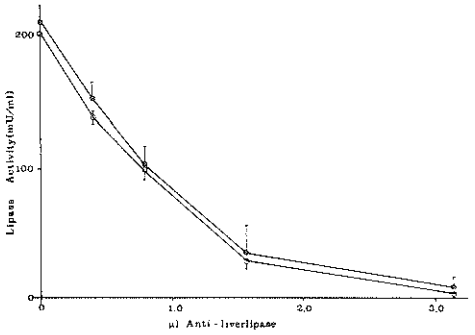

Fig. 2. Immunotitration of liver lipase from control (●) and ACTH rats (○). Aliquots (50 µl) of heparin-containing liver perfusate of control and ACTH rats were incubated for 1 min at 30°C and for 29 min at 0°C with different amounts of anti-liver lipase or 0.9% NaCl to a volume of 100 µl. After centrifugation (2 min) to remove the precipitate, lipase activities were measured. Results are expressed in mU lipase per ml liver perfusate. The results are the mean of three experiments.

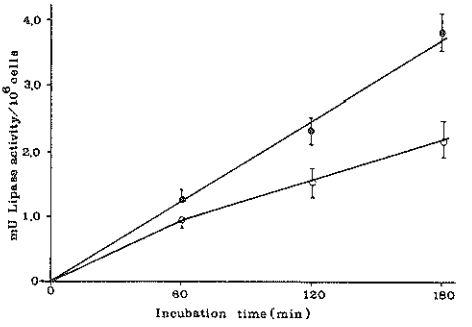

Fig. 3. Lipase activity in vitro secreted by isolated hepatocytes. Parenchymal liver cells (20–25 mg cell protein) of control rats (●) or ACTH rats (○), were suspended in 5 ml incubation medium and incubated at 37°C under an atmosphere of 95% O_2, 5% CO_2 [15]. Samples were drawn at various times and lipase activities were measured. The figure shows the mean of three separate experiments.

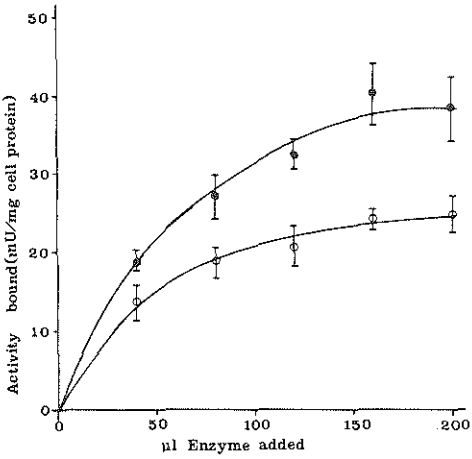

Fig. 4. Binding of different amounts of liver lipase to a fixed amount of non-parenchymal liver cells. Different amounts of isolated liver lipase (700 mU/ml) were incubated for 2 min at 25°C with 50 µl of non-parenchymal liver cell preparation (2 mg/ml) from control (●) and ACTH rats (○) [17]. The activity bound to the cells is shown in the figure and is expressed as mU lipase activity bound per mg cell protein. The mean ± S.D. of three separate experiments is given.

Binding of liver lipase activity to non-parenchymal liver cells in vitro

In vitro binding of liver lipase was studied by incubation of different amounts of partially purified liver lipase with isolated non-parenchymal cells, as described earlier [17]. Per mg of protein control cells bound maximally 40–50 mU liver lipase activity (Fig. 4). If enzyme was isolated from ACTH rats the same binding saturation curve was obtained (not shown). Non-parenchymal cells isolated from ACTH rats also showed saturation, however, at a significantly lower level (Fig. 4). In Scatchard analysis of the binding data linear curves were obtained, indicating a single set of binding sites. A maximal binding of 50 mU and of 29 mU of lipase activity per mg cell protein could be calculated for the control cells and ACTH cells, respectively (Fig. 5). The slopes of the Scatchard plots and the points at which half-saturation was obtained with either cell preparation were similar, indicating that the affinity of the binding sites for the enzyme had not changed.

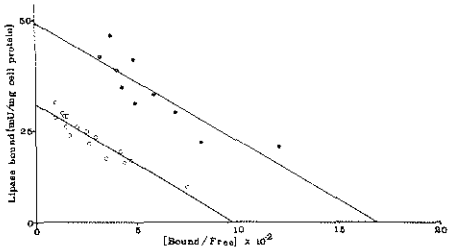

Fig. 5. Scatchard plot of specific binding of liver lipase to non-parenchymal cells isolated from control (●) and ACTH rats (○).

Discussion

Enzyme activity can be regulated by variations in the number of enzyme molecules or by changes in the specific activity by alteration of the protein moiety. For some enzymes such as liver lipase and lipoprotein lipase, which reside at the surface of cell types different from those in which they are synthesized, additional regulatory sites are possible. The secretion of the enzyme, the transport from the cells where the enzyme is secreted to the cells where the enzyme is bound and the binding of the enzyme may be affected.

Liver lipase activity is lowered during hypercortisolism [11,12]. This lowering in lipase activity seems not to be due to alterations in the protein moiety of the enzyme, since the heat stabilities, immunotitrations and the affinities for non-parenchymal cells of enzyme isolated from control or ACTH rats were identical. The amount of enzyme activity secreted by the parenchymal cells in vitro of ACTH-pretreated rats was lower than in control cells, suggesting that the synthesis and subsequent secretion of the enzyme is impaired. This lowering seems not to be due to an overall effect on protein synthesis, as the incorporation of [^{3}H]leucine and [^{3}H]glucosamine into trichloroacetic acid-precipitable material was similar with control and ACTH cells (not shown). Since corticosteroids are known effectors of mRNA formation, the lowering of the enzyme secretion may take place at the transcription level, analogously to their effect on lipoprotein lipase activity in adipose tissue [24].

Besides this lowered rate of synthesis of liver lipase less enzyme could be bound to the isolated non-parenchymal cells from ACTH-rats compared to non-parenchymal cells from control rats. This seems not to be due to a change in the binding affinity but in the number of binding sites, because the amount of enzyme that could be bound to the cells was reduced for the ACTH cells, while the slopes of the Scatchard plots and the amounts of enzyme where half-saturation was obtained were similar with both cell preparations. Therefore, it seems that the number of binding sites for liver lipase is, as shown before for lipoprotein receptors [25], under hormonal control. The decrease in secretion of liver lipase activity from the parenchymal cells as well as the decrease in the number of specific binding sites from the other cell type, the non-parenchymal, indicated that both processes are synchronized in this condition. How the synchronization is effected can only be speculated on. It may be that the hormone treatment directly affects both synthesis and binding, or that only one of these processes is affected and that by intercellular communication the other process is influenced. The latter possibility is favoured, since in recent experiments we found that the rate of enzyme synthesis is enhanced if liver lipase is removed from its binding sites by heparin (unpublished data).

In conclusion, liver lipase activity seems to be regulated at multiple sites during hypercortisolism. Whether this also holds for other hormonally induced alterations in its activity remains to be established.

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APPENDIX PAPER IV

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REGULATION OF LIVER LIPASE

II. INVOLVEMENT OF THE α_1-RECEPTOR

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The effects of different adrenergic agents on high density lipoprotein (HDL) cholesterol concentration and on the neutral NaCl-resistant triacylglycerol hydrolase (liver lipase) activity of the liver were studied in rats. Treatment of rats with the β-blockers metoprolol, atenolol or propranolol led to a lowering of the HDL-cholesterol (esterified and non-esterified) content. The α_1-antagonist prazosin had no effect. Administration of norepinephrine for 10 days resulted in an increase of HDL non-esterified cholesterol. This effect of norepinephrine was largely abolished by prazosin, but not by propranolol. In normal rats the liver lipase activity was not influenced by α- or β-blockade. Adrenergic stimulation, either short-term (by diethyl ether stress) or long-term (by norepinephrine treatment), led to a lowered liver lipase activity. The lipase activity was restored by prazosin but not by propranolol. The apparent involvement of the α_1-receptor in the regulation of liver lipase activity was further studied in vitro. Blockade of α- or β-receptors with prazosin or propranolol did not affect the secretion of the liver lipase activity by isolated parenchymal liver cells. Stimulation of α- or β-receptors by epinephrine led to a lower secreted lipase activity. Selective stimulation by isoprenaline had no effect. The effect of epinephrine could be abolished by prazosin but not by propranolol. Vasopressin and the calcium ionophore A23187 also lowered the secretion of liver lipase activity in vitro. Glucagon and/or the phosphodiesterase inhibitor Ro 20-1724 had no effect. These results indicate an involvement of the α_1-receptor in the regulation of liver lipase activity at the level of synthesis or secretion of the lipase. The effect of the α_1-receptor is presumably mediated through changes in the intracellular free calcium concentration. The effect of adrenergic modulation on HDL-cholesterol concentrations can partly be explained through modification of the liver lipase activity.

Introduction

The high-density lipoprotein (HDL) cholesterol level in the plasma seems to be related to the risk of development of coronary heart disease [1,2]. Therefore, it is of interest to study the factors that regulate the HDL concentration in the serum. An important role in the metabolism of HDL, especially in the uptake of HDL lipidic components (phospholipids, cholesterol) by the liver, may be played by liver lipase [3,4]. Therefore, liver lipase activity may, at least partly, regulate HDL levels in the plasma. This would explain the inverse relationship between HDL cholesterol levels in the plasma and postheparin liver lipase activity [5] and

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Paper I in this series is Ref. 27.

the opposite changes in both entities under different hormonal conditions [6,7]. Recently, adrenergic effects on the HDL cholesterol concentration have been described [8–11]. The mechanisms involved are not clear. In view of the proposed role of liver lipase activity in HDL metabolism, the changes in HDL levels may be secondary to alterations in liver lipase activity under the influence of adrenergic agents. However, adrenergic regulation of liver lipase activity is not known. Therefore, we studied adrenergic effects on HDL cholesterol and on liver lipase activity in intact rats. The involvement of adrenergic receptors in the synthesis and/or secretion of liver lipase activity by liver cells was established in vitro.

Materials and Methods

Normally fed, male Wistar rats were used (250–300 g). They were housed under controlled conditions; temperature 20–22 °C, lights on between 07.00–19.00 h.

β-Blockers were given for 3 weeks by a daily, subcutaneous injection of 0.1 ml 12.5 mg/ml propranolol; 6 mg/ml metoprolol; 4 mg/ml atenolol). Control animals were given 0.9% NaCl Endogenous adrenergic stimulation was induced by diethyl ether stress. This was accomplished by exposing rats for 30 s to diethyl ether/air atmosphere every hour during 6 h. Norepinephrine was administered by daily subcutaneous injections of 0.1 ml norepinephrine bitartrate during 10 days, according to the following scheme: days 1–3; 1 mg/kg body weight, days 4–6; 2 mg/kg body weight, days 7–9; 3 mg/kg body weight, day 10; 4 mg/kg body weight [12]. Prazosin-HCl was added to the drinking water (100 mg/ml). The water was changed daily.

Cell preparations and secretion studies. Parenchymal cells were isolated from rat liver by collagenase treatment, as described before [13]. The intactness of the cells was routinely checked before and after incubation by the Trypan blue exclusion test and by measuring the leakage of lactate dehydrogenase from the cells. In the experiments presented here, more than 90% of the cells excluded Trypan blue, while never more than 20% of total lactate dehydrogenase was found in the media. Experiments on the secretion of lipase activity by isolated parenchymal liver cells were carried out as described before [14].

Biochemical measurements. HDL was separated from VLDL at $d = 1.050$ g/ml, essentially as described by Bronzert and Brewer [15]. HDL non-esterified and HDL esterified cholesterol was measured by standard methods (Boehringer testkit combination). Liver lipase activity was measured using an artificial trioleoylglycerol emulsion, exactly as described before [16]. Lipase activities are expressed in milli-units (1 mU = 1 nmol free fatty acid released/min). Protein contents and lactate dehydrogenase activities were estimated by standard methods.

Materials. Propranolol and atenolol were purchased from ICI (London, U.K.). Metoprolol was purchased from Astra (Gothenburg, Sweden). Prazosin-chloride was purchased from Pfizer (Brussels, Belgium). Norepinephrine and epinephrine were from BDH Chemicals (Poole, U.K.). Isoprenalin sulphate was purchased from Boehringer (Ingelheim, F.R.G.); A23187 was from Boehringer Mannheim, (F.R.G.). Vasopressin and collagenase type I were purchased from Sigma (St. Louis, MO, U.S.A.). Glucagon was from Novo Industri A/S (Copenhagen, Denmark). Ro 20-1724 was a gift from Dr. A.N.M. Schoffelmeer, Free University, Amsterdam, The Netherlands. Ham F-10, culture medium was obtained from Gibco Europe (Glasgow, U.K.).

Statistical procedures. Results are expressed as mean $\pm$ S.D. Comparisons between groups were made with Student's t-test.

Results

Adrenergic modulation of HDL cholesterol levels

Treatment of rats for 3 weeks with the β-blockers propranolol, metoprolol and and atenolol decreased the HDL cholesterol level (Table I). The effects were more pronounced with the β_1-selective blockers metoprolol and atenolol than with propranolol. The α_1-antagonist prazosin did not affect the HDL cholesterol level. Treatment of the rats with norepinephrine significantly increased the concentration of HDL non-esterified cholesterol, but had no effect on the total HDL cholesterol (Table II). Prazosin reduced in norepinephrine-treated rats the HDL non-esterified cholesterol

TABLE I

EFFECT OF DIFFERENT β-BLOCKERS AND THE α-BLOCKER PRAZOSIN ON HDL NON-ESTERIFIED AND HDL TOTAL CHOLESTEROL LEVELS

HDL cholesterol and HDL non-esterified cholesterol were measured in the plasma of rats after separation from VLDL and LDL. *n*, number of rats.

Treatment	*n*	HDL cholesterol (mM)	HDL non-esterified cholesterol (mM)
None	6	1.20 ± 0.08	0.223 ± 0.002
Propranolol	3	1.00 ± 0.09 [a]	0.203 ± 0.010 [a]
Metoprolol	3	0.84 ± 0.08 [a]	0.211 ± 0.008 [a]
Atenolol	3	0.90 ± 0.06 [a]	0.201 ± 0.06 [a]
Prazosin	4	1.08 ± 0.04	0.220 ± 0.004

[a] Significant difference from control values, $P < 0.01$.

content without affecting the total HDL cholesterol content. In contrast, propranolol did not affect the HDL non-esterified cholesterol but decreased, as in control rats, the total HDL cholesterol content (Table II).

Adrenergic modulation of liver lipase activity

Neither α- nor β-blockade affected, in otherwise normal rats, the liver lipase activity (Table III). This indicates that, under basal physiological conditions, catecholamines are not involved in the regulation of liver lipase activity. Since this may be different during adrenergic stimulation, diethyl ether stress was used to induce an endogenously elevated level of catecholamines. In more pro-

TABLE II

EFFECT OF NOREPINEPHRINE IN THE ABSENCE OR PRESENCE OF PROPRANOL OR PRAZOSIN ON THE HDL CHOLESTEROL LEVEL

Values are given ± S.D. *n*, number of rats.

Treatment	*n*	HDL cholesterol (mM)	HDL non-esterified cholesterol (mM)
None	4	0.993 ± 0.026	0.223 ± 0.002
Norepinephrine	4	0.950 ± 0.023	0.317 ± 0.020 [b]
Norepinephrine + prazosin	4	0.967 ± 0.035	0.257 ± 0.020 [a]
Norepinephrine + propranolol	3	0.904 ± 0.041 [b]	0.290 ± 0.022 [b]

[a] $P < 0.05$.
[b] $P < 0.001$.

TABLE III

EFFECT OF DIFFERENT ADRENERGIC BLOCKERS, DIETHYL ETHER STRESS AND NOREPINEPHRINE TREATMENT ON LIVER LIPASE ACTIVITY

Rats were treated with different β-blockers and the α-blocker prazosin with norepinephrine with or without prior treatment with prazosin or propranolol or they were exposed to diethyl ether stress, also with or without prior treatment with prazosin. After the treatment rats were killed by decapitation and liver lipase activity was measured in the liver. *n*, number of rats.

Expt. No.	Treatment	*n*	Liver lipase activity	
			mU/g wet weight	mU/total liver
I	None	4	450 ± 64	4815 ± 387
	Propranolol	3	383 ± 40	4181 ± 450
	Metoprolol	3	478 ± 96	5218 ± 278
	Atenolol	3	430 ± 60	5041 ± 514
	Prazosin	4	438 ± 28	4555 ± 312
II	None	4	540 ± 20	5917 ± 288
	Ether stress	8	517 ± 27	5227 ± 207 [a]
	Ether stress + prazosin	8	545 ± 40	6057 ± 325
III	None	4	430 ± 11	4551 ± 378
	Norepinephrine	4	327 ± 13 [a]	2863 ± 253 [a]
	Norepinephrine + prazosin	4	412 ± 22	4126 ± 277
	Norepinephrine + propranolol	3	311 ± 10 [a]	2773 ± 542 [a]

[a] Values are statistically different from control values ($P < 0.01$).

TABLE IV

EFFECT OF α- AND β-ADRENERGIC DRUGS ON THE SECRETION OF LIVER LIPASE ACTIVITY

Parenchymal liver cells were incubated in the absence or presence of different drugs for 2 h. After the incubation liver lipase activity was measured in the medium. *n*, number of rats.

Additions	*n*	Liver lipase activity (mU/mg cell protein)
None	12	0.68 ± 0.04
Epinephrine (10^{-7} M)	6	0.47 ± 0.05 [a]
Isoprenalin (10^{-6} M)	4	0.73 ± 0.06
Propranolol (10^{-4} M)	3	0.56 ± 0.08
Prazosin (10^{-6} M)	2	0.69;0.57
Epinephrine + propranolol	3	0.39 ± 0.09 [a]
Epinephrine + prazosin	4	0.62 ± 0.04

[a] Values are statistically significant from control ($P < 0.01$).

longed studies rats were treated with norepinephrine. Under both conditions the liver lipase activity was significantly decreased. Diethyl ether stress for 6 h lowered the total lipase activity by 15% (Table III). Long-term treatment with norepinephrine reduced the liver lipase activity by more than 35%. The lipase activity per gram of liver was not significantly lowered in the short-term experiments, but was decreased in the norepinephrine-treated rats. To investigate whether the lowering of the lipase activity was mediated through the α- or the β-receptor, rats were, in addition to norepinephrine or diethyl ether stress, treated with the α_1-blocker prazosin or the β-blocker propranolol. The effect of the diethyl ether stress as well as of norepinephrine treatment was abolished by pretreatment of the animals with prazosin. Propranolol was without effect (Table III).

Experiments with isolated liver cells

The apparent involvement of liver lipase activity was further studied in vitro on the synthesis or secretion of liver lipase activity by isolated parenchymal liver cells. Epinephrine inhibited the secretion of liver lipase activity by 30% (Table IV). This effect could neither be obtained with the β-agonist isoprenalin nor be blocked by propranolol. In the presence of prazosin, however, the secretion was no longer inhibited by epinephrine. Prazosin alone did not influence the secretion of liver lipase activity. To study the mechanism by which stimulation of the adrenergic receptor lowers the liver lipase activity, cells were incubated in the presence of a number of drugs which enhance the intracellular calcium or cAMP concentration (Table V). No effect was obtained with glucagon alone or in combination with the phosphodiesterase inhibitor Ro 20-1724. In contrast, vasopressin and the calcium ionophore (A23187) inhibited the lipase by 30 and 60%, respectively. The effect of the calcium ionophore could only partly be ascribed to its solute ethanol, which lowered the lipase activity less than 20%.

TABLE V

EFFECT OF DIFFERENT CALCIUM-INFLUENCING HORMONES AND DRUGS ON THE SECRETION OF LIVER LIPASE ACTIVITY

Parenchymal liver cells were incubated with or without different drugs for 3 h. After incubation, liver lipase activity was measured in the medium. *n*, number of rats.

Additions	*n*	Liver lipase activity (mU/mg cell protein)
None	6	0.82 ± 0.08
Epinephrine (10^{-7} M)	4	0.55 ± 0.06 [a]
Vasopressin (12 nM)	4	0.57 ± 0.03 [a]
A 23187 (2.10^{-6} M)	3	0.35 ± 0.02 [a]
Ethanol (1‰)	3	0.60 ± 0.10 [a]
Glucagon (0.05 mg/ml)	3	0.80 ± 0.09
Ro 20-1724 (10^{-4} M)	2	0.78;0.84
Glucagon + Ro 20-1724	3	0.78 ± 0.06

[a] Significant difference from control value $P < 0.01$.

Discussion

This paper shows that in the rat HDL cholesterol levels are influenced by adrenergic modulation. As shown before in humans [8–11], β-blockers lower the total HDL cholesterol concentration in the plasma. Although no effect on total HDL cholesterol was found during adrenergic stimulation, the HDL fraction became relatively enriched in non-esterified cholesterol. This indicates a change in the metabolism of HDL under this condition. The effects of the β-blockers on HDL were not accompanied by changes in liver lipase activity. Therefore, other mechanisms, for example changes in the HDL formation rate due to impairment of the catabolism of triacylglycerol-rich lipoproteins [17,18], could be operative. The adrenergic effect on non-esterified cholesterol in HDL seems to be mediated through the α-receptor, since prazosin but not propranolol abolishes this effect.

Liver lipase activity showed, like HDL non-esterified cholesterol, an α_1-selective dependency. The changes in liver lipase activity were always inversely related to the changes in HDL non-esterified cholesterol. Earlier, we proposed a role of liver lipase in the transfer of HDL non-esterified cholesterol to the liver [4,19]. Recently, strong support for this hypothesis was provided by Bamberger et al. [20], who showed that liver lipase action induces an increased flux of non-esterified cholesterol to cultured hepatocytes. Therefore, it is likely that the changes in liver lipase activity and

HDL non-esterified cholesterol are causally related.

The primary action of the adrenergic effects may be on the liver lipase activity, as the secretion of liver lipase by isolated parenchymal liver cells was lowered by epinephrine. This in vitro effect of epinephrine could not be mimicked by the β-specific agonist isoprenalin, but was blocked by prazosin. This indicates an involvement of the α_1-receptor. Enhancement of endogenous cAMP levels by glucagon or by inhibition of phosphodiesterase does not affect the secretion rate of liver lipase. This suggests an involvement of a second messenger, different from cAMP, e.g., calcium. Stimulation of the α_1-receptor in hepatocytes results in calcium mobilization [21]. Agents known to influence the intracellular free calcium concentration inhibited the liver lipase activity (Table V). Therefore, the secretion of liver lipase activity is affected by the endogenous calcium concentration. How liver lipase is regulated by calcium is not known. One possibility is a calcium-dependent, post-translational modification of the completed enzyme, as has been suggested for lipoprotein lipase in adipose tissue [22,23]. Another possibility is a calcium-dependent intracellular catabolism of the enzyme [24]. The central role of calcium in liver lipase regulation suggests that other effectors (free fatty acids [25], cholesterol [26]) possibly influence liver lipase activity as well. During ACTH treatment of rats, liver lipase activity is also lowered. This lowering may be due to interference of corticosteroids at the transcription level [27]. Besides the secretion rate from the parenchymal cells, the number of binding sites of the enzyme at the endothelial cells can be influenced, as shown during prolonged hypercortisolism [27]. Therefore, regulation of the lipase activity may invoke effects of the intracellular calcium concentration on the secretion rate of the enzyme, the rate of transcription of the enzyme and transport and binding of the enzyme [27] to the endothelium. A lowered liver lipase activity has been suggested to be related to atherosclerosis [28]. Indeed, normolipidemic atherosclerotic patients were shown to have a lowered liver lipase activity [29]. Stress (hormones, catecholamines, corticosteroids [7]) lowers liver lipase activity, as shown in this paper. Prolonged stress in known to lead to an increased atherogenic risk. Whether there is a causal relation between it and these effects of stress remains to be elucidated.

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APPENDIX PAPER V

Effects of Hormones, Fasting and Diabetes on Triglyceride Lipase Activities in Rat Heart and Liver

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Summary

Male rats were fasted for 3 days, subjected to streptozotocin-diabetes or injected with L-thyroxine, Kenacort®-A40 (corticosteroid) and Synacthen® (ACTH). Cardiac heparin-releasable lipoprotein lipase (LPL) activity was increased after fasting, experimental diabetes and all hormone treatments. Cardiac neutral lipase activity was decreased during diabetes and enhanced in the fasted state and by L-thyroxine, corticosteroid and ACTH administration. The close correlation between vascular LPL and tissue neutral lipase with cardiac triglyceride content is in agreement with the contention that tissue neutral lipase is similar to LPL (*Hülsmann, Stam* and *Breeman* 1982). Myocardial acid lipase activity was reduced during diabetes and L-thyroxine treatment, increased during fasting and corticosteroid administration and not affected by short-term ACTH treatment.

Hepatic acid lipase activity was increased during fasting, diabetes and by L-thyroxine and reduced after corticosteroid and ACTH treatment. The alkaline liver lipase activity was depressed by fasting, experimental diabetes, corticosteroid and ACTH treatment, whereas L-thyroxine induced a slight increase in enzyme activity.

The possible mechanism underlying the observed changes in acid, neutral, alkaline, and LPL activities in heart and liver are discussed.

Key-Words: *Heart – Lipoprotein Lipase – Neutral Lipase – Acid Lipase – Liver – Alkaline Lipase – Acid Lipase – Fasting – Diabetes – L-Thyroxine – Corticosteroid – ACTH*

Introduction

Rat heart and liver contain several triglyceride (TG) lipase activities. Both organs contain an acid, lysosomal, lipase (EC 3.1.1.3) (*Wang, Menahan* and *Lech* 1977; *Hülsmann* and *Stam* 1978; *Vavrinková* and *Mosinger* 1965) and this lipase has been implicated to be involved in endogenous lipolysis (*Hülsmann, Stam* and *Geelhoed-Mieras* 1979; *Debeer, Thomas, De Schepper* and *Mannaerts* 1979; *Stam* and *Hülsmann* 1982). The rate of lipolysis of stored TG in heart and liver is modified by various hormones and may take place by effects on lysosomal autophagocytosis or by product inhibition of lipase action (*Hülsmann* et al. 1979; *Stam* and *Hülsmann* 1981; 1982a; *Debeer, Beynen, Mannaerts* and *Geelen* 1982).

Another TG lipase present in cardiac muscle is LPL (EC 3.1.1.34). LPL is synthesized in the cardiac myocytes, secreted and bound to the vascular endothelium where it is involved in the breakdown of lipoprotein TG. Upon perfusion with heparin LPL is released from the vasculature. After prolonged heparin-perfusion a small amount of lipolytic activity, with a pH optimum of 7.4, remains present in the tissue. A role of this neutral enzyme in intracellular TG hydrolysis is still controversial. The neutral lipase may be the precursor of LPL since both enzymes have many properties in common (*Hülsmann, Stam* and *Breeman* 1982).

Cardiac LPL activity seems to be under hormonal control by mechanisms involving enzyme synthesis and secretion (*Cryer* 1981). No information is available concerning the regulation of the neutral lipase activity in cardiac muscle, although cardiac lipidosis is accompanied by an increase in neutral lipase activity (*Stam* and *Hülsmann* 1982b).

Upon heparin-perfusion of the liver an alkaline lipase, different from muscle LPL (*La Rosa, Levy, Windmueller* and *Frederickson* 1972), is released. This enzyme is synthesized by the parenchymal liver cells, secreted and bound to the non-parenchymal cells where it is physiologically active (*Jansen, van Berkel* and *Hülsmann* 1978; *Jansen, Kalkman, Zonneveld* and *Hülsmann* 1979; *Schoonderwoerd, Hülsmann* and *Jansen* 1981). The enzyme may play a role in the uptake of cholesterol from high density lipoproteins (*Jansen* and *Hülsmann* 1980). In previous studies concerning hormonal modification of liver lipase, activity was determined in post-heparin serum (*Jansen* and *Hülsmann* 1975; *Hülsmann, Oerlemans, Geelhoed-Mieras* 1977). To exclude hormone-effects on heparin-releasability of the enzyme, we studied alkaline lipase activities in liver homogenates.

The scope of the present study is to (re)evaluate the effect of various hormones (L-thyroxine, corticosteroid, ACTH), fasting and experimental diabetes on the activities of acid, neutral, and alkaline TG lipase activities in heart and liver. The possible relation between rat heart LPL and neutral lipase was further explored.

Methods and Materials

Animals and hormone treatment protocol

Male Wistar rats (220–260 g body weight) were used in all experiments. They had free access to laboratory chow and water. One group of rats was fasted for 72 h. Injection of the various substances was given under ether anesthesia. Control rats received only the appropriate vehicle.

Diabetes was induced by intravenous (tail vein) injection of streptozotocin (50 mg/kg, freshly dissolved in 50 mM citrate buffer of pH

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4,5) 3 days prior to sacrifice. The serum glucose level was then increased from 10.0 ± 0.6 mM (n = 7) to 34.4 ± 3.9 mM (n = 5) at the moment of sacrifice.

Acute hyperthyroidism of the rats was achieved by intraperitoneal injections of L-thyroxine (1 mg/kg) dissolved in 0.03 N NaOH during 5 successive days at 16.00 h.

Corticosteroid effects were studied after intramuscular injection of 4 mg Kenacort®-A40 (9α-fluor-11β,16α, 17α, 21-tetrahydroxy-1,4 pregnadien-3,20dion acetonide) 4 and 2 days prior to sacrifice at 16.00 h. The acute effect of ACTH was induced by a single subcutaneous injection of 5U (50 μg) Synacthen®-Depot (the zinc complex of tetracosactide hexa-acetate).

All animals were killed between 10.00 and 11.00 h.

Perfusion protocol

Under ether anesthesia of the rats the hearts and livers were removed. The livers were weighed, frozen in liquid N_2 and stored at −20°C. The hearts were perfused retrogradely as described earlier (*Stam* and *Hülsmann* 1981). After 15 min of recovery they were perfused with buffer containing heparin (5U/ml). The first 20 ml cardiac post-heparin effluent was collected in glycerol (0°C, 20% v/v final concentration) and immediately tested for LPL activity. Heparin perfusion continued for another 25 min followed by a washout of 5 min. Then a post-nuclear supernatant (PNS) in 1 mM phosphate buffer (pH 7.4) was prepared as described earlier (*Hülsmann, Stam* and *Breeman* 1981).

Determination of lipase activities

LPL activity in post-heparin effluent was assayed using [9,10(n)-^{3}H]trioleate (TO) sonicated with 10 times diluted 20% Intralipid® or 5% (w/v) gum acacia at 21 kHz (one min for each ml). 150 μl effluent in 20% glycerol was incubated at 37°C in a final volume of 250 μl containing 4 mM [^{3}H]TO (TO/Intralipid® or TO/gum acacia), 2% (w/v) defatted bovine serum albumin (BSA), 50 mM Tris-buffer of pH 8.2 and 45 mM NaCl. Rat serum (25 μl) or apolipoprotein C_{II} (apo C_{II}, 0.75 μg) was added as activator during incubation with TO/gum acacia or TO/Intralipid®, respectively. After 30 min the fatty acids were extracted according to *Belfrage* and *Vaughan* (1969) and estimated by liquid scintillation counting. LPL activity is expressed in mU/20 ml effluent/g wet weight (gww). One mU represents one mole of fatty acid released from the TO substrate per min.

Acid and neutral lipase activities were estimated during a 30 min incubation of 0.5–0.8 mg of PNS protein in a final volume of 125 μl containing 4 mM [^{3}H]TO/gum acacia, 2% BSA and 100 mM Tris-acetate buffer of pH 4.5 or pH 7.4, respectively. PNS lipase activities are expressed in mU/mg protein.

Hepatic alkaline and acid lipase activities were determined in 2% (w/v) homogenates in 0.15 M NaCl (15 sec, Polytron setting (4) after freeze-thawing and sonication). Acid lipase activity was estimated as described for cardiac acid lipase. Alkaline lipase activity was measured at pH 8.5 during a 30 min incubation of 1 mg protein in a final volume of 250 μl containing 4 mM [^{3}H] TO/gum acacia, 50 mM Tris-buffer, 2% (w/v) BSA and 0.6 M NaCl. Acid and alkaline lipase activities are expressed in mU/mg protein.

Analytical procedures

Serum glucose was estimated according to *Werner, Rey* and *Wielinger* (1970) and PNS TG as described by *Laurell* (1966). Protein in PNS was measured by the biuret method (*Gornall, Bardawill* and *David* 1949) and in liver homogenates according to *Lowry, Rosebrough, Farr* and *Randall* (1951). BSA was used as standard.

Chemicals and reagents

Reagents (all of analytical grade) were obtained from Merck (Darmstadt, Germany), L-thyroxine and BSA (fraction V) were from Sigma Chemical Co. (St. Louis, MO, USA). [^{3}H] TO was purchased from Amersham Int. PLC (Amersham, U.K.), heparin from Organon (Oss, The Netherlands), Kenacort®-A40 from Squibb (Rijswijk, The Netherlands), Synacthen® Depot from Ciba-Geigy (Groot-Bijgaarden, Belgium) and Intralipid® from Vitrum (Stockholm, Sweden). Streptozotocin was obtained from Calbiochem (Lucerne, Switzerland). Enzymes for the glucose assay were from Boehringer (Mannheim, Germany).

Statistics

Results are expressed in mean values ($\bar{X}$) ± standard error of the mean (SEM). Significance was tested by Student's t-test (two-tailed). $P < 0.05$ was considered to be significant. n is the number of observations. The measured parameters did not differ in the various control groups and their data were combined.

Results

Growth, organ weight and tissue protein content

Table 1 shows the effects of fasting, diabetes, and of various hormone treatments (L-thyroxine, corticosteroid and ACTH) on net body weight change, organ: body weight ratios and tissue protein content.

During diabetes the loss in body weight is about 10% higher than the loss in heart weight, resulting in a small increase in the heart: body weight ratio. In contrast, the dramatic reduction in liver weight causes a drop in liver: body weight ratio.

Diabetes hardly affects heart weight but causes a sharp drop in body weight. The loss in liver weight decreases the liver: body weight ratio. Diabetes and fasting are accompanied by an increase in heart and liver weight.

L-thyroxine has little effect on body weight but heart weight increases and liver weight drops with consequent alterations in heart- and liver: body weight ratios. Thyroxine treatment only induces an increase in liver protein content.

Corticosteroid administration increases heart and liver weight concommitant with a severe loss in body weight, causing enhanced organ: body weight ratios. Protein content of heart PNS and liver is decreased.

ACTH hardly affects overnight growth of the rats. The rise in liver weight results in a higher liver: body weight ratio. Liver protein content is lowered.

Cardiac lipoprotein, acid and neutral lipases; Lipidosis

The effects of fasting, diabetes, and of L-thyroxine, corticosteroid and ACTH treatment on cardiac TG lipases is shown in Table 2.

LPL activity assays using TO/gum acacia yield lower values than using TO/Intralipid®. Fasting, diabetes and all hormone treatments lead to an increase in LPL activity. Neutral lipase activity is increased by fasting and after administration of L-thyroxine, corticosteroid and ACTH.

Diabetes is accompanied by a 45% reduction in this lipase activity. However (Fig. 1), serum-stimulated neutral lipase activity is higher in PNS from diabetic hearts. Acid lipase activity is increased during fasting and L-thyroxine treatment but depressed in the diabetic state and after corticosteroid administration. Overnight treatment with ACTH has no effect. Incubation in the presence of serum inhibits acid lipase activity (Fig. 1) in PNS from control and diabetic rats.

Table 1 Effect of fasting, streptozotocin-diabetes and various hormone treatments on net body weight changes, organ: body weight ratios and organ protein content in the rat

Treatment	Duration (days)	Net body weight[a] change (g)	Protein content (mg/g ww)		Heart:body weight (g/kg)	Liver:body weight (g/100 g)
			Heart PNS	Liver		
Control	–	–	62.2±1.5 (19)[b]	181±2 (8)	3.67±0.05 (19)	3.86±0.16 (8)
Fasting	3	−53.2	78.8±0.8 (4)[•,c]	223±8 (4)[•]	3.99±0.09 (4)[□]	2.62±0.04 (4)[•]
Diabetes	3	−56.2	73.3±1.9 (5)[•]	207±3 (4)[•]	4.15±0.12 (5)[•]	3.21±0.10 (4)[•]
L-Thyroxine	5	−7.5	66.7±4.3 (4)[ns]	215±2 (4)[•]	5.26±0.08 (4)[•]	3.67±0.07 (4)[*]
Kenacort®	4	−63.2	53.6±1.6 (4)[•]	150±3 (4)[•]	5.23±0.10 (4)[•]	5.67±0.13 (4)[•]
Synacthen®	1[d]	−2.5	60.3±3.7 (4)[ns]	159±4 (4)[•]	3.88±0.10 (4)[ns]	4.59±0.16 (4)[•]

a. Net body weight (bw) change is defined as the mean difference in growth between the experimental group of rats and the appropriate controls during the indicated period of treatment, i.e. $(bw_{end}-bw_{start})$ experimental rats $-(bw_{end}-bw_{start})$ control rats

b. $\bar{X}$ ± SEM (n)

c. *: $P < 0.05$; □: $P < 0.02$; •: $P < 0.001$ *vs* control; ns: not significant

d. overnight

Table 2 Effect of fasting, streptozotocin-diabetes and various hormone treatments on rat heart heparin-releasable lipoprotein lipase (LPL) activity, myocardial post-nuclear supernatant (PNS), acid and neutral lipase activities, and PNS triglyceride (TG) content

Treatment	n	LPL activity (mU/20 ml/g ww)		PNS lipase activity (mU/mg protein)		PNS TG (mg/mg protein·10^{-2})
		TO/Intralipid®	TO/gum acacia	Neutral (pH 7.4)	Acid (pH 4.5)	
Control	19	30.8±4.9[a]	12.5±1.8	0.68±0.02	0.53±0.02	1.51±0.18
Fasting	4	84.5±4.6[•,b]	33.7±2.9[•]	0.89±0.09[○]	0.79±0.08[•]	0.89±0.09[○]
Diabetes	5	125.7±5.0[•]	74.8±6.0[•]	0.31±0.03[•]	0.11±0.02[•]	2.13±0.16[○]
L-Thyroxine	4	64.8±4.0[•]	19.1±2.2[*]	1.27±0.04[•]	0.70±0.03[○]	2.25±0.18[•]
Kenacort®	4	98.9±4.8[•]	27.0±2.1[○]	0.93±0.04[•]	0.43±0.02[*]	3.19±0.89[•]
Synacthen®	4	115.4±10.4[•]	24.1±3.0[□]	1.08±0.02[•]	0.59±0.02[ns]	4.06±0.99[•]

a. $\bar{X}$ ± SEM

b. *: $P < 0.05$; □: $P < 0.02$; ○: $P < 0.005$; •: $P < 0.001$ *vs* control; ns: not significant

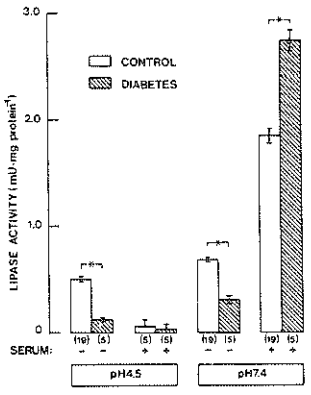

Fig. 1 Effect of serum on acid and neutral lipase activity in myocardial post-nuclear supernatant from control and streptozotocin-diabetic rats. $\bar{X}$ ± SEM. n is given in brackets under each bar. *: $P < 0.05$.

PNS TG are increased under all conditions of enhanced LPL activity except during fasting when they are decreased. Not considering the data from the fasted group, the LPL activity correlates well with PNS TG content. From the linear regression curve (not shown) a correlation coefficient (r) is obtained of 0.87 ($P < 0.02$), while correlation of LPL and neutral lipase activities of all groups, except values in diabetics, reveals $r = 0.67$ ($P < 0.05$). Neutral lipase also correlates with PNS TG content in all groups of fed rats ($r = 0.64$, $P < 0.05$).

Hepatic acid and alkaline lipase activities

Table 3 shows the effect of diabetes, fasting and hormones on hepatic acid and alkaline lipase activities. Except for the action of ACTH which depresses, and of diabetes, which enhances hepatic acid lipase activity, the effect of fasting, L-thyroxine and corticosteroid is similar in liver and heart. The alkaline lipase activity, expressed on mg protein basis is not altered by L-thyroxine but depressed after corticosteroid and ACTH administration. Since, like LPL, the hepatic alkaline lipase is functionally active at the extracellular compartment (vascular lumen) it may be of greater physiological significance to express the enzyme activity on tissue wet weight basis. As Table 3 indicates, in that case,

Table 3 Effects of fasting, streptozotocin-diabetes and various hormone treatments on rat liver acid and alkaline lipase activity

Treatment	n	Acid lipase activity (mU/mg protein)	Alkaline lipase activity (mU/mg protein)	(mU/g ww)
Control	8	0.41±0.02[a]	3.35±0.06	606±12
Fasting	4	0.55±0.06[■,b]	1.64±0.17[●]	366±38[●]
Diabetes	4	0.98±0.03[●]	1.36±0.30[●]	281±63[●]
L-Thyroxine	4	1.03±0.15[●]	3.21±0.16[ns]	690±34[■]
Kenacort®	4	0.29±0.09[*]	0.92±0.06[●]	138±9[●]
Synacthen®	4	0.24±0.01[●]	2.19±0.03[●]	349±7[●]

a. $\bar{X}$ ± SEM

b. *: $P < 0.05$; ■: $P < 0.01$; ●: $P < 0.001$ *vs* control; ns: not significant

L-thyroxine increases its activity by 15%, whereas the effects in the other experimental groups are similar.

Discussion

Alteration of enzyme activities by hormones can be achieved by several mechanisms. First, by a change in enzyme activity without affecting the amount of enzyme, and second by alteration of enzyme content, either by altered rates of protein synthesis or degradation. Although our study has not dealt with the mechanisms of hormone action, we shall discuss our data in the light of the above mentioned possibilities.

Acid lipase of heart and liver

It has been previously shown that overnight starvation did not affect acid lipase activity in rat heart (*Stam* and *Hülsmann* 1982b). Prolonged fasting, however, is accompanied by an enhanced acid lipase activity in heart and liver (Table 2 and 3). This may be a reflection of an increased rate of autophagocytosis in the fasted state (*Aronson* 1980). *Vavrinková* and *Mosinger* (1971), indeed, reported increased liver acid lipase activity by the high glucagon:insulin ratio occurring during fasting. The decrease in cardiac acid lipase activity during diabetes, first described by *Rösen, Budde* and *Reinauer* (1981), can be explained by enhanced protein degradation in diabetic hearts, since it has been shown (*Rannels, Kao* and *Morgan* 1975) that insulin restricted the rate of protein degradation in rat heart. In contrast, insulin may repress lysosomal autophagocytosis of lipid droplets in liver. The absence of such an action in the diabetic state will induce an enhanced acid lipase activity in liver.

After L-thyroxine treatment heart and liver acid lipase activities increase. This observation is new for the heart enzyme and confirms earlier studies on the liver enzyme (*Coates, Brown, Lau, Krulich* and *Koldovsky* 1978). Thyroxine effects may be accounted for by increased protein synthesis since administration of the hormone is accompanied by an increased RNA:DNA ratio (*Bressler* and *Wittels* 1966) and this effect was inhibited by the simultaneous administration of actinomycin D (*Coates, Lau, Krulich, Brown* and *Koldovsky* 1979).

After corticosteroid injections heart and liver acid lipase activity is reduced. This action cannot be explained by its lysosomal membrane stabilizing property for we disrupted the lysosomes thoroughly. Based on the decrease in liver and heart PNS protein content, increased protein degradation is more likely involved.

Overnight ACTH treatment did not alter heart acid lipase activity, whereas the livers early responds with a decreased activity. The similar effects of corticosteroid and ACTH on liver acid lipase suggest a common mechanism of action.

Lipoprotein lipase, neutral lipase and TG in heart

LPL activity towards TO/Intralipid® is higher than towards TO/gum acacia. This finding is in line with observations of *Hülsmann, Stam* and *Breeman* (1982) who showed that LPL requires both phospholipids and apo C_{II} for full activity. Our data concerning the changes in heparin-releasable LPL activity during fasting, diabetes, and after administration of L-thyroxine and ACTH confirm earlier reports (see for review *Cryer* 1981). Next we now demonstrate a marked stimulation of LPL after corticosteroid treatment, recently also described by *Pedersen, Wolf* and *Schotz* (1981). Hormonal activation of LPL activity occurs via changes in enzyme synthesis (*Cryer* 1981). It must be taken into account, however, that enhanced tissue perfusion increases the release of LPL during heparin-perfusion. The higher coronary flow rates of diabetic and fasted hearts (*Stam* and *Hülsmann* 1977) induce a better LPL washout and may hereby contribute to an overestimation of LPL activity.

Except for fasting, all conditions of enhanced LPL activity result in cardiac lipidosis. This correlation is in line with the role of this enzyme in the uptake of serum lipoprotein TG.

In contrast with LPL, less is known about hormonal regulation of the neutral lipase present in heart after long-term heparin-perfusion. The enzyme is probably not affected by the adenylate cyclase-cyclic AMP-protein kinase system as is accepted for adipose tissue neutral lipase (*Rösen, Budde* and *Bernauer* 1981). Our data demonstrate that, except during diabetes, all conditions of enhanced vascular LPL activity also reveal a higher neutral lipase activity. This confirms the possibility that LPL and neutral lipase are indeed similar enzymes. Moreover, the higher serum-activated neutral lipase activity in diabetic hearts may be explained by the lack of activating apo C_{II} in homogenates of these hearts. The higher coronary flow rates in diabetic hearts may cause a better wash-out of plasma components from the vascular and interstitial space, and hereby a lower

residual homogenate content in comparison with control homogenates. The presence of plasma components in the interstitial (lymphatic) space was shown by us before (*Stam, Jansen* and *Hülsmann* 1980).

Alkaline lipase of liver

The effects of fasting, diabetes, corticosteroid and ACTH on the alkaline lipase activity can be measured in whole liver homogenates and in post-heparin serum (*Jansen* and *Hülsmann* 1975; *Hülsmann, Oerlemans* and *Geelhoed-Mieras* 1977). Comparison of our and these data indicate that differences in heparin-releasability are not responsible for the observed lowering of alkaline lipase activity, and that reduced enzyme synthesis is more likely. Furthermore, L-thyroxine treatment only increased the alkaline lipase activity when expressed on g wet weight basis. Due to liver hypertrophy and increased liver protein content the activity on mg protein basis is not altered. The activity on g wet weight basis, however, may be of greater physiological significance, since the vascular lumen is the site at which alkaline liver lipase is functioning.

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