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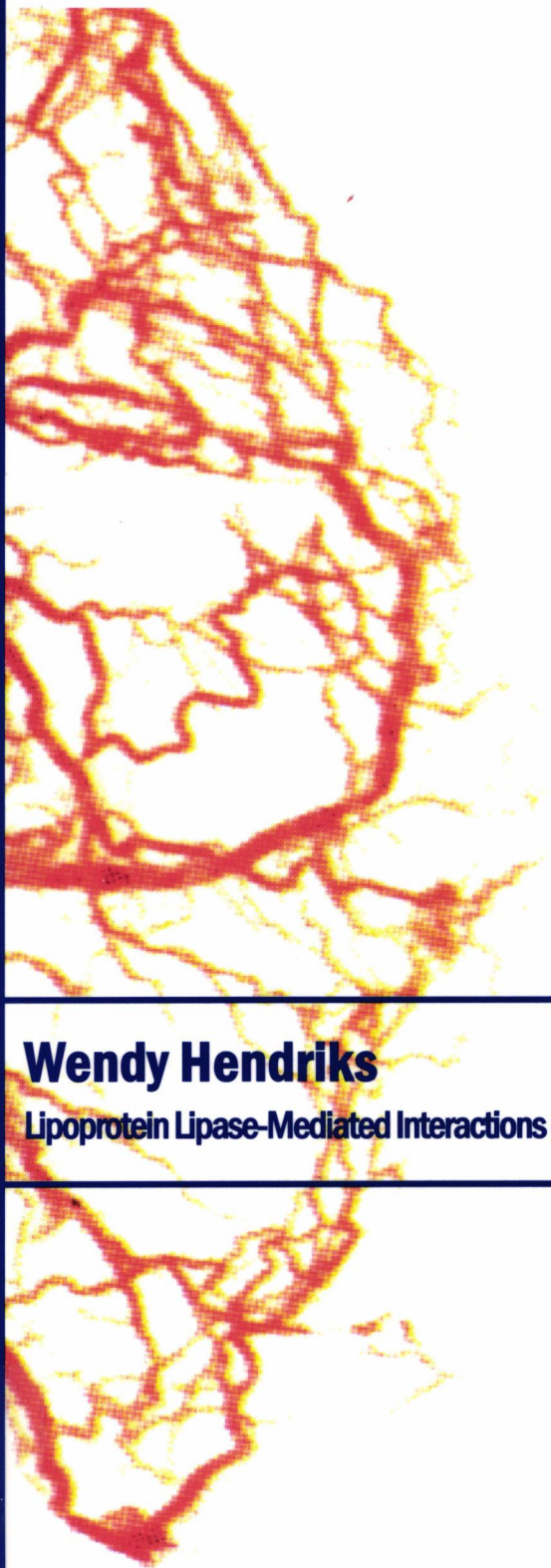

Wendy Hendriks

Lipoprotein Lipase-Mediated Interactions of Lipoproteins with Macrophages

Lipoprotein Lipase-Mediated Interactions of Lipoproteins with Macrophages

Proefschrift

ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden,
op gezag van de Rector Magnificus Dr. W.A. Wagenaar,
hoogleraar in de faculteit der Sociale Wetenschappen,
volgens besluit van het College voor Promoties
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door

Wilhelmina Leonie Hendriks

geboren te Weert in 1970

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Stellingen behorende bij het proefschrift:
'Lipoprotein Lipase-Mediated Interactions of Lipoproteins with Macrophages'

1. De karakterisering van LPL als 'good guy' of 'bad guy' is afhankelijk van de plaats in het lichaam waar het tot expressie komt.
   Dit proefschrift

2. Het feit dat apoE-deficiënte muizen ernstige atherosclerose ontwikkelen toont aan dat de opname van lipoproteïnen door macrofagen kan plaatsvinden in afwezigheid van zowel apoE als apoB100, en dus dat andere processen hieraan ten grondslag liggen.
   Dit proefschrift

3. Het feit dat incubatie van J774 macrofagen met geoxideerd VLDL leidt tot minder accumulatie van triglyceriden dan incubatie met natief VLDL, geeft aan dat extracellulaire lipolyse van belang is voor de opname van triglyceriden door deze cellen.
   Dit proefschrift

4. Aangezien in het C-terminale deel van LPL de bindingsplaatsen voor lipoproteïne-receptoren zo dicht bij de bindingsplaatsen voor lipoproteïnen zijn gelokaliseerd, moet LPL eerst een conformatieverandering ondergaan alvorens de monomeer een brug kan vormen tussen deze receptoren en de lipoproteïnen.
   Dit proefschrift

5. Het introduceren van een gen via de 'knock-in' techniek hoeft niet hetzelfde effect te hebben als het tot expressie brengen van datzelfde gen via adenovirus-gemedieerde gen-transfer op een nul-achtergrond.
   P.M. Sullivan et al. J. Biol. Chem. 272: 17972-17980 (1997); K. Tsukamoto et al. J. Clin. Invest. 100: 107-114 (1997)

6. Het feit dat een groot deel van de Nederlandse bevolking leeft volgens de dieetregels van Montignac, toont het gemak aan waarmee de sinds decennia voorgeschreven richtlijnen voor de goede voeding volgens de 'schijf van vijf', overboord gegooid worden.

7. Het mogelijk preventieve effect van het drinken van 2 tot 3 glazen rode wijn per dag op het ontstaan van hart- en vaatziekten, moet worden afgewogen tegen de kans daardoor alcoholist te worden.

8. Aangezien apoE zoveel verschillende functies heeft, is voorzichtigheid geboden bij de interpretatie van de fenotypes van apoE knock-out en apoE transgene muizen.

9. Het huidige beleid van subsidieverleners om met name direct klinisch relevant onderzoek te steunen, ten koste van strategisch wetenschappelijk onderzoek, zal er op den duur toe leiden dat er geen innovatief klinisch onderzoek meer mogelijk is.

10. Citatie van literatuur, die de eigen hypothese tegenspreekt, gebeurt met minder enthousiasme dan citatie van literatuur, waarin de eigen hypothese wordt onderbouwd.

11. Bloedheet = 37°C.
Loesje

12. When the going gets tough, the tough go shopping.
P. Brard

Leiden, 29 april 1998

Wendy Hendriks

Aan mijn ouders
Voor Bob

CONTENTS

Chapter 1

GENERAL INTRODUCTION

1.1 Introduction

Cardiovascular disease, and particularly atherosclerosis, is the main cause of death in the Western society. Next to age, gender, hypertension, obesity, smoking, alcohol consumption, physical activity and a positive family history of coronary artery disease, elevated levels of cholesterol and/or triglycerides in the blood are important risk factors for the development of atherosclerosis. Cholesterol and triglycerides are transported in the blood by lipoproteins. Knowledge of the synthesis, processing, and catabolism of these lipoprotein particles by various lipoprotein receptors in different tissues, is currently under investigation by many researchers.

1.2 Lipoprotein Metabolism

The major lipids occurring in the blood are cholesterol, cholesteryl esters, triglycerides and phospholipids. Cholesterol is essential for the growth and synthesis of cellular membranes, for the production of steroid hormones and vitamins and as a precursor for the bile acid synthesis. Triglycerides can either be stored in the adipose tissue or used as an energy source in muscles. Since the lipids are highly insoluble in an aqueous compartment such as blood, they must be converted into a water-soluble transport form. In the plasma, lipids are therefore complexed with proteins and phospholipids, to form the so-called lipoproteins. Lipoproteins are sperical particles with a core consisting of the hydrophobic triglycerides and cholesteryl esters, and a surface monolayer of phospholipids, unesterified cholesterol and proteins, the latter called apolipoproteins. These apolipoproteins interact with various enzymes, lipid transfer proteins and cell surface receptors, and thus determine largely the metabolic fate of the particle.

The lipoproteins have been sub-divided into five major classes, according to their density as determined by density gradient ultracentrifugation: 1, chylomicrons; 2, very low density lipoproteins (VLDL); 3, intermediate density lipoproteins (IDL); 4, low density lipoproteins (LDL); and 5, high density lipoproteins (HDL). Besides differing in

density, lipoproteins also differ in apolipoprotein composition, electrophoretic mobility and size, as shown in Table I [1,2].

Based on their site of synthesis and their function, the lipoprotein metabolism can be divided into three different pathways [2,3,4,5,6]: 1, the exogenous pathway, in which intestinally derived lipoproteins are transported to the liver; 2, the endogenous pathway, which involves the transportation of hepatically derived lipoproteins to peripheral tissues; and 3, the reversed cholesterol transport from peripheral tissues back to the liver (Figure 1 and Figure 2).

Table I. Physical properties and composition of human plasma lipoproteins [1,2]

	Chylomicron	VLDL	IDL	LDL	HDL
Diameter (nm)	75-1200	30-80	25-35	19-25	5-12
Mobility*	origin	pre-ß	pre-ß/ß	ß	α
Density (g/ml)	< 0.96	0.96-1.006	1.006-1.019	1.019-1.063	1.063-1.210
Composition					
Protein	1-2	6-10	11	21	45-55
Triglyceride	88	56	29	13	15
Phospholipid	8	20	26	28	45
Cholesteryl ester	3	15	34	48	30
Free cholesterol	1	8	9	10	10
Apolipoproteins	A1, A4, B48 C1, C2, C3, E	B100, C1, C2, C3, E	B100,E	B100	A1, A2, E

*According to electrophoretic mobility of plasma α- and ß-globulins on agarose gel electrophoresis. The values given for protein, triglyceride, phospholipid, cholesteryl ester, and free cholesterol are expressed as the percentage of total weight.

1.2.1 The Exogenous Pathway

In the exogenous lipid transport (Figure 1), dietary lipids are absorbed by the intestinal mucosa, packaged into large triglyceride-rich particles called chylomicrons and subsequently secreted into the bloodstream via the thoracic duct lymph. Chylomicrons possess primarily apolipoprotein (apo) A1, apoA4, and apoB48. After entering the bloodstream the chylomicrons release apoA1 and part of apoA4, while they acquire apoE, apoC1, apoC2 and apoC3 from HDL. Furthermore, the enzyme

lipoprotein lipase (LPL) hydrolyses most of the triglyceride-rich core of the chylomicrons after activation by apoC2. LPL is attached to the heparan sulphate proteoglycans on the vascular endothelial cells [7,8]. The free fatty acids generated by the hydrolysis of triglycerides are either stored as triglycerides in the adjacent adipose tissue, or taken up by the muscle tissue as an energy supply. As the particle becomes smaller due to the hydrolysis of triglycerides, a part of the surface components become superfluous, and excess phospholipids and apolipoproteins are transferred to HDL. The residual particles, now referred to as chylomicron remnants, are smaller, and have become relatively enriched in cholesterol and apoE. They are rapidly removed from the circulation via uptake by the liver through apoE-specific recognition sites [9,10]. The liver uptake of remnant lipoproteins is thought to involve a so-called secretion re-capture process [11,12,13]. Firstly, the remnants pass through the fenestrae of hepatic endothelial cells and accumulate in the space of Disse, where they become enriched in apoE excreted by the hepatocytes. The increased amounts of surface apoE enhance the binding of the remnants to the cells, in a process which is suggested to be mediated by heparan sulphate proteoglycans (HSPG) [14,15]. Secondly, the apoE-enriched remnants are taken up by the liver via the LDL receptor [16] and/or a specific remnant- or apoE receptor, either involving a proteoglycan-receptor complex or the receptor alone [17]. One likely candidate for the remnant receptor is the LDL receptor-related protein (LRP), originally described by Herz *et al* [18].

Hepatic lipase or lipoprotein lipase are also described to play a role in the uptake of remnant lipoproteins by the liver, in a process involving both heparan sulphate proteoglycans and LRP. It is postulated that LPL and HL can enhance the uptake of the remnants by the receptor, in a process which is initiated by the bridging of LPL or HL between the proteoglycans and the lipoproteins [19,20,21,22,23].

The cholesterol which has entered the liver through the uptake of the chylomicron remnants can be used in the hepatocyte for different purposes, including membrane synthesis and VLDL production, or it can be excreted in the bile duct as free cholesterol or bile acid.

1.2.2 The Endogenous Pathway

In the endogenous pathway (Figure 1), cholesterol and triglycerides are secreted into the plasma by the liver, packaged in the form of VLDL. The cholesterol is derived from either *de novo* synthesis, or from the chylomicron remnants taken up from the circulation. The triglycerides present in VLDL are derived from *de novo*

synthesised fatty acids from carbohydrates, from plasma free fatty acids, or from the incoming chylomicron remnants. The VLDL particles contain apoB100, apoE and apoC's. Like chylomicrons, the VLDL also change their apolipoprotein composition in the course of triglyceride hydrolysis by LPL. This results in the formation of smaller, cholesterol-rich and triglyceride-poor particles, known as VLDL remnants or intermediate density lipoprotein (IDL).

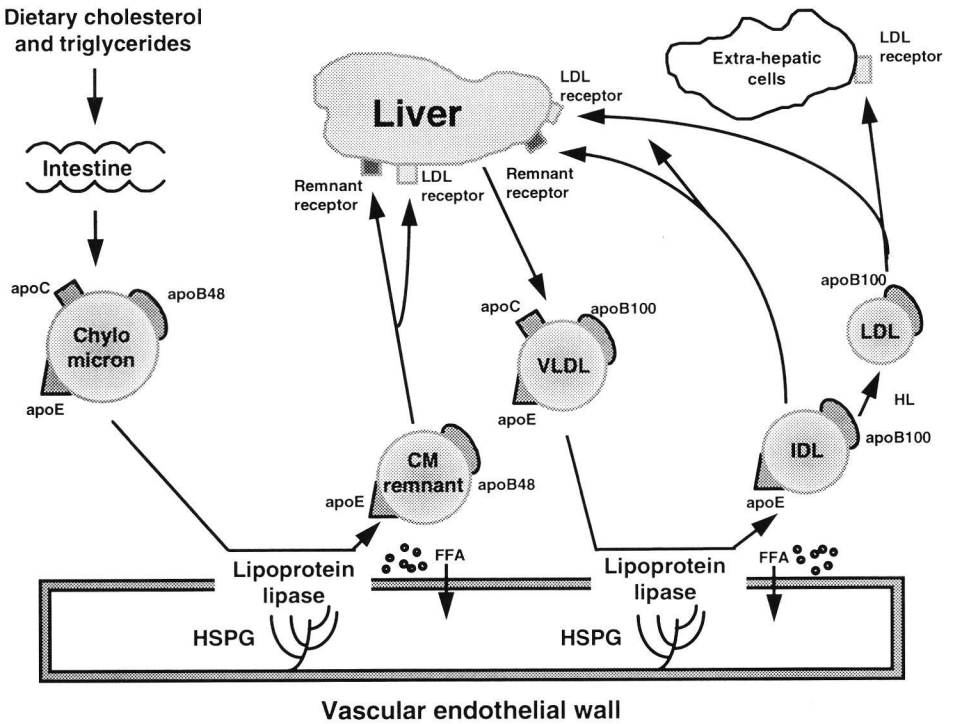

Figure 1. Exogenous and endogenous pathways of lipoprotein metabolism

LPL, lipoprotein lipase; HL, hepatic lipase; HSPG, heparan sulphate proteoglycans; apoE, apolipoproteinE, etc.

The IDL particles are either removed from the circulation by the hepatic LDL receptors and/or remnant receptors, or converted into LDL, losing apoC's and apoE in the latter process. The removal of the C-peptides and the further conversion into LDL is associated with the action of LPL or the hepatic triglyceride hydrolase [24]. LDL is rich in cholesteryl esters and contains apoB100 as the sole protein constituent, which is the ligand for the LDL receptors present on the liver and extra-hepatic tissues. A

small portion of the LDL particles are cleared from the circulation via LDL receptor-independent pathways, such as the scavenger receptor present on cells of reticuloendothelial origin, *i.e.* macrophages. This receptor, in contrast to the LDL receptor, is not regulated by intracellular cholesterol levels. Furthermore, it does not recognise native LDL, but efficiently takes up LDL which has undergone (oxidative) modification. It is commonly assumed that high concentrations of (modified) LDL in the intima of the vessel wall, will result in cholesterol loading of the macrophages, and subsequently their transformation into the so-called foam cells. This is assumed to be one of the initial and crucial steps in the development of atherosclerotic lesions [25]. This process will be discussed further in Chapter 1.5.

1.2.3 Reverse Cholesterol Transport

Excess of cholesterol in peripheral tissues can be transported back to the liver via a process referred to as reverse cholesterol transport (RCT). The liver, in turn, removes this excess cholesterol from the body, by conversion of cholesterol into bile acids and secretion directly into the bile (Figure 2). Reverse cholesterol transport involves (i) the uptake of free cholesterol from peripheral tissues by HDL, (ii) esterification of cholesterol by the enzyme lecithin: cholesterol acyltransferase (LCAT), which uses apoA1, the major protein constituent of HDL, as a cofactor, and (iii) the transfer of cholesteryl esters to VLDL, IDL and LDL by the cholesteryl ester transfer protein (CETP). The latter process allows the uptake of cholesterol esters by the liver via the LDL receptor pathway. An alternative pathway for the removal of HDL-derived cholesterol from the circulation is via apoE-mediated uptake of HDL by the liver. Finally, HDL associated cholesteryl esters can also selectively be taken up by the hepatocytes via the scavenger receptor SR-BI [26].

This mechanism of RCT is the explanation for the inverse relationship between the HDL concentration on one hand, and the risk of coronary heart disease on the other hand, as found in numerous epidemiological studies.

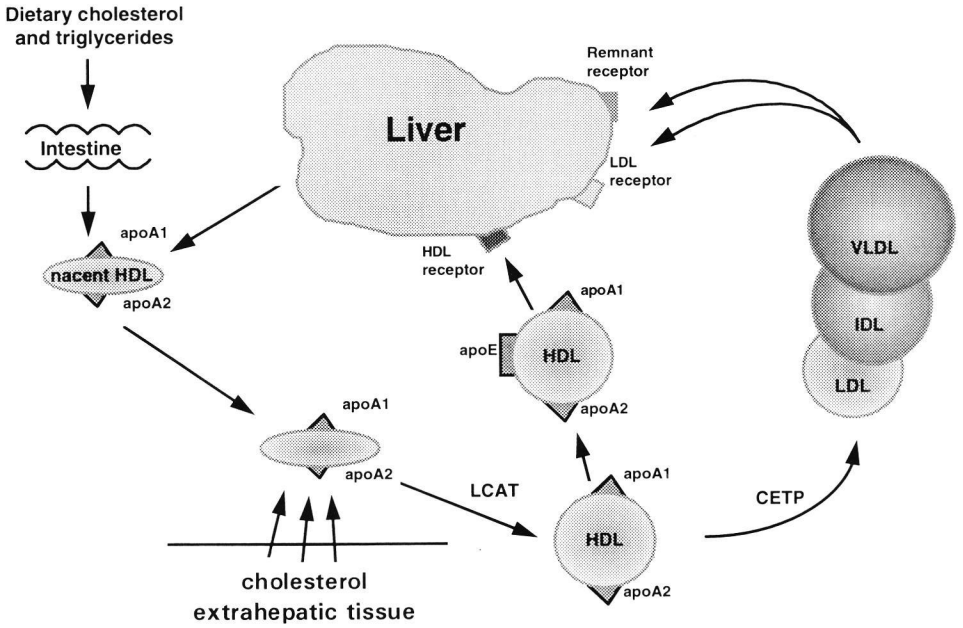

Figure 2. Reverse cholesterol transport

LCAT, lecithin:cholesterol acyltransferase; CETP, cholesteryl ester transfer protein, apoA1, apolipoproteinA1, etc.

1.3 Lipoprotein Lipase (LPL)

1.3.1 Structure and functions of LPL

LPL belongs to a conserved lipase gene family, which also includes hepatic lipase (HL) and pancreatic lipase (PL). Comparison of the amino acid sequences within this family, has revealed extensive homology between species and also between LPL, HL and PL [27]. The structure, function and role of the plasma lipases is reviewed in [7,8,28,29,30,31].

Active lipase is a non-covalently linked dimer of two glycoprotein chains of equal size. The single subunit molecular weight varies between 34 and 77 kDa, depending upon the species and tissue of origin. For human LPL, two potential N-glycosylation sites, Asn^{43} and Asn^{359}, have been reported [32].

The three-dimensional crystallographic structure of human PL has been solved [33], and it appears that the structure of LPL is very similar to that of PL. The protein consists of two domains; a larger NH_2-terminal domain (amino acids 1-312), and a smaller COOH-terminal domain (amino acids 313-448). The catalytic function of the

enzyme is conferred to the amino-terminal domain of LPL: the catalytic triad (Ser^{132}, Asp^{156} and His^{241}), as well as the site of interaction of LPL with its cofactor apoC2 [34] have been found to be localised in this domain [35]. As in PL, LPL contains a lid consisting of residues 217 to 238, which covers the catalytic triad and restricts the access of the substrate to the active site [36,37].

LPL is localised on the surface of the capillary endothelium via ionic interactions with surface heparan sulphate proteoglycans [38], and it is widely distributed in extrahepatic tissue including heart, skeletal muscle and adipose tissue. Small amounts are also found in mammary gland, brain, spleen, lung, diaphragm, aorta, kidney, uterus and intestine [39]. It is furthermore synthesised by monocytes, macrophages and smooth muscle cells present in atherosclerotic lesions [40,41]. Since LPL binds to the heparan sulphate proteoglycans on the endothelium of the vessel wall, heparin binding is an essential function of LPL. Although most of the heparin binding is mediated by the carboxy-terminal part of LPL [35], it has been shown that the amino-terminal domain of LPL also contributes to the heparin binding properties of the protein [42,43]. The carboxy-terminal domain of LPL also appears to play a major role in the initial interaction of LPL with lipoproteins, which is necessary for the subsequent hydrolysis of circulating triglycerides.

1.3.2 Role of LPL in cellular uptake of lipoproteins

Recently, it has been shown that the COOH-terminal domain of LPL also contains binding sites for different lipoprotein receptors, including the LDL receptor [44,45], the LRP [19,46,47,48] and the VLDL receptor [49]. Numerous studies have been published, supporting a role for LPL, independent of its hydrolytic function, in the binding and uptake of lipoproteins by different cell types [44-48,50,51,52]. LPL can enhance the cellular binding and uptake of different lipoproteins (LDL, VLDL, ß-VLDL, Lp(a) [53,54] and chylomicrons), via bridging of LPL between the lipoproteins and the proteoglycans and/or receptors.

The precise mechanism is still unclear, but it is proposed that LPL increases the binding of the lipoproteins to cell surface heparan sulphate proteoglycans, which, in turn, may facilitate the transfer of the LPL-lipoprotein complexes to either the LRP or the LDL receptor for internalisation. In contrast to these findings, Rumsey *et al* [50] and Fernandez-Borja *et* al [55] reported that LPL-mediated binding, internalisation and degradation of LDL by human fibroblasts and THP-1 cells occurs independent of any cell surface receptors. According to these investigators, LPL mediates the uptake of lipoproteins through a slow but high capacity metabolic pathway, involving

internalisation and degradation of the whole lipoprotein-LPL-HSPG complex. Beisiegel *et al* [19] reported that LPL can enhance the binding of apoE-containing lipoproteins directly to the receptor, in this case LRP. In Figure 3, the different proposed mechanisms for LPL-mediated uptake of lipoproteins by cells are illustrated.

To perform its enzymatic function as a lipase, it is shown that LPL should be in the dimeric state [56,57]. However, for LPL-mediated uptake of lipoproteins by cells, controversy exists whether LPL should have the monomeric or dimeric form. Krapp *et al* [58] and Nykjær *et al* [47,59] found that for this function LPL should be in the dimeric state, whereas Williams *et al* [48] and Chappell *et al* [60] proposed that LPL monomers are also able to mediate uptake of lipoproteins into cells.

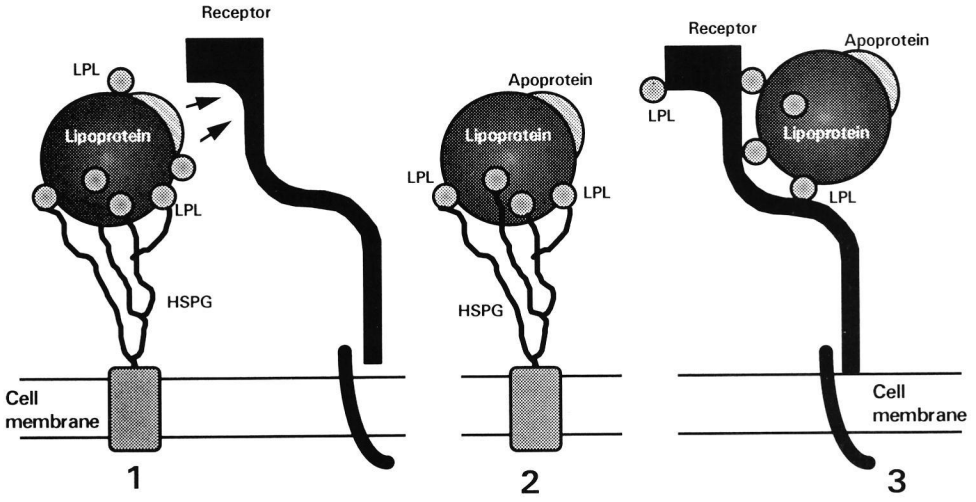

Figure 3. Mechanisms of LPL-mediated uptake of lipoproteins by cells

1. LPL increases binding of lipoproteins to proteoglycans which, in turn, facilitate transfer of the LPL-lipoprotein complex to the receptor for internalisation; 2. LPL-mediated uptake of lipoproteins via heparan sulphate proteoglycans, through a slow but high capacity pathway, involving uptake of the whole lipoprotein-LPL-HSPG complex; 3. LPL enhances directly the binding and uptake of lipoproteins by the receptor.

1.3.3 LPL and atherosclerosis

If the proposed mechanism for LPL-mediated uptake of lipoproteins takes place in the liver, the role of LPL in lipoprotein uptake would be anti-atherogenic due to the clearance from the plasma of atherogenic lipoproteins such as LDL, VLDL and VLDL remnants. On the other hand, in the intima of the vessel wall, LPL may serve as an atherogenic protein by stimulating the uptake of atherogenic lipoproteins by smooth muscle cells and macrophages, leading to foam cell formation. Thus, depending on its location, LPL might have an anti-atherogenic as well as an atherogenic function in the body [53].

There are several lines of evidence that LPL may indeed be important in the metabolic pathways that lead to atherosclerosis. It has been shown that LPL is secreted *in vitro* by both of the predominant cell types present in atherosclerotic lesions, *i.e.* macrophages and smooth muscle cells [61,62,63,64]. Moreover, LPL mRNA and LPL protein were detected in association with macrophage-derived foam cells, endothelial cells, adventitial adipocytes, medial smooth muscle cells, and, to a lesser extent, with intimal smooth muscle cells from rabbit and human atherosclerotic plaques [40,41].

Smooth muscle cells, endothelial cells and macrophages present in the intima also synthesise HSPG, which can bind apoB-containing lipoproteins such as VLDL and LDL [65,66]. As described above, LPL can enhance the interaction of LDL with proteoglycans [67,68,69], which leads to an increased susceptibility of LDL for oxidation because of (i) a longer residence time of LDL-complexes in the intima [67], and (ii) a higher oxidation rate of LDL-HSPG complexes as compared to free LDL [70]. The ability of LPL to enhance LDL retention in vascular tissue has also been shown in *in vivo* experiments by Rutledge *et al* [71]. Increased oxidation of LDL results in an increased lipid accumulation in the macrophages, via the uptake of oxidized LDL by the scavenger receptor [72]. The findings that oxidative stress can enhance the production of LPL by macrophages [73], and that LPL can enhance the uptake of lipoproteins by macrophages *in vitro*, further emphasises the importance of these processes in atherogenesis. Thus, LPL within the vascular tissue, and not on the luminal endothelial surface, potentates atherosclerosis by (i) enhancing the uptake of atherogenic lipoproteins by macrophages and smooth muscle cells, and (ii) stimulating the association of LDL with proteoglycans, leading to enhanced LDL oxidation.

1.4 Proteoglycans

1.4.1 Introduction

Proteoglycans are macromolecules containing one or more glycosaminoglycan (GAG) chains, covalently linked to a protein core. They are widely distributed in all connective tissues and appear to be synthesised by virtually all types of cells, including those of the blood vessel wall. Proteoglycans have various biological functions, ranging from relatively simple mechanical support functions and maintenance of the structure of various connectives tissues, to effects on more dynamic processes such as cell adhesion, differentiation and morphogenesis.

GAG chains are polysaccharide chains composed of repeating disaccharides, and are highly negatively charged due to the presence of sulphate groups and carboxyl groups. They can be divided into six different classes, on the basis of the number and location of their sulphate groups and their sugar residues: 1, hyaluronic acid; 2, chondroitin sulphate; 3, dermatan sulphate; 4, heparan sulphate; 5, heparin; and 6, keratan sulphate. The number of GAG chains on a core protein may vary from 1 to more than 100. For an overview of proteoglycan structure and function, see [74,75,76].

1.4.2 Role of proteoglycans in cellular uptake of lipoproteins

There are several lines of evidence that proteoglycans, and in particular heparan sulphate proteoglycans, play a role in lipoprotein clearance by the liver. It has been postulated that the uptake of remnant lipoproteins by liver is mediated by a so-called secretion-recapture process [11-15,17], involving HSPG. This process is described in Chapter 1.2.1. It has been reported that there is a variable interaction of mutant forms of apoE with HSPG [77]. The mutants associated with a dominant form of type III hyperlipoproteinemia (apoE3-Leiden, apoE(Arg$^{142}\rightarrow$ Cys)) showed a much lower binding affinity for HSPG than mutant forms of apoE associated with a recessive form (apoE2(Arg^{158} $\rightarrow$ Cys)). Thus, according to these and other [78] investigators, a correlation exists between the amount of HSPG-mediated binding and uptake of remnants containing a specific apoE-mutation on one hand, and the mode of expression of type III hyperlipoproteinemia on the other hand, suggesting an important role for HSPG in remnant clearance *in vivo*. The importance of the role of HSPG in remnant clearance *in vivo,* has been shown in studies by Ji *et al* [79]. They

reported that heparinase injections into mice to remove hepatic HSPG, resulted in a decreased remnant clearance by the liver of these mice.

1.4.3 Proteoglycans and atherosclerosis

Proteoglycans are synthesised by macrophages and smooth muscle cells which are present in the intima of the vessel wall. It has been found that cholesterol enrichment of both rabbit aorta smooth muscle cells [80], and pigeon peritoneal macrophages [81] results in an increased production and excessive intracellular accumulation of proteoglycans, indicating that in atherosclerotic lesions proteoglycan production may be increased. Proteoglycans can bind to apoB containing lipoproteins such as LDL, and it has been shown that lipoprotein-proteoglycan complexes are taken up more avidly by macrophages and smooth muscle cells than native lipoproteins [82,83]. Furthermore, Vijayagopal and co-workers have shown that uptake of lipoprotein-proteoglycan complexes induces cholesteryl ester accumulation in human monocytes/macrophages [84] and in rabbit foam cells [66]. In line with this, Seo *et al* [85] showed that heparan sulphate proteoglycans can mediate the uptake and degradation of ß-VLDL by pigeon macrophages, leading to cholesterol accumulation.

There is yet another aspect to the atherogenicity of LDL-binding to intimal proteoglycans. Complex formation of LDL with proteoglycans leads to a prolonged retention time of LDL in the intima, which increases the susceptibility of the particle to oxidation. Furthermore, complexes of LDL with proteoglycans are more susceptible to *in vitro* oxidation than native LDL [70]. Oxidation of LDL leads to foam cell formation via uptake by the scavenger receptor, that is not regulated by intracellular cholesterol levels. The formation and processing of oxidized LDL, and its role in atherogenesis will be discussed in Chapter 1.5.

Although these findings suggest a major contribution of proteoglycans to the formation of foam cells and thus atherogenesis [86], Lookene *et al* [87] reported that the binding of chylomicrons, VLDL and LDL to heparan sulphate proteoglycans, as measured by the surface plasmon resonance technique, is very low. Addition of lipoprotein lipase markedly enhanced the binding of these lipoproteins to the proteoglycans. However, the binding of ß-VLDL to HSPG was substantial, even in the absence of LPL. These results once more demonstrate an important role of lipoprotein lipase, or molecules with similar properties (apoE, hepatic lipase), in the binding of lipoproteins to proteoglycans.

1.5 Modified low density lipoprotein

1.5.1 Introduction

Hypercholesterolemia is most commonly associated with an elevation of plasma LDL, and LDL is the ultimate source of the cholesterol that accumulates in developing foam cells [88]. However, numerous studies indicate that uptake of LDL via the LDL receptor does not lead to the accumulation of cholesteryl esters in smooth muscle cells and macrophages [89,90,25]. The fact that the LDL receptor is regulated by intracellular cholesterol levels, explains these observations: it is downregulated whenever the intracellular cholesterol pools start to increase [91,92,93], and it thus prevents cholesterol-overloading of the cell. Furthermore, patients and animals that completely lack the LDL receptor, do develop foam cells. These findings suggest that the accumulation of cholesteryl esters in cells, and thus the formation of foam cells, occurs via a distinct mechanism. Since foam cells develop *in vivo* when plasma LDL levels are elevated, it is postulated that the LDL particle must be modified before it can contribute to the atherogenic process [72].

Goldstein *et al* [89] first described a modified form of LDL that could rapidly be taken up by macrophages, converting them into foam cells. They found that chemical acetylation of LDL resulted in an enhanced uptake of this modified form of LDL via a novel receptor, called the 'acetyl LDL receptor' or the 'scavenger receptor'. This receptor has a broad ligand specificity, but it does not recognise native LDL. Furthermore, the number of receptors on the cell surface is not regulated by intracellular cholesterol levels. It has particularly been found on sinusoidal endothelial cells in the liver [94], but also on monocyte/macrophages, Kupffer cells [95], fibroblasts [96], and vascular endothelial cells [97,98]. However, there is little or no evidence that acetylation of LDL occurs *in vivo*. Therefore, the search for biological modifications of LDL continued, and since then there have been a number of publications about modifications of LDL which enhance its uptake by macrophages *in vitro*. These include (i) self-aggregation [99], (ii) complex-formation with heparan sulphate proteoglycans [70,82,83], (iii) immune complex formation [100,101,102], and (iv) oxidative modification. The latter is the best studied modification of LDL (for review see [72,103,104,105,106,107]), and leads to foam cell formation via uptake by a receptor not regulated by intracellular cholesterol content.

1.5.2 The hypothetical role of oxidized LDL in atherosclerosis

Oxidation of LDL can be induced by incubation of LDL with endothelial cells [108,109,110,111], smooth muscle cells [112], or macrophages [113,114,115], or by incubation with a heavy metal ion such as copper. In addition, LDL can be oxidized by incubation with lipoxygenase [116].

Oxidation of LDL is a complex process, involving oxidative modifications of both the protein and lipid moiety of LDL. The extent of changes in the LDL particle induced by oxidation, depends on the conditions used and the length of time the particle is exposed to those conditions. For a detailed description of the oxidation process of LDL, finally resulting in the generation of 'new' epitopes on apoB which are recognised by macrophage receptors, see [103,117].

As compared to native LDL, oxidized LDL shows a reduced rate of uptake through the LDL receptor, and an increased rate of uptake and degradation by the scavenger receptor, leading to foam cell formation [72]. Oxidized LDL has an increased negative charge, increased density, increased lysolecithin content, increased content of oxidized forms of cholesterol, and a decreased content of polyunsaturated fatty acids, when compared with native LDL. It furthermore displays fragmentation of apoB100. Finally, oxidized LDL is chemotactic for circulating human monocytes [118], it is cytotoxic (in the absence of serum) [112], and it inhibits the motility of resident macrophages [119].

Some of these properties suggest a pro-atherogenic role for oxidized LDL. The postulated mechanisms of the contribution of oxidized LDL to atherogenesis are shown in Figure 4. In the presence of a high plasma concentration of LDL, the concentration of LDL in the intima also increases. LDL is retained in the intima by binding to connective tissue components *i.e.* proteoglycans. Thereafter, it is oxidized by the present endothelial cells, smooth muscle cells or macrophages. Subsequently, monocytes enter the intima, recruited by the chemotactic factor which is present in oxidized LDL, but absent in native LDL. The recruitment of the monocytes is furthermore induced by monocyte chemoattractant protein-1 (MCP-1), which is expressed by macrophages at an enhanced rate due to the presence of oxidized LDL [120]. Once within the arterial wall, the monocytes differentiate into tissue macrophages. Oxidized LDL inhibits the motility of these resident macrophages, and therefore their ability to leave the intima. The resident macrophages take up oxidized LDL at an increased rate via various scavenger receptors, leading to the formation of foam cells. The activated macrophages furthermore express various enzymes, receptors and inflammatory mediators, that further stimulate the recruitment of cells, and also further increase LDL oxidation [104], resulting in the progression of the

lesion. Finally, the cytotoxicity of oxidized LDL, which has been found to be caused mainly by 7ß-hydroperoxycholest-5-en-3ß-ol [121], leads to loss of endothelial integrity, facilitating the entering of even more monocytes into the intimal space.

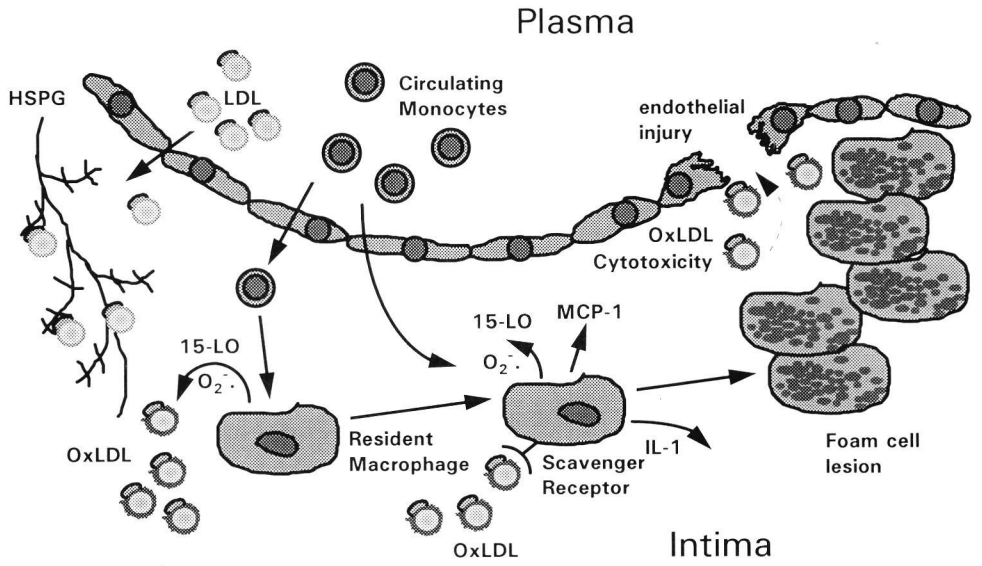

Figure 4. Mechanisms for the role of oxidized LDL in atherogenesis
OxLDL, Oxidized LDL; HSPG, Heparan sulphate proteoglycans; 15-LO, 15 Lipoxygenase; IL-1, Interleukin 1; MCP-1, Monocyte chemotactic protein. See text for explanation.

1.5.3 Evidence for the presence of oxidized LDL *in vivo*

The hypothesis as described above for the formation of fatty streaks mediated by oxidized LDL, is primarily based on the results of cell culture studies, and the main question remains whether oxidized LDL is present *in vivo*. There are several lines of evidence that oxidized LDL does occur *in vivo*. First, it has been demonstrated that oxidized LDL is present in extracts of atherosclerotic lesions from both rabbit and human lesions [122]. In this study it was shown, that the physical, chemical and biological properties of LDL extracted from human and rabbit atherosclerotic lesions, greatly resemble those of *in vitro* oxidized LDL. Furthermore, Hoff and O'Neil [123] found that LDL isolated from aortic atherosclerotic plaques showed an increased susceptibility for aggregation, similar to that of oxidized LDL, but not native LDL. This resulted in an enhanced uptake by macrophages via phagocytotic processes. Second, antibodies directed against oxidized LDL and malondialdehyde-conjugated

LDL, react with materials in atherosclerotic lesions of rabbits [124,125,126]. Third, the presence of autoantibodies against oxidized LDL has been demonstrated in different species [125,127,128,129]. Finally, probably the most important line of evidence is the fact that intervention with anti-oxidants to prevent oxidative modification of LDL, can slow the progression of atherosclerosis in different species [130,131,132,133,134,135,136], although there are some reports which do not show any effect of anti-oxidants on lesion progression [133,134,137,138,139].

1.5.4 Receptors for oxidized LDL

In addition to the scavenger receptor [108], and in particular the scavenger receptor class A type I/type II (SR-AI/II) [140], several other macrophage or Kupffer cell membrane proteins have been described as possible receptors for oxidized LDL. It was found that as much as 30 to 70% of the binding and uptake of oxidized LDL by mouse peritoneal macrophages [141,142] or Kupffer cells [95], could not be competed for with acetyl LDL, indicating there had to be (an)other receptor(s) also mediating the uptake of oxidized LDL. The existence of different receptors recognising acetylated LDL or oxidized LDL, or both, was also shown by Ueda *et al* [143]. Furthermore, studies by Lougheed *et al* [144], showing high affinity, saturable uptake of oxidized LDL by macrophages from SR-AI/II knockout mice, provide direct evidence that a receptor other than the SR-AI/II is responsible for most of the uptake of oxidized LDL in murine macrophages. Several approaches have been used to identify these candidate receptors for oxidized LDL.

Endemann and co-workers used a mouse cDNA library and an expression cloning strategy with fluorescently labelled oxidized LDL. They identified two proteins mediating the uptake of oxidized LDL but not acetylated LDL. The first was found to be the FcγRII-B2 receptor [145]. Although a monoclonal antibody directed against FcγRII-B2 blocked the uptake of oxidized LDL by transfected cells, the same antibody was unable to significantly inhibit the uptake of oxidized LDL by mouse peritoneal macrophages. Therefore, it probably does not contribute to oxidized LDL uptake by macrophages. The second was shown to be the murine homologue of CD36 [146]. It was shown that transfected cells expressing CD36 bind and take up oxidized LDL, however the degradation was very low. Hence, from these experiments the importance of CD36 as receptor for oxidized LDL is not clear. Using an antibody against CD36, Nicholson *et al* [147] showed that the degradation of oxidized LDL by human monocyte/macrophages is reduced by 22%. More quantitative data arise from a study by Nozaki *et al* [148], who found that the binding and uptake of oxidized LDL

by monocyte/macrophages from patients totally lacking CD36, was inhibited by approximately 40% as compared to control monocyte/macrophages. From these data they conclude that CD36 is one of the receptors mediating the binding and uptake of oxidized LDL. However, since the reduction of the binding was only 40% in spite of complete lack of CD36, other proteins may play a role in the uptake of oxidized LDL as well. Another aspect of the role of CD36 in foam cell formation, is the finding that macrophage expression of CD36 mRNA was increased by LDL, acetylated LDL and also by oxidized LDL, with oxidized LDL causing the greatest induction [149]. These data suggest a new mechanism for the contribution of lipoproteins to atherogenesis, by upregulating a major receptor for oxidized LDL.

Another binding protein for oxidized LDL was identified in mouse peritoneal macrophages [150,151] and in Kupffer cells [152]: macrosialin, the mouse homologue of human CD68. The main part of this protein is intracellular, however a significant amount is detected on the cell surface of macrophages [153]. It was found that monoclonal antibodies against CD68, inhibited the uptake and degradation of oxidized LDL by THP-1 cells by 30 to 50%.

Recently, the cDNA of a new scavenger receptor has been cloned [154]. It is a new member of the CD36 family (class B scavenger receptors), named SR-BI. Cell lines transfected with hamster SR-BI, bound modified lipoproteins, such as acetylated LDL and oxidized LDL, but also native LDL and HDL.

Recently, Lougheed and Steinbrecher [155] reported that the degree of LDL oxidation greatly determines the pathway by which it is taken up. Therefore, conclusions regarding the uptake mechanisms of oxidized LDL, which are based on competition experiments between oxidized LDL and other scavenger receptor ligands, should be drawn with caution.

Thus, it seems that different macrophage receptors are involved in the binding and uptake of oxidized LDL. The relative contribution of these different binding sites to the uptake of oxidized LDL by macrophages *in vivo,* remains to be determined.

1.6 Apolipoprotein E

1.6.1 Introduction

Apolipoprotein (apo) E, first described by Shore and Shore [156] is a protein with a relative molecular mass (M_r) of 34.000 It is formed mainly in the liver, but a large concentration of apoE mRNA is also found in the brain [157,158]. Other organs have also been reported to produce apoE, including spleen, lung adrenal, ovary, skin, kidney and muscle [159,160]. In addition, macrophages produce large amounts of apoE, especially in response to peripheral nerve injury [161].

In humans, there is a marked genetic polymorphism of the apoE gene. There are three alleles (E*2, E*3, E*4), the respective proteins differing with regard to only one single amino acid [162,163]. The most frequent allele is E*3, which codes for cysteine and arginine at amino acid position 112 and 158, respectively. In E*4, the cysteine residue in position 112 is replaced by arginine, in E*2 the arginine at position 158 is replaced by cysteine.

1.6.2 Role of apoE in the cellular uptake of lipoproteins

ApoE is the major protein constituent of several lipoproteins, including chylomicrons, VLDL, their remnants, and HDL. An important role for apoE is to mediate the interaction of these lipoproteins with two different receptors, the LDL receptor and the remnant receptor. It therefore plays an important role in the clearance of remnant lipoproteins from the plasma. As described in chapter 1.2.1, clearance of remnants from the circulation is thought to be mediated via a so-called secretion-recapture process, in which the remnants are enriched in liver-derived apoE in the space of Disse. This increases the binding of the remnants to the hepatocytes, in a process also involving HSPG, whereafter they are taken up by the liver via the LDL receptor or the LRP. ApoE is furthermore a ligand for the VLDL receptor [164], thereby probably facilitating LPL-mediated hydrolysis of VLDL-triglycerides and the subsequent uptake of free fatty acids by the adipose tissue, heart, and muscle tissue [165].

The isoforms of apoE differ in their affinity for the LDL receptor. The apoE2 isoform was found to be defective in binding to this receptor [166], while the apoE3 and apoE4 isoforms show high binding affinities for the LDL receptor. Subjects with homozygosity for the E*2 allele display an impaired uptake of remnant lipoproteins by the liver, and approximately 4% of the E2/E2-subjects will develop familial

dysbetalipoproteinemia, mostly at older age [167]. In addition to the difference in binding to the LDL receptor, the isoforms furthermore differ from each other with respect to their affinity for heparan sulphate proteoglycans [77,78], probably explaining the mode of expression of type III hyperlipoproteinemia.

1.6.3 Role of apoE in lipid redistribution

ApoE is also thought to be involved in the local redistribution of lipids among cells within a tissue and in intracellular lipid transport [168]. It also modulates the cellular metabolism of both triglycerides and cholesteryl esters, distinct from its enhancing effect on particle uptake [169]. Furthermore, Lombardi *et al* [170] postulated that apoE might interfere with the efficiency of cellular degradation of VLDL and VLDL remnants. They found a retarded transport to the lysosomal compartment in HepG2 cells of VLDL and VLDL remnants as compared to LDL, and it was hypothesised that the polyvalent binding of apoE to the LDL receptor inhibited the intracellular acid-mediated release of the ligand from the receptor. These results are in accordance with those of Chen *et al* [171], who found that in contrast to apoB, substantial amounts of apoE are not degraded after uptake by J774 macrophages and human fibroblasts.

1.6.4 Role of macrophage-derived apoE on cholesterol homeostasis in the vessel wall

Cholesterol loading of macrophages enhances macrophage apoE synthesis and secretion by these cells [172]. In line with this, it has been found that apoE is abundantly present in atherosclerotic lesions, and the macrophage is the major source of apoE in these lesions [173,174]. Recently, it has been shown that the production of apoE by these macrophages can be stimulated by oxidized LDL [175,176,177], and inhibited by lipoprotein lipase [178]. Cholesterol loading of the cells attenuated the inhibitory effect of LPL on apoE secretion. The precise consequences of these findings have to be further evaluated, however, they stimulated further research into the role of apoE in cholesterol homeostasis of the vessel wall, as reviewed in [179].

Using apoE knockout mice [180,181] as recipients, the effect of bone marrow transplantation from normal mice on lipoprotein profile and atherosclerosis has been studied by different groups [182,183]. Bone marrow transplanted from wild type mice

to apoE knockout mice was demonstrated to reduce circulating cholesterol levels and reduce the extent of atherosclerosis, even though circulating apoE levels were only 10 to 15% of normal. These results indicate that macrophage-derived apoE may play a significant role in the regulation of the cholesterol balance in the vessel wall. However, these studies do not prove a direct protective effect of macrophage-derived apoE at the vessel wall, since the results could also be explained by an effect of apoE on circulating lipoproteins. Strong evidence that macrophage-derived apoE is anti-atherogenic, even in the presence of high levels of circulating atherogenic lipoproteins, was provided by the use of transgenic mice overexpressing high levels of apoE in the arterial wall, on a background of normal hepatic apoE expression [184], and by the use of apoE knockout mice with macrophage-specific expression of human apoE [185]. It was demonstrated that overexpression of apoE in the vessel wall resulted in a resistance to the development of atherosclerosis, in the latter study even in the presence of high levels of plasma lipoproteins. It was concluded that apoE prevents atherosclerosis by promoting cholesterol efflux from the cells of the vessel wall. This was sustained by performing the opposite transplantation, *i.e.* transplantation of apoE null macrophages into control mice [186]. It was found that at the same level of circulating lipoproteins, the mice transplanted with apoE null macrophages developed significantly more diet-induced atherosclerosis than mice transplanted with control macrophages. Thus apoE null macrophages are more prone to become foam cells than wild type macrophages in a similar atherogenic environment, indicating a role of apoE in delaying atherosclerosis by promoting cholesterol efflux from macrophages.

Another anti-atherogenic role for apoE has been postulated by Saxena *et al* [187]. They found that apoE could reduce the lipoprotein lipase-mediated retention of LDL by the subendothelial matrix, and that the amount of reduction was dependent on the apoE isoform [188]. ApoE3 was the most effective in decreasing LDL retention, followed by apoE4; apoE2 was the least effective.

The above shows that apoE has several functions in lipoprotein metabolism, and further research is required to establish the overall role of apoE.

1.7 Outline of this thesis

The finding that lipoprotein lipase (LPL) enhances the uptake of lipoproteins by various cell types including macrophages, suggested that LPL may play a role in the formation of foam cells, and ultimately, in the formation of atherosclerotic plaques. The atherogenicity of oxidized LDL *in vitro,* and its presence in atherosclerotic

plaques *in vivo*, had also already been established. In view of this, we decided to investigate the effect of LPL on the binding and uptake by J774 macrophages of LDL which had been chemically oxidized to different degrees (Chapter 2). At that time, little was known about oxidation of VLDL. We thought it likely that also VLDL, similar to LDL, may undergo oxidative modification *in vivo,* and therefore we found it of physiological relevance to examine the cellular processing of oxidized human VLDL by J774 macrophages (Chapter 3).

The role of apoE in lipoprotein metabolism is further addressed in this thesis. First, in Chapter 4 we investigated the affinity of VLDL particles, containing different apoE-isoforms, for proteoglycans. Also the role of LPL in this process was studied. This was done using both isolated HSPG molecules coated on tissue culture wells, and cell-bound proteoglycans to J774 macrophages.

We also studied the role of apoE in atherogenesis using apoE-deficient mice. These mice display a marked accumulation in the plasma of VLDL lipoproteins which are deficient in both apoE and apoB100. Since apoE-deficient mice develop severe atherosclerotic lesions with lipid laden macrophages, we reasoned that the uptake of lipoproteins by intimal macrophages can take place in the absence of both apoE and apoB100, which are the common ligands for receptor-mediated uptake. To obtain more insight into the mechanism of foam cell formation in apoE-deficient mice, we measured the interaction of apoE-null VLDL with J774 macrophages in the presence and in the absence of LPL (Chapter 5).

The major part of our reports also focus on the bridging function of LPL, by which it enhances the uptake of lipoproteins by cells. For these studies, we use LPL isolated from bovine milk by heparin sepharose chromatography. In Chapter 6, we examined the role of a 37 kDa protein copurifying with LPL during the isolation, which seemed to be obligatory for the bridging activity of the LPL sample.

1.8 References

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Chapter 2

Lipoprotein Lipase Stimulates the Binding and Uptake of Moderately Oxidized Low Density Lipoprotein by J774 Macrophages

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Lipoprotein Lipase Stimulates the Binding and Uptake of Moderately Oxidized Low Density Lipoprotein by J774 Macrophages

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ABSTRACT

Lipoprotein lipase (LPL) stimulates the uptake of low density lipoprotein (LDL) and very low density lipoprotein (VLDL) in different cell types, including macrophages, through bridging of LPL between lipoproteins and extra-cellular heparan sulphate proteoglycans (HSPG). Since macrophages produce LPL and since modified lipoproteins are present in the arterial wall *in vivo*, we wondered whether LPL also enhances the uptake of oxidized LDL by J774 macrophages. LDL samples with different degrees of oxidation, as evaluated by relative electrophoretic mobility (REM) as compared to native LDL are used, as well as native and acetylated LDL. Addition of 5 µg/ml of LPL to the J774 cell culture medium, stimulated the binding of both native LDL and moderately oxidized LDL (REM < 3.5) 50-100-fold, and their uptake was stimulated approximately 20-fold. The LPL-mediated binding of native LDL and moderately oxidized LDL was dose-dependent. Preincubation of the cells with heparinase (2.4 units/ml) inhibited the stimulatory effect of LPL, indicating that this LPL-mediated stimulation was due to bridging between the lipoproteins and HSPG. The binding to J774 macrophages of severely oxidized LDL (REM = 4.3) was stimulated less than 3-fold by LPL, whereas its uptake was not stimulated significantly. The binding and uptake of acetylated LDL (AcLDL) both were not stimulated by LPL, although the LPL-molecule itself does bind to AcLDL. Measurements of the cellular lipid content showed that addition of LPL also stimulated the accumulation in the cells of cholesteryl ester derived from both native LDL and moderately oxidized LDL in a dose-dependent manner. We conclude that our results present experimental evidence for the hypothesis that in the vessel wall, LPL serves as an atherogenic component.

Keywords: Lipoprotein receptors, heparan sulphate proteoglycans, lipoprotein lipase, macrophages, hypercholesterolemia, foam cell formation.

Abbreviations used in this paper: LPL, Lipoprotein Lipase; LDL, Low Density Lipoprotein; VLDL, Very Low Density Lipoprotein; AcLDL, Acetylated Low Density Lipoprotein; OxLDL, Oxidized Low Density Lipoprotein; HSPG, Heparan Sulphate Proteoglycans; FCS, Fetal Calf Serum; HSA, Human Serum Albumin; DMEM, Dulbecco's Modified Eagle's Medium; LPDS, Lipoprotein Deficient Serum; PBS, Phosphate Buffered Saline; REM, Relative Electrophoretic Mobility; CE, Cholesteryl Ester.

INTRODUCTION

It has been shown that the enzyme lipoprotein lipase (LPL) enhances the cellular binding and uptake of very low density lipoproteins (VLDL) and low density lipoproteins (LDL) by different cell types, including HepG2 cells, fibroblasts and THP-1 monocytes and macrophages. The increased binding and uptake of LDL and VLDL is due to the bridging of LPL between the lipoproteins and the heparan sulphate proteoglycans (HSPG) which are present on the plasma membrane [1-4].
If the proposed mechanism for LPL-mediated uptake of lipoproteins takes place in the liver, the role of LPL in lipoprotein uptake would be anti-atherogenic due to the enhancing of the hepatic uptake of atherogenic lipoproteins such as LDL, VLDL and VLDL remnants. On the other hand, in the intima of the vessel wall, LPL may serve as an atherogenic protein by stimulating the uptake of atherogenic lipoproteins by smooth muscle cells and macrophages, leading to foam cell formation. Thus LPL might have a dual function, depending on its location [5].
There are several reports showing that in the arterial wall LPL is associated with the atherosclerotic process. It has been reported that macrophages in atherosclerotic plaques synthesize LPL and that the concentration of LPL in the vessel wall is related to the concentration of cholesterol in the vessel wall [6]. Furthermore, smooth muscle cells, endothelial cells and macrophages present in the intima synthesize HSPG, depending on the amount of intracellular cholesterol ester which has accumulated [7]. Cellular HSPG can bind apo-B containing lipoproteins, after which these lipoprotein-proteoglycan complexes can be taken up by macrophages mainly via a receptor-mediated pathway [8,9] and result in the formation of foam cells. Edwards *et al* [10] suggest that in the atherosclerotic artery, the LPL produced by macrophages and smooth muscle cells binds to the proteoglycans, thereby increasing the interaction of LDL with proteoglycans. Complex formation of LDL with HSPG leads to an increased oxidation of LDL, because of (i) a longer residence time of LDL-complexes in the intima [11] and (ii) a higher oxidation rate of LDL-HSPG complexes as compared to

free LDL [12]. Subsequently, increased oxidation of LDL results in an increased lipid accumulation in the macrophages, via the uptake of oxidized LDL by the scavenger receptor. Furthermore, Ylä-Herttuala *et al* [13] have shown that oxidized LDL is indeed present in atherosclerotic lesions *in vivo*.

All these facts together, point to an important role for LPL in the atherosclerotic process. However, no studies have yet been made to determine whether LPL might also stimulate the binding and uptake of oxidatively modified LDL by macrophages directly. It also remains to be determined whether a stimulation of the binding of native LDL and possibly oxidized LDL to macrophages is accompanied by an enhanced accumulation of cholesteryl esters in these cells. In the present study, we found that in J774 macrophages, LPL indeed enhances the cellular binding and uptake of LDL that is partly oxidized. However, the uptake of severely oxidized LDL and acetylated LDL is not stimulated by the presence of LPL. We also found that LPL stimulated the cellular cholesteryl ester content in a dose-dependent manner after incubation of the J774 macrophages with both native LDL and moderately oxidized LDL. Hence, our findings provide experimental evidence for the hypothesis that in the vessel wall, LPL serves as an atherogenic factor.

MATERIALS AND METHODS

Cells

Murine macrophage-like J774 cells were cultured in 75 cm^2 flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 0.85 g/L $NaHCO_3$, 4.76 g/L Hepes, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamin. The cells were incubated at 37°C in an atmosphere containing 5% CO_2 in air. For each experiment, cells were plated in 12-well plates. The cells were fed every 3 days, and used for experiments within 7 days after plating. Twenty-four hours prior to each experiment, the cells were washed with DMEM/1% HSA and further incubated with DMEM containing 5% (v/v) of lipoprotein deficient serum (LPDS) instead of FCS.

Lipoproteins

Blood was obtained from healthy volunteers. Serum was separated from the cells by centrifugation at 3000 rpm for 10 minutes. LDL was isolated from the serum by density gradient ultracentrifugation according to Redgrave *et al* [14]. The protein content of the LDL fraction was determined using the method of Lowry *et al* [15]. After isolation, a part of the LDL sample was oxidized or acetylated as described below. LDL samples were iodinated, using the ^{125}I-monochloride method described by Bilheimer *et al* [16]. The specific activity ranged from 50 to 300 cpm/ng of protein. After iodination, the LDL samples were extensively dialysed against phosphate buffered saline (PBS, pH 7.4), stored at 4°C and used within 2 weeks.

Whenever unlabelled lipoproteins were used, immediately after isolation, dialysis against PBS and, subsequently, DMEM was performed at 4°C.

Lipoprotein deficient serum was prepared by ultracentrifugation of the serum at d = 1.21 g/ml and removal of the lipoprotein-containing supernatant, followed by extensive dialysis against PBS.

Modification of LDL

Oxidation. LDL was oxidized by using $CuSO_4$. LDL (600 µl, 0.25 µg/ml), $CuSO_4$ (22.5 µl, 1.6 mM) and PBS (277.5 µl) were incubated at 37°C. Various degrees of oxidation were obtained by inhibiting the oxidation reaction at different time points, using an excess of EDTA after 4 hours, 8 hours, 16 hours or 24 hours of oxidation. Immediately after oxidation, lipoproteins were dialysed against PBS at 4°C. The degree of oxidation was visualized by means of agarose gel electrophoresis (100 V, 30 minutes, Paragon Lipoprotein Electrophoresis kit, Beckman Instruments), as shown in Figure 1. Subsequently, the electrophoretic mobility relative to native LDL (REM) of the different oxidized LDL fractions (and VLDL and HDL as references) was determined.

Acetylation. LDL with known concentration (A ml, B mg of protein) was acetylated using an equal volume (A ml) of saturated sodium acetate and 1.5 x B µl acetic anhydride. The latter was added in portions of 2 µl for a period of 60 minutes, with continuous stirring on ice, and then extensively dialysed against PBS. The conversion of LDL to acetylated LDL (AcLDL) was confirmed by agarose gel electrophoresis (Figure 1), and the REM was determined subsequently.

Lipoprotein lipase

LPL was partially purified from fresh bovine milk using heparin sepharose chromatography. After centrifugation (Sorvall GSA rotor, 30 minutes, 8000 rpm, 4°C), the skimmed milk was filtered and adjusted to 0.4 M NaCl. Heparin-Sepharose (CL-6B, Pharmacia), swollen according to the manufacturer's protocol, was equilibrated with 0.4 M NaCl and added to the milk. The mixture was shaken gently for 3 hours at 4°C. After washing with 0.5 M NaCl and subsequently with 0.75 M NaCl, 10 mM KH_2PO_4, pH 6.8, the mixture was applied to a column and washed with 0.75 M NaCl, 10 mM KH_2PO_4, pH 6.8 at a flow rate of 0.3 ml/min. Thereafter, 1.5 M NaCl, 10 mM KH_2PO_4, pH 6.8 was used as an eluent buffer at a flow rate of 1.5 ml/min. LPL-containing fractions were pooled and an equal volume of 10 mM KH_2PO_4, pH 6.8 was added. The pooled fractions were applied to a 5 ml HiTrap Heparin affinity column (Pharmacia) equilibrated with 0.75 M NaCl, and eluted using a linear gradient of 0.75 M to 2 M NaCl, 10 mM KH_2PO_4, pH 6.8 at a flow rate of 1 ml/min. The LPL-containing fractions were pooled and dialysed against 3.6 M $(NH_4)_2SO_4$, pH 6.8. The precipitated protein was collected after centrifugation (Sorvall SS-34 rotor, 30 minutes, 20000 rpm, 4°C), resuspended in 1-2 ml 20 mM NaH_2PO_4, 50 % glycerol and stored in aliquots at -80°C.
Prior to the experiments LPL was heat-inactivated by incubation in a water bath at 56°C for 30 minutes.

Binding and uptake of lipoproteins by J774 macrophages

J774 macrophages were cultured in 12-well plates as described above. Twenty-four hours before the start of the experiment, DMEM supplemented with 5% (v/v) of LPDS instead of FCS was added to the cells. The binding of ^{125}I-labelled LDL, oxidized LDL and acetylated LDL to the cells in the absence or in the presence of the indicated amounts of LPL, was determined after a 3 hour incubation at 4°C with 10 µg/ml of ^{125}I-labelled lipoprotein, either in the presence or in the absence of a 20-fold excess of unlabelled lipoprotein. The receptor mediated (specific) cell-binding was calculated by subtracting the amount of labelled lipoproteins that was cell-bound after incubation in the presence of a 20-fold excess of unlabelled lipoprotein (aspecific) from the amount of labelled lipoprotein that was cell-bound

after incubation in the absence of unlabelled lipoprotein (total cell binding). After removing the medium the cells were washed 4 times with ice-cold PBS containing 0.1% (w/v) of bovine serum albumin (BSA), and subsequently with PBS without BSA. Cells were then dissolved in 1 ml of 0.2 N NaOH. Protein content was measured according to Lowry *et al* [15]. In an aliquot, the radioactivity represented the amount of cell-bound lipoprotein.
To measure the effect of LPL on the association and degradation of ^{125}I-labelled LDL, oxidized LDL and acetylated LDL, cells were incubated for 3 hours at 37°C with 10 µg/ml ^{125}I-labelled lipoprotein either in the absence or in the presence of 5 µg/ml LPL. At the end of the incubation period, a fraction of the medium was removed to determine the amount of lipoprotein degraded as described previously [17,18]. After removing the rest of the medium, the cells were washed four times with ice-cold PBS/BSA 0.1% (w/v), and subsequently with PBS without BSA. The cell-associated (bound plus internalized) lipoprotein fraction was determined exactly as described previously [18]. In the respective figures, lipoprotein uptake is expressed as the sum of cell-associated and degraded lipoproteins.
Treatment with heparinase was performed by preincubating the cells for 2 hours in the presence of 2.4 units/ml heparinase (Sigma, catalog number H2519) at 37°C prior to the experiment. The 2.4 units/ml heparinase were also present during the 3 hours of incubation of the cells with labelled lipoproteins in order to prevent regeneration of HSPG on the cellular membrane during the experiment.

Binding of lipoproteins to plastic tissue culture wells
After iodination, the lipoproteins were dissolved in DMEM/1% HSA and incubated for 3 hours in plastic tissue culture wells without cells at 4°C, either in the presence or in the absence of 5 µg/ml of LPL. The wells had been incubated with DMEM/1% HSA 24 hours prior to the experiment. After 3 hours, the binding of the lipoproteins to the plastic was measured as described above for binding to the cells.

Determination of the cellular lipid content
J774 cells were cultured in 6-well plates as described above. Twenty-four hours before the start of the experiment, DMEM supplemented with 5% (v/v) of LPDS instead of FCS was added to the cells. At the start of the experiment, fresh DMEM media containing 1% HSA (v/v) and 100 µg/ml of lipoprotein either in the presence or in the absence of the indicated amounts of LPL were added in triplicate dishes of cells and incubated for 24 hours at 37°C. Control incubations were performed with DMEM/1% HSA (v/v) without any further additions. At the end of the incubation period, the cells were washed four times with 1.5 ml of PBS containing 0.1% (w/v) of BSA, followed by one wash with PBS. Intracellular lipid content was determined as described by Havekes *et al* [19]. Briefly, the cells were harvested by scraping with a rubber policeman and resuspended by three successive slow passages through a syringe needle (G25). Samples (100 µl) were taken for measurement of protein according to Lowry *et al* [15]. Lipids were extracted from the cell suspension using methanol/chloroform (2:1, v/v) as described by Bligh and Dyer [20], after addition of cholesteryl acetate (2 µg) as an internal standard. The lipids were separated using high performance thin layer chromatography (HPTLC) and subsequently, the lipid bands were quantified densitometrically.

RESULTS

Effect of LPL on the binding and uptake of lipoproteins

LDL was oxidized to different degrees by incubation with $CuSO_4$ during different periods. For all experiments presented in this paper, LDL was isolated from one and the same subject in order to standardize the oxidation procedure. In Figure 1 it is shown that on agarose gel, the electrophoretic mobility of LDL increases with oxidation time. AcLDL, VLDL and HDL are shown as reference samples. The relative electrophoretic mobilities (REM) of the different lipoprotein samples, as compared to native LDL, are presented in Figure 1.

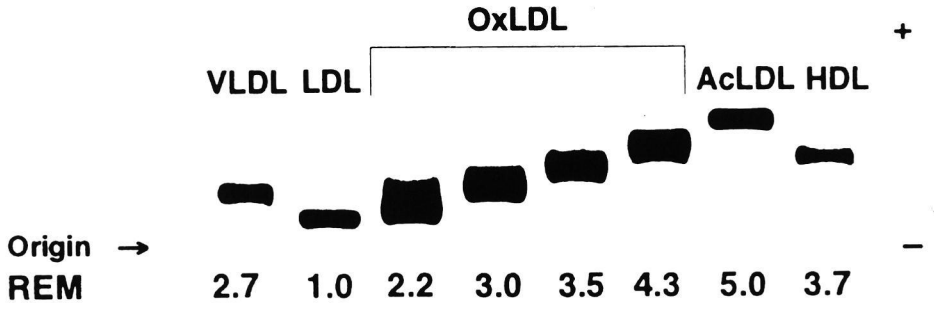

Figure 1 Agarose gel electrophoresis of different lipoproteins
LDL was oxidized or acetylated as described under 'Materials and Methods'. Native LDL, HDL and VLDL are shown as reference samples. The electrophoretic mobility relative to native LDL (REM) of each lipoprotein fraction was determined, by measuring the distance from the origin to the centre of each band. REM increases with increasing oxidation times. Oxidized LDL having an REM of 2.2, 3.0, 3.5 and 4.3 represent LDL which has been oxidized by $CuSO_4$ for 4, 8, 16 and 24 hours, respectively.

As can be seen in Table 1, 5 μg/ml of LPL stimulated both the binding and uptake by J774 macrophages of native LDL and oxidized LDL having an REM of 2.2 and 3.0 (OxLDL2.2 and OxLDL3, respectively). The binding of these lipoprotein fractions was stimulated 50- to 100-fold, and the uptake approximately 20-fold upon the addition of LPL. In contrast, the binding of oxidized LDL having REMs of 3.5 and 4.3 (OxLDL3.5 and OxLDL4.3) was stimulated by LPL only by a factor 6 and 3, respectively, whereas their uptake was not stimulated significantly. Also the binding and uptake of AcLDL were hardly stimulated by LPL, or not at all.

Table 1 **The effect of 5 µg/ml LPL on the binding and uptake of different ^{125}I-labelled lipoproteins by J774 cells**

	Binding *(ng/mg cell protein)*		Uptake *(ng/mg cell protein)*	
Lipoprotein	- LPL	+ LPL	- LPL	+ LPL
LDL	18.8 ± 5.9	1061.7 ± 80.6	39.2 ± 13.4	773.5 ± 91.7
OxLDL2.2	9.4 ± 7.1	991.1 ± 42.3	40.4 ± 15.9	744.5 ± 199.8
OxLDL3	14.5 ± 4.8	842.0 ± 79.0	36.2 ± 15.0	770.7 ± 75.2
OxLDL3.5	110.9 ± 12.5	651.0 ± 143.0	821.0 ± 285.0	988.2 ± 404
OxLDL4.3	154.2 ± 32.1	407.3 ± 71.8	1179.3 ± 236.1	1433.1 ± 202.1
AcLDL	217.3 ± 36.2	304.4 ± 12.5	5082.5 ± 382.4	5000.1 ± 646.4

Lipoprotein binding and uptake (expressed as cell-associated plus degraded lipoprotein) were measured after a 3 hour incubation of the cells with 10 µg/ml of labelled lipoproteins at 4°C and 37°C, respectively, either in the absence or in the presence of 5 µg/ml of LPL. Specific binding and uptake are expressed as ng labelled lipoprotein per mg of cell protein and were determined as described under 'Materials and Methods'. The values represent the mean ± SD of triplicate experiments.

As has been shown before for fibroblasts [3], THP-1 macrophages [2] and HepG-2 cells [1,4], the LPL-mediated binding of LDL and OxLDL2.2 by J774 cells could, at least partly, be prevented by pretreating the cells with heparinase (Figure 2). This indicates that also in J774 cells, the stimulating effect of LPL is due to its bridging between HSPG and the lipoprotein particles. We wondered whether LPL stimulates the binding of lipoproteins to cells in a dose-dependent way. Therefore, the LPL-mediated binding of native LDL, moderately oxidized LDL and severely oxidized LDL to J774 cells was determined at concentrations of LPL of 0, 1.25, 2.5 and 5 µg/ml. Figure 3 shows that increasing concentrations of LPL result in an increasing stimulation of the binding to J774 macrophages of both native LDL and moderately oxidized LDL (OxLDL2.2). For native LDL this dose-dependency is slightly stronger than for OxLDL2.2. The binding of severely oxidized LDL (OxLDL4.3) to J774 cells was not stimulated by LPL, at any LPL concentration applied.

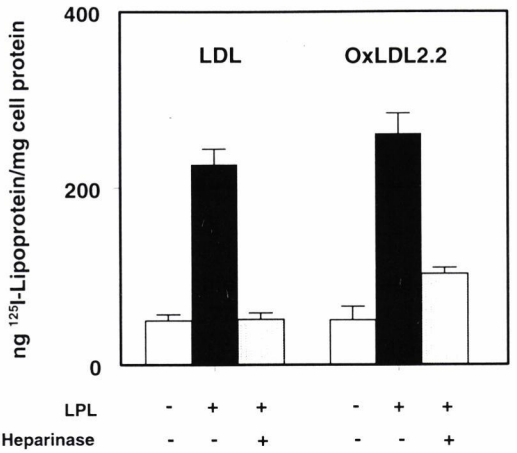

Figure 2 The effect of heparinase on LPL-mediated binding to J774 cells of ^{125}I-LDL and ^{125}I-OxLDL2.2

The cells were incubated for 3 hours at 4°C with 10 µg/ml ^{125}I-LDL or ^{125}I-OxLDL2.2 in the absence (open bars) or in the presence (solid bars) of 5 µg/ml LPL. For the heparinase treatment, the cells were preincubated for 2 hours with 2.4 units/ml heparinase at 37°C (hatched bars). The heparinase was also present during the 3 hour incubation with labelled lipoproteins in order to prevent regeneration of HSPG. The presence or absence of LPL and heparinase is also indicated by + and -, respectively. Specific binding is determined as described under 'Materials and Methods'. The values represent the mean ± SD of triplicates.

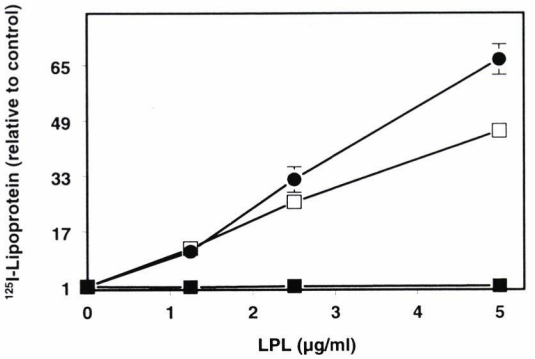

Figure 3 Dose-response relationship of LPL-mediated binding of different ^{125}I-labelled lipoproteins by J774 cells

Lipoprotein binding was measured after a 3 hour incubation of the cells at 4°C with 10 µg/ml of ^{125}I-LDL (●), ^{125}I-OxLDL2.2 (□) or ^{125}I-OxLDL4.3 (■) in the presence of 0, 1.25, 2.5 or 5 µg/ml of LPL. The amount of lipoprotein bound in the presence of LPL is indicated relative to the amount of lipoprotein bound in the absence of LPL. The amount of lipoprotein bound in the absence of LPL was taken as 1 (control value). The values of the binding in the absence of LPL are 13.2 ± 4, 14.5 ± 0.8 and 366 ± 27.1 ng/mg cell protein for LDL, OxLDL2.2 and OxLDL4.3 respectively. Specific binding is expressed as ng labelled lipoprotein per mg cell protein and is determined as described under 'Materials and Methods'. The values represent the mean ± SD of triplicate experiments.

The results presented in Table 2 show that LPL stimulated the binding of native LDL to plastic, by bridging between the plastic and the LDL particle. Furthermore, the binding to plastic of differently oxidized LDL and of AcLDL was also stimulated by LPL, although longer oxidation times resulted in a less pronounced stimulation.

Table 2 **Effect of LPL on the binding of different ^{125}I-labelled lipoproteins to plastic**

	Lipoprotein *(ng/well)*					
	LDL	OxLDL2.2	OxLDL3	OxLDL3.5	OxLDL4.3	AcLDL
-LPL	0.5 ± 0.2	0.6 ± 0.2	0.9 ± 0.2	1.0 ± 0.6	1.1 ± 0.1	1.3 ± 0.7
+LPL	640 ± 13	584 ± 17	420 ± 12	266 ± 7	36 ± 3	466 ± 10

Cell culture wells without cells were incubated with DMEM/1% HSA 24 hours prior to the experiment. The wells were thereafter incubated for 3 hours with 10 µg/ml ^{125}I-labelled lipoproteins dissolved in DMEM/1% HSA at 4°C, in the presence or in the absence of 5 µg/ml of LPL. Binding of the lipoproteins to the plastic of the well is expressed as ng lipoprotein/well and measured as described in 'Materials and Methods'. Values are expressed as mean ± SD of triplicate experiments.

Effect of LPL on the accumulation in J774 cells of cholesteryl esters derived from native and modified LDL samples

The effect of LPL on the cellular cholesterol content in J774 macrophages was determined after incubation of the cells with LDL, OxLDL2.2, OxLDL4.3 and AcLDL. Under all conditions applied, the cellular free cholesterol content did not change significantly (data not shown). The results presented in Table 3 demonstrate that the cholesteryl ester (CE) accumulation in J774 macrophages after incubation of the cells with 100 µg/ml of LDL, is significantly increased in the presence of LPL, in a dose-dependent way. Similarly, LPL also significantly enhanced the accumulation of CE after incubation of the cells with OxLDL2.2. As expected, incubation of the J774 cells with 100 µg/ml of OxLDL4.3 and AcLDL resulted in a rather dramatic increase in cellular cholesteryl ester content, which was not significantly influenced by simultaneous addition of LPL.

Table 3 **Effect of LPL on the cholesteryl ester content in J774 cells after incubation with different lipoproteins**

	Cholesteryl ester accumulated (µg per mg cell protein)				
LPL (µg/ml)	Control	LDL	OxLDL2.2	OxLDL4.3	AcLDL
0	0.45 ± 0.03	0.93 ± 0.08	0.83 ± 0.06	8.83 ± 0.17	8.78 ± 0.63
5	N.D.	1.80 ± 0.39*	1.03 ± 0.28	8.38 ± 0.46	11.04 ± 2.79
10	N.D.	2.49 ± 0.33*	1.45 ± 0.24*	N.D.	N.D.

Cellular cholesteryl ester content was measured after a 24 hour incubation at 37°C with 100 µg/ml of lipoprotein in the absence or in the presence of the indicated amounts of LPL. Control incubations were performed in DMEM/1% HSA without any further additions. Cholesteryl ester content is expressed as µg CE per mg of cell protein and was determined as described under 'Materials and Methods'. The values represent the mean ± SD of triplicates. * indicates a significant difference between CE content after incubation in the presence of LPL and in the absence of LPL ($P < 0.05$, Student's t-test). N.D.: not detected.

DISCUSSION

It is known that LPL stimulates the uptake of VLDL and LDL in different cell types, including HepG2 cells, fibroblasts and THP-1 monocytes and macrophages, due to a bridging by LPL between LDL and HSPG [1-4]. It is also known that oxidized LDL is present in atherosclerotic lesions *in vivo*, and that it causes lipid accumulation in macrophages [21, 22, 23, 24]. Since cells of the arterial wall, including macrophages, have been shown to produce and secrete LPL [6], we wondered whether LPL could also stimulate the uptake of oxidized LDL by macrophages, and whether that leads to increased cellular cholesteryl ester levels. Such an effect would imply that in the vessel wall, LPL serves as an atherogenic factor.

In the present study, we showed that also in J774 cells LPL stimulates the binding and uptake of both native LDL and moderately oxidized LDL 20-100-fold, due to a bridging of LPL between HSPG and the lipoproteins. On the contrary, the binding by J774 cells of severely oxidized LDL (REM ≥ 3.5) and acetylated LDL (REM = 5) was stimulated only minimally by LPL, whereas the subsequent uptake of these lipoproteins was not stimulated at all upon the addition of LPL. In accordance with this, Obunike *et al* [2] found that in THP-1 macrophages, LPL caused a 3-fold increase in the binding of acetylated LDL, whereas LPL did not increase the degradation of acetylated LDL.

The absence of an LPL-mediated stimulation of the binding and uptake of severely oxidized LDL can be only partly explained by a defective binding of LPL to these

lipoproteins, as indicated by the fact that increasing degrees of oxidation resulted in decreasing ability of LPL to stimulate the binding of these lipoproteins to plastic (Table 2). This hypothesis is supported by the fact that apoB contains LPL-binding sites [25] that could be damaged by fractionation of apoB upon oxidation [26]. However, the fact that the binding of severely oxidized LDL (REM = 3.5) to plastic was still stimulated some 200-fold by LPL, indicates that additional but unknown mechanisms underlay the absence of an LPL-mediated stimulation of the binding of severely oxidized LDL.

The lack of an LPL-mediated cellular binding and uptake of AcLDL, can not be explained by a defective binding of LPL to AcLDL, since LPL was able to stimulate its binding to plastic, in a similar way as that of native LDL. We propose the following mechanism for the lack of an LPL-mediated binding and uptake of AcLDL and OxLDL3.5: LPL enhances the binding and uptake of LDL and moderately oxidized LDL (REM $\leq$ 3) by forming a bridge between the lipoprotein and the negatively charged HSPG, which are present on the plasma membrane. However, due to the pronounced negative charges of both OxLDL3.5 and AcLDL (REM = 5), the formed complexes of LPL with these lipoproteins may not be able to bind to the negatively charged HSPG because of electrostatic repulsion forces.

In order to extrapolate the present results of LPL-mediated binding and uptake of lipoproteins to the *in vivo* situation in the vessel wall, it is necessary to speculate about the degree of oxidation of LDL in the atherosclerotic plaque. Steinbrecher and Lougheed [27] reported that LDL isolated from plaques or fatty streaks exhibited variable but usually only modest signs of oxidative change, including slightly increased electrophoretic mobility. Morton *et al* [28] described an LDL-sized lipoprotein particle isolated from homogenates of human aortic atherosclerotic plaques, which migrated with a pre-beta electrophoretic mobility similar to that of VLDL (REM about 2). Similarly, Ylä-Herttuala *et al* [13], also isolated lesion LDL having a relative electrophoretic mobility of 2 as compared to plasma LDL. These facts together suggest that the *in vivo* presence of severely oxidized LDL having a relative electrophoretic mobility of more than 3, is highly unlikely. Thus, extrapolating the results obtained with severely oxidized LDL and AcLDL to the *in vivo* situation regarding the effect of LPL, would not be appropriate.

The LPL-mediated binding of native LDL and moderately oxidized LDL was dose-dependent and occurred already at LPL-concentrations of about 1 µg/ml. According to Babirak *et al* [29] the LPL mass in postheparin plasma of normal controls was approximately 200 ng/ml. Several other groups reported that macrophages synthesize LPL, and that the amount is related to the amount of intracellular cholesterol [6]. Ylä-Herttuala *et al* [30] showed that in atherosclerotic lesions, LPL protein is especially high in macrophage-rich intimal regions. Furthermore, Goldberg *et al* [31] showed that

in addition to synthesis and secretion of LPL activity, monocyte-derived macrophages have LPL attached to their cell membranes. This suggests that the local concentration of LPL in atherosclerotic lesions may be much higher than the LPL concentrations found in plasma after heparin injection. Hence, the results obtained in the present study may be relevant for the situation in the intima *in vivo*.

Although, even in the presence of LPL, the uptake of native LDL and moderately oxidized LDL is low as compared to that of severely oxidized LDL and acetylated LDL (Table 1), our results indicate that the LPL-mediated binding and uptake by J774 cells of both native LDL and moderately oxidized LDL lead to a stimulation of cholesteryl ester accumulation in these cells (Table 3). From our results we conclude therefore, that further experimental evidence is given for the hypothesis that LPL plays an important role in the formation of foam cells, a process which is considered to be one of the initial steps in atherogenesis.

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Chapter 3

Oxidized Very Low Density Lipoprotein Induces Triglyceride Accumulation in J774 Macrophages via Receptor-Mediated Uptake and Not via Extracellular Lipolysis

Submitted

Oxidized Very Low Density Lipoprotein Induces Triglyceride Accumulation in J774 Macrophages via Receptor-Mediated Uptake and not via Extracellular Lipolysis

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ABSTRACT

We investigated the interaction of oxidatively modified very low density lipoprotein (OxVLDL) with J774 macrophages. VLDL was oxidized for maximally 4 hours, resulting in an increase in thiobarbituric acid-reactive substances (TBARS) and an increased electrophoretic mobility on agarose gel. The lipid composition of the VLDL samples did not change during oxidation. The uptake of ^{125}I-labelled VLDL by the J774 cells increased with oxidation time. Despite the increased uptake of OxVLDL protein, the cell association of triglyceride (TG)-derived fatty acids by the J774 macrophages was less pronounced after incubation with OxVLDL than with native VLDL. Also the *de novo* synthesis of TG by the cells, as measured by the incorporation of [^{3}H]glycerol, was approximately three-fold less efficient after incubation with OxVLDL than after incubation with native VLDL. In the presence of polyinosinic acid (PolyI), the receptor-mediated uptake of OxVLDL is completely blocked, indicating that OxVLDL is taken up by the cells via the scavenger receptor. Upon incubation with 100 µg/ml of OxVLDL-TG, the endogenous *de novo* TG synthesis in J774 cells is increased 10-fold as compared to that in the absence of OxVLDL. This OxVLDL-induced synthesis of TG is fully prevented upon simultaneous incubation with PolyI. This indicates that OxVLDL induces endogenous TG synthesis only through uptake of OxVLDL via the scavenger receptor, and not via extracellular lipolysis of OxVLDL-TG, as is the case for native VLDL. *In vitro* lipolysis experiments using purified bovine lipoprotein lipase (LPL), showed that the suitability of VLDL as a substrate for LPL decreases with oxidation time. Thus, upon oxidation, VLDL becomes less efficient in inducing TG-accumulation in J774 cells, as a consequence of an impaired extracellular lipolysis.

Keywords: Oxidized VLDL, lipid accumulation, lipolysis, macrophages

Abbreviations used in this paper: TG, Triglyceride; CE, Cholesteryl Ester; PL, Phospholipid; FFA, Free Fatty Acid; LPL, Lipoprotein Lipase; OxVLDL, Oxidized Very Low Density Lipoprotein; DMEM, Dulbecco's Modified Eagle's Medium; FCS, Foetal Calf Serum; BSA, Bovine Serum Albumin; PBS, Phosphate Buffered Saline; TBARS, Thiobarbituric Acid Reactive Substances; REM, Relative Electrophoretic Mobility.

INTRODUCTION

The role of triglyceride (TG)-rich lipoproteins in the development of atherosclerosis is currently under investigation. Several groups of investigators have shown that human VLDL is capable of inducing cholesteryl ester and TG accumulation in different macrophage culture systems [1-4]. It has been postulated that the mechanisms by which VLDL stimulates cellular lipid accumulation involve two different pathways [2,4]: (i) the receptor-mediated uptake of intact VLDL particles and (ii) the direct uptake of free fatty acids (FFA) as generated by the extracellular lipoprotein lipase (LPL)-mediated hydrolysis of VLDL-TG, followed by intracellular re-esterification into TG. The resulting cholesterol-enriched remnant particles are thereafter taken up via a receptor-mediated process.

In analogy with LDL [5,6], it has been shown that *in vitro* exposure of ß-VLDL to endothelial cells causes oxidation of this lipoprotein [7,8]. This results in a two- to threefold increased degradation by mouse peritoneal macrophages [7] and rabbit smooth muscle cells [8] as compared to unoxidized ß-VLDL, and in a two- to threefold increased intracellular cholesterol esterification rate. Isolated human VLDL was shown to be effectively oxidized *in vitro* upon incubation with free radicals, in a similar manner to human LDL [9]. The degradation of oxidized human VLDL (OxVLDL) by mouse peritoneal macrophages was also increased twofold when compared with native VLDL [10]. Furthermore, it was shown that the uptake of human OxVLDL by macrophages was competitively inhibited by an excess of acetyl LDL, indicating that the cellular uptake of OxVLDL is mediated by the macrophage scavenger receptor [10]. Thus, from these results it has been suggested that oxidation of VLDL may augment its atherogenic potential and may contribute to foam cell formation in humans.

However so far, little is known about the effect of oxidized VLDL on the accumulation of lipids in macrophages. Therefore, in the present study we examined the processing of oxidatively modified forms of human VLDL in the murine macrophage cell line J774. Despite the enhanced cellular (protein) uptake, we found that OxVLDL induces less lipid accumulation in these macrophages than native VLDL. The difference between OxVLDL

and normal VLDL in this respect, appeared to be due to an impaired LPL-mediated extracellular lipolysis of OxVLDL-TG.

MATERIALS AND METHODS

Cells

Murine macrophage-like J774 cells were cultured in 75 cm^2 flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 0.85 g/L $NaHCO_3$, 4.76 g/L Hepes, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM glutamin. The cells were incubated at 37°C in an atmosphere containing 5% CO_2 and 95% air. For each experiment, cells were plated in 6- or 24-well plates. The cells were fed every 3 days, and used for experiments within 7 days after plating. Twenty-four hours prior to each experiment, the cells were washed with DMEM containing 1% (w/v) bovine serum albumin (BSA) and further incubated with DMEM containing 5% (v/v) of lipoprotein deficient serum (LPDS d < 1.21 g/ml) instead of FCS. The experiments were subsequently conducted in DMEM containing 1% (w/v) of BSA.

Lipoproteins

Human VLDL was isolated according to Redgrave *et al* [11] by density gradient ultracentrifugation of pooled plasma obtained from healthy normolipidemic volunteers after an overnight fast. Immediately after isolation, the VLDL fractions were extensively dialysed against phosphate buffered saline (PBS), containing 10 µM EDTA, at 4° C. The protein content of the VLDL samples was determined using the method of Lowry *et al* [12].

After isolation, part of the VLDL sample was labelled with glycerol tri[1-^{14}C]oleate (Amersham, specific activity 61 mCi/mmol)) as described by Groener *et al* [13]. In brief, 5 µCi glycerol tri[1-^{14}C]oleate was added to 12.8 µl phosphatidylcholine (100 mg/ml) and 10 µl butylated hydroxytoluene in chloroform (1 mM). After the evaporation of the chloroform under a stream of nitrogen, 1 ml of 50 mM Tris/HCl, pH 7.5 containing 0.01% of EDTA was added. The suspension was sonicated twice for 5 minutes under nitrogen, using a Labsonic 1510 sonicator. The sonicated lipids were added to a mixture of 6.24 ml of lipoprotein deficient serum (LPDS), 0.22 ml 5% EDTA and 0.94 ml 10 mM 5,5-dithiobis(2-nitrobenzoic acid) (DTNB). Subsequently, 5 mg of VLDL-TG was added and the mixture was incubated for 40 hours at 37 °C. The VLDL was re-isolated by ultracentrifugation as described above. The specific activity ranged from 1500 to 2500 dpm/µg TG.

Oxidation of labelled and unlabelled VLDL (d< 1.006 g/ml, 0.5 mg/ml) was performed by the addition of a 16 mM aqueous solution of the thermolabile peroxyl radical generator 2,2'-azobis(2-amidino-propane hydrochloride) (AAPH) (TNO, Zeist, The Netherlands), and incubation at 37°C. At various time intervals 2 ml aliquots of the reaction solution were withdrawn and the reaction was terminated by adding cold PBS containing 2 mM ascorbic acid. Oxidized lipoproteins were carefully dialysed overnight at 4°C against 0.1 M Tris/HCl containing 0.1% (w/v) EDTA, pH 8.5, or against PBS containing 10 µM of EDTA. Lipid peroxidation was determined by measuring thiobarbituric acid-reactive substances (TBARS) [14], using thiobarbituric acid (in 20% trichloroacetic acid) with fresh malonaldehyde-tetramethyl-acetal as standard. Furthermore, the degree of oxidation was determined by agarose gel electrophoresis (100 V, 30 min, Paragon Lipoprotein Electrophoresis kit, Beckman Instruments). Subsequently, the electrophoretic mobility relative to native VLDL (REM) of the different oxidized VLDL fractions was determined.

Total and free cholesterol, triglyceride (TG) without free glycerol, phospholipid (PL) and free fatty acid (FFA) contents of the lipoprotein samples were measured enzymatically, using commercially available kits (236691 and 310328: Boehringer Mannheim GmbH, Mannheim, Germany; 337-B: Sigma Diagnostics, St. Louis, MO; 990-54009 and Nefa-C: Wako Chemicals GmbH, Neuss, Germany, respectively).
Radio-iodination of VLDL and oxidized VLDL samples was performed using the ^{125}I-monochloride method described by Bilheimer *et al* [15]. The specific activity ranged from 100 to 300 cpm/ng of protein. After iodination, the lipoprotein samples were extensively dialysed against PBS, stored at 4 °C and used within 2 weeks.

Interaction of VLDL and OxVLDL with J774 macrophages

Lipoprotein uptake. The J774 cells were cultured in 24-well plates as described above. The association and degradation of ^{125}I-labelled VLDL and oxidized VLDL (OxVLDL), was determined after a 3 hour incubation at 37°C with 10 µg/ml of ^{125}I-labelled lipoprotein protein either in the absence or in the presence of a 20-fold excess of the respective unlabelled lipoprotein. The receptor-mediated (specific) cell-association and degradation was calculated by subtracting the amount of labelled lipoproteins that was associated or degraded after incubation in the presence of the excess of unlabelled lipoprotein (non-specific) from the amount of labelled lipoprotein that was cell-bound after incubation in the absence of unlabelled lipoprotein (total). At the end of the incubation period, a fraction of the medium was removed to determine the amount of lipoprotein degraded as described previously [16]. After removing the remaining portion of the medium, the cells were washed four times with ice-cold PBS containing 0.1% (w/v) of BSA, and subsequently, with PBS without BSA. To measure the cell-associated lipoprotein fraction, the washed cells were dissolved in 1 ml 0.2 M NaOH, and an aliquot of the cell lysate was counted for radioactivity [16]. Another aliquot was used for protein determination according to Lowry *et al* [12]. In the respective figures, lipoprotein uptake is expressed as the sum of cell-associated and degraded lipoproteins.
The effect of polyinosinic acid (Polyl) on the receptor-mediated uptake of ^{125}I-labelled OxVLDL, was determined in competition experiments. Therefore, J774 cells were incubated for 3 hours at 37°C with 10 µg/ml of ^{125}I-labelled OxVLDL in the presence of different concentrations of unlabelled Polyl, as described in the text and the figure legends. Thereafter, the association and degradation of ^{125}I-OxVLDL were determined exactly as described above.
Effect on cellular and medium lipids. J774 cells were cultured in 6-well plates and VLDL was labelled in the TG with glycerol tri[1-^{14}C]oleate and subsequently oxidized as described above. The effect of ^{14}C-TG-VLDL and ^{14}C-TG-OxVLDL (dissolved in DMEM containing 1% (w/v) of free fatty acid (FFA)-free BSA) on the lipid content of the cells and the medium was determined after incubation of the J774 cells at 37°C with 50 µg/ml of VLDL-TG for different time periods. Incubation volumes were 1 ml/well. After the incubation period, culture dishes were placed on ice, and 800 µl of the medium was used for lipid extraction. Thereafter, the rest of the medium was removed and the cells were washed four times with PBS containing 0.1% (w/v) of BSA, and subsequently, once with PBS without BSA. The cells were harvested in 1 ml PBS using a rubber policeman, and resuspended by three successive slow passages through a syringe needle (G25). Samples (100 µl) were taken for protein determination, lipid extraction (800 µl), and 50 µl of the cell lysate was counted for determination of total uptake of ^{14}C-oleate. Cellular and medium lipids were extracted using methanol/chloroform (2:1, v/v) as described by Bligh and Dyer [17], after the addition of glycerol tri[1-^{3}H]oleate as an internal standard for cellular TG, and [^{3}H]palmitic acid as an internal standard for FFA in the medium. The lipids were separated by thin-layer chromatography with hexane/diethylether/acetic acid (83:16:1) as developing solvent. Bands were visualised by phospho-imaging, and those representing the FFA, TG,

phospholipids, and cholesteryl ester fraction were scraped into vials and counted for radioactivity.

***De novo* triglyceride synthesis.** The J774 cells were cultured in 6-well plates as described above. The *de novo* synthesis of TG was measured after a 4-hour incubation of the cells at 37°C, with 1 ml of DMEM containing 1% (w/v) of FFA-free BSA in the presence or in the absence of VLDL or OxVLDL (100 µg TG/ml), with or without the addition of Polyl (100 µg/ml), as described in the text and figure legends. During the incubation, [1(3)- ^{3}H]glycerol (4.4 µCi/ml, 25 µmol/l at final concentration) was present in the medium in each well. After the incubation period, culture dishes were placed on ice, the medium was removed and the cells were washed four times with PBS containing 0.1% (w/v) of BSA, and subsequently, once with PBS without BSA. The cells were suspended in 1 ml of PBS as described above, and samples (100 µl) were taken for the measurement of protein. Subsequently, lipids were extracted from the cell suspension (800 µl) as described above, except that glycerol tri[1-^{14}C]oleate was added as an internal standard. After iodine staining, the spots containing the TG fraction were scraped into vials and assayed for radioactivity by scintillation counting.

***In vitro* lipolysis of VLDL and oxidized VLDL**

In vitro lipolysis experiments were performed as described by Jong *et al* [18]. Briefly, VLDL samples were incubated at 37°C in 0.1 M Tris/HCl, pH 8.5, in the presence of 2% (w/v) albumin (essentially FFA-free) and 0.2 units of commercially available bovine lipoprotein lipase (LPL, Sigma Chemical Co., St. Louis, MO, USA). After either 5 or 10 minutes, the reaction was stopped by the addition of 50 mM KH_2PO_4, 0.1% Triton-X100, pH 6.9 (Merck, Darmstadt, Germany). The assay was performed using 4 different concentrations of VLDL-TG in the range of 0.1 to 0.5 mM with duplicate FFA measurements. FFA were quantified as described above.

RESULTS

Characterisation of VLDL samples

Human VLDL was incubated with the peroxyl radical generator AAPH for different time periods (0, 1, 2, 3 and 4 hours of incubation; VLDL, OxVLDL1, OxVLDL2, OxVLDL3 and OxVLDL4, respectively). The oxidative properties of VLDL were analysed by TBARS assay and electrophoretic mobility on agarose gel. As shown in Table 1, the oxidative changes in VLDL were accompanied by a gradual increase in TBARS/mg of VLDL protein, and an increase in relative electrophoretic mobility (REM) on agarose gel, as compared to that of native VLDL. The VLDL-cholesterol, TG and phospholipid composition did not change significantly during 4 hours of incubation with AAPH (Table 1). Furthermore, SDS-PAGE and Western blot analysis of OxVLDL showed breakdown of apoB, apoE and apoC, similarly as previously described [10] (data not shown). Unless otherwise stated, VLDL which was oxidized for 4 hours was used in the respective experiments.

Table 1 **Chemical composition and relative electrophoretic mobility of VLDL samples after oxidation**

	TBARS	REM	FC	CE	TG	PL
	μmol/mg		*μmol/mg*	*μmol/mg*	*μmol/mg*	*μmol/mg*
VLDL	0.02 ± 0.48	1.0	0.46 ± 0.26	0.47 ± 0.17	2.11 ± 0.78	0.73 ± 0.42
OxVLDL1	2.52 ± 1.05	1.1	0.46 ± 0.23	0.54 ± 0.12	2.15 ± 0.57	0.78 ± 0.38
OxVLDL2	4.86 ± 1.18	1.2	0.47 ± 0.22	0.60 ± 0.29	2.11 ± 0.66	0.84 ± 0.36
OxVLDL3	6.53 ± 0.26	1.3	0.45 ± 0.16	0.55 ± 0.24	2.06 ± 0.42	0.80 ± 0.36
OxVLDL4	7.28 ± 0.64	1.5	0.46 ± 0.15	0.54 ± 0.07	2.03 ± 0.28	0.81 ± 0.30

FC, free cholesterol; CE, cholesteryl ester; TG, triglyceride; PL, phospholipids. The free cholesterol (FC), cholesterol ester (CE), triglycerides (TG) and phospholipids (PL) content of native VLDL and oxidized VLDL (the samples were oxidized for 1,2,3 or 4 hours, respectively) was measured as described in the Methods section. The rate of oxidation was determined by measuring the thiobarbituric acid-reactive substances (TBARS). The relative electrophoretic mobility (REM) is the mobility of the samples on agarose gel relative to that of unoxidized VLDL. All values are the mean ± S.D. of four experiments.

Interaction of VLDL and OxVLDL with J774 cells

The uptake (expressed as the sum of the cell-associated and degraded lipoprotein) of ^{125}I-labelled native and oxidized VLDL by J774 cells was determined after a 3-hour incubation at 37°C. As shown in Figure 1, the uptake of OxVLDL1 and OxVLDL4 by J774 cells was 1.3- and 2.5-fold higher than that of native VLDL, respectively.

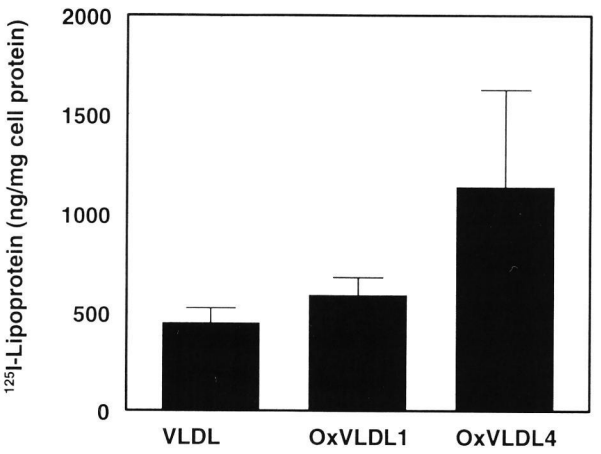

Figure 1 Uptake of VLDL and oxidized VLDL by J774 cells

The J774 cells were incubated for 3 hours at 37°C in DMEM/1% BSA containing 10 µg/ml ^{125}I-VLDL, ^{125}I-OxVLDL1, or ^{125}I-OxVLDL4. The uptake (expressed as cell-associated plus degraded lipoprotein) is determined as described in 'Materials and Methods'. The values represent the mean ± S.D. of four different experiments.

To examine the effect of native VLDL and OxVLDL on the accumulation of TG-derived lipids in J774 cells, we used VLDL that was labelled in the TG with glycerol tri[1-^{14}C]oleate, and subsequently oxidized (^{14}C-TG-VLDL and ^{14}C-TG-OxVLDL4). As shown in Figure 2, after incubation with OxVLDL4 the total uptake of ^{14}C-oleate by J774 cells is decreased as compared to that after incubation with native VLDL (9.2 ± 0.3 vs 15.6 ± 1.2 µl/mg cell protein, respectively). Lipid extraction of the cell lysate and subsequent analysis by TLC, showed that the lower amount of cellular accumulation of ^{14}C-oleate upon incubation with OxVLDL4, was reflected by a strong decrease in all intracellular lipid fractions tested: triglycerides (TG), phospholipids (PL), free fatty acids (FFA) and cholesteryl esters (CE) (Figure 2). Thus, in contrast to the protein uptake, the TG-derived fatty acid uptake by J774 cells is reduced after incubation with OxVLDL as compared to native VLDL.

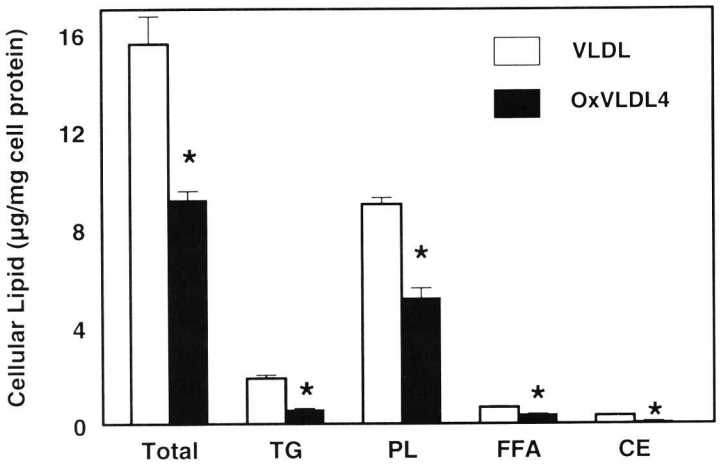

Figure 2 Effect of VLDL and oxidized VLDL on cellular lipid content in J774 cells
VLDL and VLDL oxidized for 4 hours (OxVLDL4) were labelled in the TG with glycerol tri[1-^{14}C]oleate as described in 'Materials and Methods'. After a 4-hour incubation at 37°C with labelled VLDL (open bars) or OxVLDL4 (solid bars, 50 µg TG/ml of medium), the J774 cells were washed and resuspended in 1 ml of PBS. Subsequently, 50 µl of the cell lysate was counted to determine total ^{14}C-oleate uptake. Lipids were extracted from the cell suspension, separated by thin layer chromatography, and visualized by phospho-imaging as described in 'Materials and Methods'. Bands representing triglycerides (TG), phospholipids (PL), free fatty acids (FFA), and cholesteryl esters (CE) were scraped into vials and counted for radio-activity. Values represent the mean ± S.D. of three experiments.

To investigate the mechanisms underlying the decreased cellular uptake of TG-derived oleate upon incubation with OxVLDL4, the relative contribution of the different pathways through which lipid accumulation is thought occur, was assessed for OxVLDL4. We found that the uptake (association and degradation) of ^{125}I-labelled OxVLDL4 was completely inhibited by an excess of unlabelled Polyl (Figure 3). Thus, by inhibiting the receptor-mediated uptake of OxVLDL4 via Polyl, the role of extracellular lipolysis of OxVLDL on the intracellular lipid accumulation can be determined.

The OxVLDL-induced intracellular lipid accumulation was investigated by measuring the *de novo* synthesis of TG by J774 macrophages after incubation with VLDL and OxVLDL4, the latter in the absence or in the presence of Polyl. In this experimental approach, newly formed FFA from either extracellular lipolysis or intracellular lysosomal hydrolysis of VLDL-TG, are re-esterified with [^{3}H]glycerol into [^{3}H]TG. In accordance with the decreased TG accumulation after incubation with OxVLDL4 (Figure 2), Figure 4 shows that the *de novo* synthesis of TG by J774 macrophages after incubation with

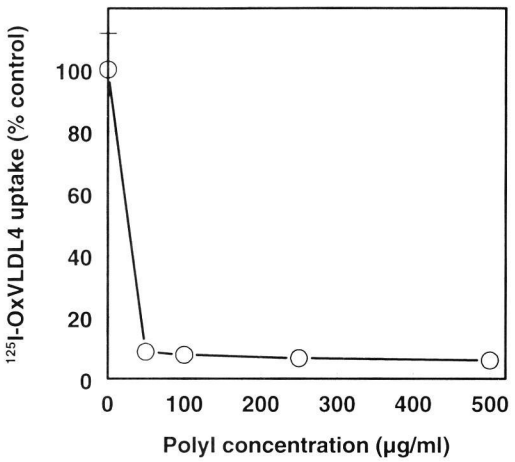

Figure 3 Competition for the uptake by J774 cells of ^{125}I-labelled OxVLDL4 by polyinosinic acid (Polyl)
The J774 cells were incubated for 3 hours at 37°C with 10 µg/ml of ^{125}I-OxVLDL4 in the presence or in the absence of different concentrations of Polyl. The specific association and degradation was determined as described in 'Materials and Methods'. 'Uptake' represents the association and the degradation, since these showed similar results. Values are given as a percentage of the uptake in the absence of any competitor, and represent the mean ± S.D. of three experiments.

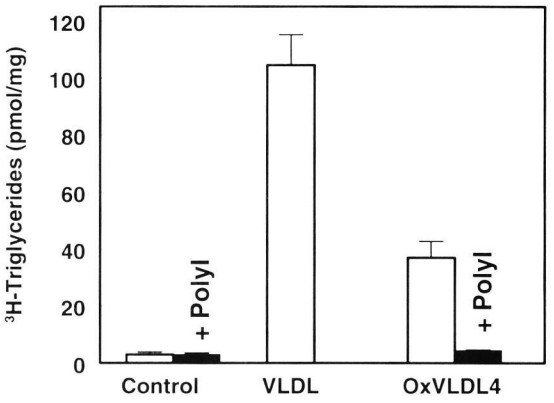

Figure 4 Effect of inhibition of receptor-mediated uptake of OxVLDL4 on the *de novo* triglyceride synthesis in J774 cells
J774 cells were incubated for 4 hours at 37°C with DMEM/1% BSA containing [1(3)-^{3}H]glycerol (4.4 µCi/ml, 25 µmol/l), either in the absence of lipoproteins (control) or in the presence of VLDL or OxVLDL4 (100 µg TG/ml of medium), with (solid bars) or without (open bars) the addition of Polyl (100 µg/ml). After the incubation, the J774 cells were washed and resuspended in 1 ml of PBS. Subsequently, lipids were extracted from the cell suspension, separated by thin layer chromatography, and visualized by iodine staining as described in 'Materials and Methods'. The bands representing the TG fraction were scraped into vials and counted for radio activity. The values represent the mean ± S.D. of three experiments.

OxVLDL4, is approximately 2.5-fold lower as compared to that after incubation with VLDL. In addition, inhibition of the receptor-mediated uptake of OxVLDL4 by Polyl, reduced the *de novo* synthesis of TG to control levels (*i.e.* in the absence of lipoproteins). These results indicate that extracellular lipolysis, mediating the intracellular TG accumulation, may be impaired for OxVLDL.

Lipolysis of oxidized VLDL

To investigate whether the extracellular lipolysis of OxVLDL4 is indeed hampered, we measured the amount of FFA released into the medium during a 4-hour incubation of the J774 cells with ^{14}C-TG-VLDL and ^{14}C-TG-OxVLDL4 (labelled in oleate). As shown in Figure 5, significantly lower amounts of ^{14}C-oleate appear in the medium after incubation with OxVLDL4 as compared to VLDL, indeed suggesting an impaired extracellular lipolysis of OxVLDL-TG.

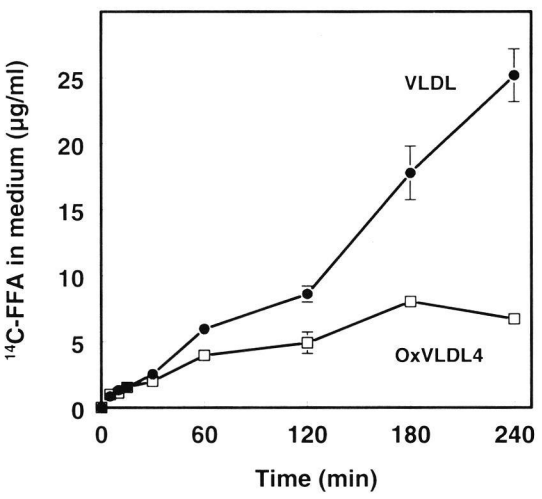

Figure 5 Release of FFA in the medium after incubation of J774 cells with VLDL or oxidized VLDL
VLDL and VLDL oxidized for 4 hours (OxVLDL4) were labelled in the TG with glycerol tri[1-^{14}C]oleate as described in 'Materials and Methods'. J774 cells were incubated during different time periods at 37°C with labelled VLDL (●) or OxVLDL4 (□, 50 µg TG/ml of medium). After the incubation period, free fatty acids (FFA) were extracted from the medium, and after thin layer chromatography, visualized by phospho-imaging and counted for radio-activity as described in 'Materials and Methods'.

To determine directly the effect of oxidation on the LPL-mediated hydrolysis of VLDL-TG, different VLDL samples with various degrees of oxidation were incubated with purified bovine LPL. Figure 6 shows that the amount of FFA released after the addition of LPL, decreases with oxidation time. Thus oxidation decreases the suitability of VLDL as substrate for LPL, leading to a defective TG hydrolysis.

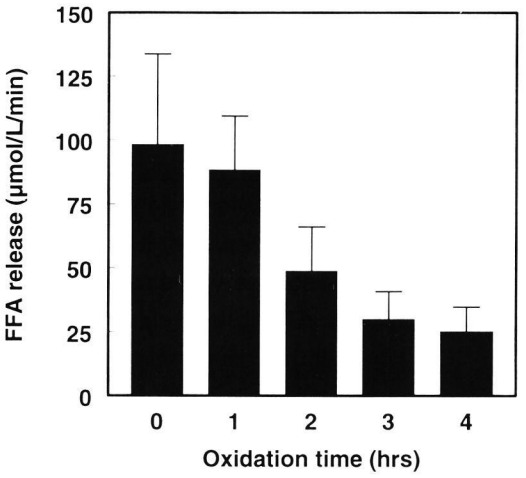

Figure 6 *In vitro* lipolysis of VLDL and oxidized VLDL

VLDL was oxidized for 0, 1, 2, 3, or 4 hours as described in 'Materials and Methods' and dialyzed against 0.1 M Tris/HCl, pH 8.5. The VLDL samples (0.1, 0.2, 0.3, 0.4, and 0.5 mM of VLDL-TG) were incubated at 37°C in the presence of 2% (w/v) FFA-free albumin and 0.2 units of lipoprotein lipase. The amount of FFA was measured after 5 and 10 minutes as described in 'Materials and Methods'. Values are expressed per unit LPL. The results for 0.3 mM TG are shown; similar results were obtained with other TG concentrations.

DISCUSSION

Since little is known about the effect of oxidized VLDL on the accumulation of lipids in macrophages, we examined the processing of oxidatively modified forms of human VLDL in the murine macrophage cell line J774. We showed that the uptake of OxVLDL protein by J774 macrophages was increased about 2.5-fold when compared with native VLDL (Figure 1). However, despite this increased protein (*i.e.* particle number) uptake, incubation of J774 cells with OxVLDL4 resulted in a 2-fold less efficient accumulation of TG (Figure 2), as well as in a 2.5-fold decreased *de novo* synthesis of TG (Figure 4), when compared with native VLDL. It is proposed that triglyceride-rich lipoproteins induce TG accumulation in cells via two different pathways [2,4,19,20]: (i) receptor-mediated uptake of the intact VLDL particle and (ii) uptake of free fatty acids generated by extracellular lipolysis of VLDL-TG, followed by intracellular re-esterification into TG. Our results indicate that the selective uptake of TG after incubation with native VLDL, is primarily mediated by extracellular lipolysis of VLDL-TG, rather than by uptake of the whole lipoprotein particle. Thus, in addition to its so-called 'bridge-function', through which LPL can enhance the cellular uptake of lipoproteins [21-26], LPL also plays an important role in the cellular lipid accumulation by mediating the extracellular hydrolysis of VLDL-TG.

We presented evidence that oxidation of VLDL results in a less efficient stimulation of intracellular TG when compared with that of native VLDL. This is due to a decreased

suitability of OxVLDL as a substrate for LPL, leading to an impaired LPL-mediated TG hydrolysis (Figures 5 and 6). It is proposed that both the lipid and the apolipoprotein composition of VLDL affect the efficiency of lipolysis by LPL. Since the lipid composition of OxVLDL is similar to that of the native VLDL samples (Table 1), and the action of lipoprotein lipase requires intact apoCII [27], the impaired TG-hydrolysis of OxVLDL may be due to a breakdown or conformational change of apoCII during oxidation. Thus, from our results we conclude that in contrast to native VLDL, OxVLDL induces TG accumulation in J774 macrophages only via receptor-mediated uptake of the whole particle, and not via the extracellular lipolysis of OxVLDL-TG.

Our results regarding the inhibiting effect of VLDL oxidation on VLDL-mediated cellular TG accumulation, are confirmed in a study by Whitman *et al* [28]. They also showed that oxidation of ß-VLDL isolated from type III hyperlipidemic patients, resulted in a decreased accumulation of TG in J774 cells when compared with native type III VLDL. Considering the facts that oxidized apoE2/E2 VLDL is taken up at an enhanced rate via the scavenger receptor, whereas the receptor-mediated uptake of native apoE2/2 VLDL is severely hampered due to a defect of apoE2 to bind to the LDL receptor, their results further sustain the important role of extracellular lipolysis in the intracellular accumulation of TG.

Whether OxVLDL, like OxLDL [29], is also present *in vivo*, remains to be determined. It has been reported that in the atherosclerotic plaque, the degree of oxidation of LDL is usually very modest, with an electrophoretic mobility on agarose gel of about twice that of native LDL [29,30,31]. Since it has been shown that VLDL can undergo oxidation *in vitro* [9,28], we suggest that VLDL oxidation may occur in the intima *in vivo,* probably to a similar modest degree as that of LDL. Since moderately oxidized VLDL is still lipolysed by LPL to some extent, extracellular lipolysis in the intima may be involved in foam cell formation.

Thus, the role of LPL in atherogenesis may be dual, depending on its location in the body. If the LPL-mediated uptake of lipoproteins occurs in the liver, the role of LPL would be anti-atherogenic, by enhancing the hepatic uptake of atherogenic lipoproteins. On the other hand, in the intima of the vessel wall LPL serves as a pro-atherogenic factor. Through bridging between the heparan sulphate proteolgycans and lipoproteins, LPL enhances the uptake of these lipoproteins by smooth muscle cells and macrophages, leading to foam cell formation. In addition to its bridge function, our results indicate that its enzymatic function as a lipase also plays an important role in the involvement of VLDL in the atherogenic process.

With respect to the direct stimulation of TG accumulation by cells, we would suggest that OxVLDL is less atherogenic than native VLDL. However, considering the fact that oxidized lipoproteins play a role in atherogenesis in various other ways, including the

stimulation of monocyte recruitment into the intima [32,33], and by their cytotoxic effects on the endothelium of the vessel wall, leading to loss of the endothelial integrity [34,35], it is not allowed to directly compare native VLDL with OxVLDL regarding their atherogenic potential.

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Chapter 4

Binding of ß-Very Low Density Lipoproteins to Heparan Sulphate Proteoglycans requires Lipoprotein Lipase, whereas Apolipoprotein E only Modulates Binding Affinity

Submitted

Binding of ß-Very Low Density Lipoproteins to Heparan Sulphate Proteoglycans requires Lipoprotein Lipase, whereas Apolipoprotein E only Modulates Binding Affinity

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ABSTRACT

The binding of ß-VLDL to heparan sulphate proteoglycans (HSPG) has been reported to be stimulated by both apolipoprotein E (apoE) and lipoprotein lipase (LPL). In the present study we investigated the effect of the isoform and the amount of apoE per particle, as well as the role of LPL on the binding of ß-VLDL to HSPG. Therefore, we isolated ß-VLDL from transgenic mice, expressing either APOE*2(Arg158→Cys) or APOE*3-Leiden (E2-VLDL and E3Leiden-VLDL, respectively), as well as from apoE-deficient mice containing no apoE at all (Enull-VLDL). In the absence of LPL, the binding affinity and maximal binding capacity of all ß-VLDL samples for HSPG coated to microtiter plates was very low. Addition of LPL to this cell-free system resulted in a 12- to 55-fold increase in the binding affinity and a 7- to 15-fold increase in the maximal binding capacity. In the presence of LPL, the association constant (K_a) tended to increase in the order Enull-VLDL < E2-VLDL < E3Leiden-VLDL, whereas the maximal binding (B_{max}) increased in the reversed order: E3Leiden-VLDL ≈ E2-VLDL < Enull-VLDL. Addition of LPL resulted in a marked stimulation of both K_a and B_{max} of ß-VLDL samples to J774 cells similar as found for the binding to HSPG-LPL complexes. Our results indicate that both K_a and B_{max} of ß-VLDL to HSPG are more than one order of magnitude increased upon addition of LPL. In addition, for the binding of ß-VLDL to HSPG-LPL complexes, the presence of apoE is not a prerequisite, but results in an increased binding affinity, depending on the apoE isoform used.

Key words: heparan sulphate proteoglycans; lipoprotein lipase; apolipoprotein E; ß-VLDL

Abbreviations used in this paper: B_{max}, maximum binding; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HSPG, Heparan sulphate proteoglycans; K_a, association constant; LPL, Lipoprotein lipase; SRM-A, standard rat mouse diet.

INTRODUCTION

Heparan sulphate proteoglycans (HSPG) are negatively charged polysaccharides consisting of a core protein and the glycosaminoglycan (GAG) heparan sulphate. In addition to their role in cell adhesion and cell growth [1], HSPG are suggested to be involved in the metabolism of very low density lipoproteins (VLDL). The interaction of VLDL with HSPG has been reported to be mediated in at least two ways. First, via apolipoprotein E (apoE), which contains heparin binding sites [2]. The importance of apoE for this interaction has been described in several reports [3-9]. Mahley *et al.* [3,5,10] have postulated that HSPG may mediate the clearance of VLDL and chylomicron remnants in a so-called secretion-recapture process. After entering the space of Disse, remnant particles become enriched in apoE excreted by the hepatocytes. The increased amounts of apoE enhance the binding of the remnants to HSPG on the surface of hepatocytes, followed by internalization of the particles via either the low density lipoprotein receptor (LDLR) or the LDL receptor-related protein (LRP). Ji *et al.* [4] showed that the addition of apoE enhances the binding of rabbit ß-VLDL to HSPG present on various cell types. Furthermore, it was shown that different variants of apoE display a variable affinity for HSPG: rabbit ß-VLDL enriched in apoE2(Arg158→Cys) bound to HSPG with increased affinity, whereas addition of the dominant apoE3-Leiden variant did not lead to increased binding to HSPG [6].

In addition to apoE, there is strong evidence that the binding of VLDL to HSPG is mediated by lipoprotein lipase (LPL, EC 3.1.1.34). In 1977 it was suggested that LPL, which is anchored to HSPG on the luminal surface of endothelial cells [11, 12], functions as a bridge between VLDL and HSPG during lipolysis of VLDL-triglycerides [13]. More recently, it was demonstrated by several investigators (for a review, see [14]) including our own group [15-17], that LPL can enhance the binding and uptake of several classes of lipoproteins by different cell types through bridging between the lipoproteins and HSPG. The hydrolytic activity of LPL is not required for this bridging function.

Recently, we developed a cell-free system in which HSPG is coated to microtiter plates to investigate the direct interaction of lipoproteins with HSPG [18]. In the present study, the binding of mouse ß-VLDL was studied to further elucidate the

effect of the amount of apoE per particle and the apoE isoform, as well as the role of LPL on the interaction of ß-VLDL with HSPG. We found that both the binding affinity and maximal binding of ß-VLDL directly to HSPG was very low and that addition of LPL resulted in a marked stimulation. Furthermore, it appeared that for the binding of ß-VLDL to HSPG-LPL complexes, the presence of apoE is not a prerequisite, but results in an increased binding affinity.

MATERIALS AND METHODS

Cells

Murine macrophage-like J774 cells were cultured in 75-cm^2 flasks in DMEM supplemented with 10% (vol/vol) FCS, 0.85 g/L $NaHCO_3$, 4.76 g/L HEPES, 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mmol/L glutamine. The cells were incubated at 37°C in an atmosphere containing 5% CO_2 in air. For each experiment, cells were plated in 24-well plates. The cells were fed every 3 days and used for experiments within 7 days of plating.

Animals

APOE*3-Leiden and APOE*2 transgenic mice [19,20] were cross-bred with *apoe-/-* mice [21-23] to obtain mice that produce VLDL that contains apoE3-Leiden or apoE2 without the endogenous mouse apoE protein. Subsequently, these APOE*3-Leiden•*apoe-/-* and APOE*2•*apoe-/-* mice were cross-bred with LDL receptor-deficient (*Ldlr-/-*) mice (Jackson Laboratory, Bar Harbor, ME) to obtain mice that accumulate ß-VLDL in high amounts. Mice were housed under standard conditions with free access to water and food. All mice were fed a SRM-A diet (Hope Farms, Woerden, The Netherlands).

Lipoproteins

After a 4-hour fasting period, blood was collected from 10-25 mice by orbital puncture. Serum was separated from the blood cells by centrifugation at 1500 ***g*** for 15 min at room temperature. Pooled sera were ultracentrifuged to isolate ß-VLDL ($d < 1.006$ g/mL).

Protein content of the ß-VLDL samples was determined by the method of Lowry *et al.* [24]. Triglyceride (TG), total cholesterol (TC), free cholesterol (FC) and phospholipid (PL) content of the ß-VLDL fractions was measured enzymatically, using commercially available kits (337-B: Sigma Chemical Co., St. Louis, MO; 236691 and 310328: Boehringer Mannheim, Mannheim, Germany; and 990-54009: Wako Chemicals, Neuss, Germany, respectively). The cholesterol ester (CE) content was calculated by subtracting the concentration of FC from the concentration of TC.

ß-VLDL apoE levels were determined using an enzyme-linked immunosorbent assay (ELISA) as described previously [25]. ß-VLDL particle size was determined by photon correlation spectroscopy using a Malvern 4700 C system (Malvern Instruments, UK). Measurements were performed at 25°C and a 90° angle between laser and detector. The number of apoE molecules per ß-VLDL particle was calculated from the total lipoprotein mass and ß-VLDL particle size, with the assumption that the particles were spherical in shape and their density was 1.006 g/mL.

The ß-VLDL apoB content was determined using SDS-polyacrylamide gradient gels (4-20%) [26]. After electrophoresis, gels were stained with Coomassie Brilliant blue and subsequently

scanned with a HP ScanJet Plus (Hewlett Packard, Santa Clara, CA) to calculate the amount of apoB (B100+B48) relative to total protein content.
ß-VLDL samples were iodinated using the ^{125}I-monochloride method of Bilheimer *et al.* [27]. After iodination, ß-VLDL was dialyzed extensively at 4°C against PBS for 24 hours and thereafter stabilized with 1% (wt/vol) BSA (fraction V, Sigma). The specific radioactivity ranged from 110-240 cpm/ng of protein. The stabilized ^{125}I-labelled ß-VLDL was stored at 4°C and used within two weeks.

Lipoprotein lipase
Lipoprotein lipase (LPL) was purified from fresh bovine milk as described previously [17]. The isolated fraction was resuspended in 20 mmol/L NaH_2PO_4, 50% glycerol and stored in aliquots at -80°C.

Binding to HSPG or HSPG-LPL complexes
Microtiter plates (Greiner GmbH, Frichenhausen, Germany) were coated with commercially available HSPG (Sigma, H4777) as described previously [18]. Briefly, wells were incubated with 0.5 µg HSPG in PBS for 18 hours at 4°C. Non-specific binding sites were blocked with PBS containing 1% (wt/vol) BSA for 1 hour at 37°C. Thereafter, wells were incubated with 1.5 µg LPL in 0.1 mol/L Tris, 20% (vol/vol) glycerol, pH 8.5 for 3 hours at 4°C and subsequently washed two times with ice-cold PBS to remove unbound LPL.
The binding of ^{125}I-labelled ß-VLDL isolated from *apoe-/-* mice and transgenic mice expressing apoE2 or apoE3-Leiden without endogenous apoE to plates coated with HSPG or HSPG-LPL complexes was determined by incubating the plates for 3 hours at 4°C with the indicated amounts of ^{125}I-labelled ß-VLDL, either in the presence or in the absence of a 20-fold excess of unlabelled ß-VLDL. Thereafter, plates were washed two times with ice-cold PBS containing 0.1% (wt/vol) BSA and subsequently with PBS without BSA. The ^{125}I-labelled ß-VLDL bound to HSPG or to HSPG-LPL complexes was dissolved in 0.2 mol/L NaOH for quantitation of the binding.

Binding to J774 macrophages
J774 cells were cultured in 24-well plates as described above. Twenty-four hours before each experiment, cells were washed with DMEM containing 1% (wt/vol) BSA and further incubated with DMEM containing 5% (vol/vol) of lipoprotein-deficient serum (d < 1.21 g/mL) instead of FCS. The binding of ^{125}I-labelled ß-VLDL to J774 cells in the absence or presence of 5 µg/mL of LPL was determined after a 3-hour incubation at 4°C with the indicated amounts of ^{125}I-labelled ß-VLDL, either in the presence or absence of a 20-fold excess of unlabelled ß-VLDL. The receptor-mediated (specific) cell binding was calculated by subtracting the amount of labelled ß-VLDL that was cell bound after incubation in the presence of the excess of unlabelled ß-VLDL (non-specific) from the amount of labelled ß-VLDL that was bound after incubation in the absence of unlabelled ß-VLDL (total cell binding). After removal of the medium, the cells were washed four times with ice-cold PBS containing 0.1% (wt/vol) BSA and subsequently once with PBS without BSA. Cells were then dissolved in 1 mL of 0.2 mol/L NaOH. Protein content was measured by the method of Lowry *et al.* [24]. In an aliquot, the radioactivity represented the amount of cell-bound ß-VLDL.

RESULTS

ß-VLDL composition

To obtain ß-VLDL particles containing high amounts of apoE2(Arg158→Cys), apoE3-Leiden, or no apoE at all, ß-VLDL was isolated from APOE*2•*apoe-/-*•*Ldlr-/-* mice (E2-VLDL), APOE*3-Leiden•*apoe-/-*•*Ldlr-/-* mice (E3Leiden-VLDL) and *apoe-/-* mice (Enull-VLDL), respectively. As shown in Table 1, ß-VLDL isolated from these transgenic mice were all cholesterol ester-rich particles. Particle size measurements showed that both E2-VLDL and E3-Leiden-VLDL were enlarged as compared to Enull-VLDL. In addition, these particles were very rich in apoE: 48 and 66 apoE molecules per particle, respectively, *versus* 2 apoE molecules in human apoE3/3 VLDL.

Binding of ß-VLDL to HSPG in the absence or presence of LPL

To investigate the effect of the amount of apoE and the apoE isoform on the interaction of ß-VLDL with HSPG, we determined the binding of ß-VLDL containing no apoE at all (Enull-VLDL) and of ß-VLDL rich in apoE (E2-VLDL and E3Leiden-VLDL) to microtiter plates coated with HSPG. In the absence of LPL, the binding to HSPG of all ß-VLDL particles was very low (Figure 1A). Nevertheless, we performed Scatchard analysis [28], revealing that both the association constant (K_a) and the maximal binding (B_{max}) increased in the order E2-VLDL < Enull-VLDL < E3Leiden-VLDL (Table 2).

Addition of LPL to this cell-free system resulted in a marked increase in the binding of all ß-VLDL particles to HSPG, as compared to that in the absence of LPL (compare Figure 1B with 1A). The values of the K_a for the binding of the particles in the presence of LPL were 12- to 55-fold higher than in the absence of LPL and tended to increase in the order Enull-VLDL < E2-VLDL < E3Leiden-VLDL (Table 2). The values of the B_{max} were also increased as compared to those in the absence of LPL (7- to 15-fold). B_{max} values in the presence of LPL increased in the reversed order: E3Leiden-VLDL ≈ E2-VLDL < Enull-VLDL, probably due to the size of the particles (Table 1).

These results indicate that, even despite very high amounts of apoE2 on the surface of the ß-VLDL particle, the binding affinity and maximal binding capacity of ß-VLDL to HSPG coated to microtiter plates were very low, as compared to that in the presence of LPL. Strikingly, in the cell-free system, the binding affinity and maximal binding capacity of Enull-VLDL to HSPG was in the same order of magnitude as that of the two apoE-containing particles, both in the absence and in the presence of LPL.

Table 1 ß-VLDL composition

ß-VLDL	TC	CE	FC	TTG	PL	Particle size	Number of human apoE molecules per VLDL particle*
	µmol/mg protein					*nm*	
Enull-VLDL	17.0 ± 0.5	16.2 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	2.5 ± 0.1	49 ± 11	-
E2-VLDL	16.4 ± 0.1	15.5 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	2.8 ± 0.1	71 ± 12	48 ± 0.2
E3Leiden-VLDL	9.5 ± 0.8	8.3 ± 0.8	1.2 ± 0.1	6.6 ± 0.3	2.2 ± 0.2	96 ± 26	66 ± 1.3

TC indicates total cholesterol (free plus esterified); CE, cholesterol ester; FC, free cholesterol; TTG, total triglycerides; PL, phospholipids; and PS, particle size. ß-VLDL was isolated from pooled serum by ultracentrifugation. Lipid levels, particle size and number of apoE molecules per ß-VLDL particle were measured as described in the Methods section. The CE content was calculated by subtracting the amount of FC from the amount of TC. Values are expressed as micromoles of lipid per milligram of lipoprotein protein and represent the mean ± S.D. of three measurements.

*2 apoE molecules per VLDL particle in human apoE3/E3 VLDL.

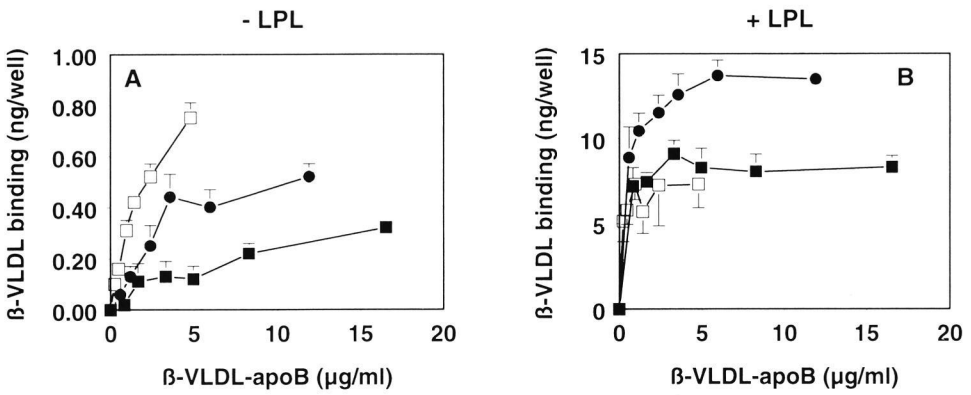

Figure 1 **Binding of ß-VLDL to HSPG in the absence or presence of LPL**
Wells of microtiter plates were preconditioned with HSPG or HSPG-LPL complexes described in the Methods section. Binding of VLDL to HSPG (A) or HSPG-LPL complexes (B) was measured after a 3-hour incubation with the indicated amounts of ^{125}I-labelled ß-VLDL (expressed as ß-VLDL-apoB concentration) at 4°C, either in the presence or in the absence of a 20-fold excess of unlabelled ß-VLDL. The specific binding is expressed as ng of labelled ß-VLDL per well. The values represent the mean ± S.D. of three measurements. (●) Enull-VLDL, (■) E2-VLDL, (□) E3Leiden-VLDL.

Table 2 **Binding affinity of ^{125}I-labelled ß-VLDL to HSPG in the presence and absence of LPL**

	K_a	B_{max}	K_a	B_{max}
ß-VLDL	- LPL		+ LPL	
	ml/µg	*ng/well*	*ml/µg*	*ng/well*
Enull-VLDL	0.12 ± 0.03	1.0 ± 0.1	2.6 ± 0.5	14.6 ± 0.2
E2-VLDL	0.06 ± 0.02	0.7 ± 0.2	3.3 ± 1.2	9.7 ± 1.2
E3Leiden-VLDL	0.39 ± 0.11	1.2 ± 0.2	4.5 ± 1.6	8.4 ± 0.4

The specific binding of ^{125}I-labelled ß-VLDL to microtiter plates coated with HSPG or HSPG-LPL complexes was determined as described in the Methods section. Association constants were calculated by Scatchard analysis [28] based on the amount of apoB protein reflecting particle number. The values represent the mean ± S.D. of three measurements.

Binding of ß-VLDL to J774 macrophages in the absence or presence of LPL

To determine whether similar results could be obtained in a more physiological system, we performed binding experiments using J774 macrophages instead of HSPG coated to microtiter plates. Like other macrophages [29,30], J774 cells express an extracellular matrix containing HSPG [17], whereas they do not secrete apoE [31,32]. As expected, binding of all ß-VLDL samples to J774 cells in the absence of LPL, was higher as compared to that in the cell-free system (Figure 2A). This is due to the presence of lipoprotein receptors on the cell membrane. As expected from previous results [20], Enull-VLDL and E2-VLDL both displayed a very low affinity to these receptors, as compared to E3Leiden-VLDL (Table 3).

Addition of LPL resulted in a 6- to 11-fold stimulation of the maximum binding to J774 cells of all ß-VLDL particles tested (Figure 2 and Table 3), similar as found for the HSPG-LPL complexes coated to microtiter plates (Figure 1 and Table 2). Furthermore, Table 3 shows that the K_a for the binding of ß-VLDL to J774 cells in the presence of LPL tended to increase in the same order as found for the binding of ß-VLDL to HSPG-LPL in microtiter plates: Enull VLDL< E2-VLDL < E3Leiden-VLDL. Again, as for the cell-free system, the values for the B_{max} increased in the reversed order.

These results show that the data obtained using the HSPG coated to microtiter plates were similar to those obtained using a cell culture system, supporting the suitability of this cell-free system for testing the binding affinities of lipoproteins to HSPG.

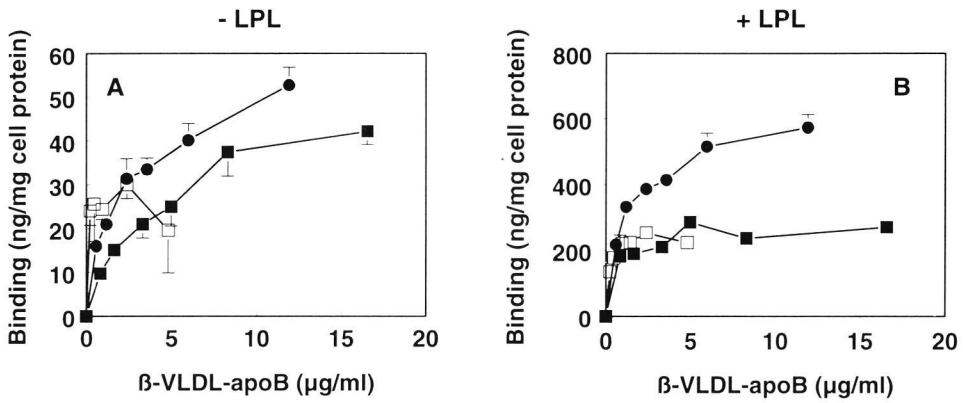

Figure 2 **Binding of ß-VLDL to J774 macrophages in the absence or presence of LPL**

Binding of ß-VLDL to J774 cells was measured after a 3-hour incubation of the cells with the indicated amounts of ^{125}I-labelled ß-VLDL (expressed as ß-VLDL-apoB concentration) at 4°C, either in the absence (A) or in the presence (B) of 5 µg/ml of LPL. Specific binding is expressed as ng labelled lipoprotein per mg cell protein and was determined as described in Methods section. Values represent the mean ± S.D. of three measurements. (●) Enull-VLDL, (■) E2-VLDL, (□) E3Leiden-VLDL.

Table 3 **Binding affinity of ^{125}I-labelled ßVLDL to J774 macrophages in the presence and absence of LPL**

	K_a	B_{max}	K_a	B_{max}
ß-VLDL	- LPL		+ LPL	
	ml/µg	*ng/mg*	*ml/µg*	*ng/mg*
Apoe-/-	0.6 ± 0.1	56.8 ± 7.3	0.9 ± 0.1	604 ± 33
APOE*2	0.3 ± 0.1	53.2 ± 7.7	1.2 ± 0.3	314 ± 9
APOE*3-Leiden	4.0 ± 2.1	39.8 ± 6.6	4.0 ± 1.3	274 ± 21

The specific binding of ^{125}I-labelled ß-VLDL to J774 macrophages in the presence and absence of LPL was determined as described in the Methods section. Association constants were calculated by Scatchard analysis [28] based on the amount of apoB protein reflecting particle number. The values represents the mean ± S.D. of three measurements.

DISCUSSION

It has previously been shown for all lipoproteins, except rabbit ß-VLDL, that the binding to HSPG is very low, whereas addition of LPL increases this binding several fold [33]. In addition to LPL, an excess of apoE results in a marked enhancement of the binding of rabbit ß-VLDL to HSPG [4]. Furthermore, different isoforms of apoE display variable interactions with HSPG: addition of apoE2(Arg158→Cys) results in a marked stimulation of the binding of rabbit ß-VLDL to HSPG present on hepatocytes and fibroblasts, whereas addition of apoE3-Leiden does not stimulate the binding [6].
In the present study we investigated the influence of LPL as well as the effect of high amounts of apoE isoforms on the binding of mouse ß-VLDL to HSPG, as compared to the binding of ß-VLDL containing no apoE at all. For this purpose, binding experiments and Scatchard analyses were performed using both HSPG coated to microtiter plates and HSPG present on J774 cells, either in the absence or presence of LPL. We found that in the absence of LPL, the association constant (K_a) and the maximal binding capacity (B_{max}) for the binding of all ß-VLDL particles to HSPG coated to microtiter plates were in the same order of magnitude, but very low. These results indicate that in the presently used system, apoE is not an essential factor for the binding of ß-VLDL to HSPG.
Surprisingly, the K_a for the binding to HSPG of E2-VLDL, containing high amounts of apoE2, was even decreased as compared to E3Leiden-VLDL or Enull-VLDL, respectively. This is in contrast with the data of Ji *et al.* [6] and Mann *et al.* [7], who showed that rabbit ß-VLDL enriched in apoE variants associated with a dominant mode of inheritance of type III hyperlipoproteinemia (*i.e.* apoE3-Leiden) bound less efficiently to HepG2 cells, McA-RH7777 cells and isolated HSPG, as compared to rabbit ß-VLDL enriched in apoE variants associated with the recessive form (*i.e.* apoE2(Arg158→Cys)). This discrepancy can be explained by several reasons. First, in our study isolated mouse ß-VLDL that only contained the respective apoE variants was used, whereas in the experiments of Ji *et al.* [6] cells were incubated with rabbit ß-VLDL that contained endogenous apoE in addition to the respective exogenously added apoE variants. Second, Ji and co-workers [6] also used McA-RH7777 cells transfected with human apoE isoforms to test the effect of apoE secretion on the binding of rabbit ß-VLDL. They found that in comparison with the non-transfected cells, the apoE2-secreting cells displayed a 2-fold enhancement in the binding of ß-VLDL, whereas there was no enhancement of the binding of ß-VLDL to the apoE3-Leiden secreting cells. Therefore, we hypothesize that the presence of free apoE during the binding experiments and the method used to enrich ß-VLDL with apoE is of importance for the outcome of the experiments. This hypothesis is supported by data

of Mann *et al.* [7], who found that the presence of free apoE enhances the binding of apoE-enriched ß-VLDL to HSPG to a larger extent, as compared to apoE-enriched ß-VLDL that has been re-isolated prior to the binding experiment. Third, Ji *et al.* [6] performed a dot blot assay, using HSPG coated to nitrocellulose membranes, to test direct binding of ß-VLDL to HSPG. This difference in methodology may also (partly) explain the difference between their and our results.

Rabbit ß-VLDL has been shown to bind directly to HSPG [4,33], whereas in the present study the binding of mouse ß-VLDL was low. Whether this discrepancy could be explained by the presence of LPL on rabbit ß-VLDL particles, whereas mouse ß-VLDL does not contain LPL, is at present only subject to speculation. In this respect, it is important to note that several studies have indicated that in human pre- and postheparin plasma LPL is attached to LDL-like particles [34-36] and VLDL [37].

In accordance with Lookene *et al.* [33], we showed that addition of LPL markedly enhanced K_a and B_{max} values of all ß-VLDL particles, including that of apoE-deficient VLDL, to both HSPG coated to microtiter plates and HSPG present on the plasma membrane of J774 cells. Furthermore, the presence of apoE was not a prerequisite, but resulted in an increased binding affinity. For both systems, in the presence of LPL, the values for the K_a tended to increase in the order Enull-VLDL < E2-VLDL < E3Leiden-VLDL, whereas the values for the B_{max} decreased in this order. The latter can be explained by differences in steric hindrance, due to a different size of the particles (Table 1). Calculations revealed that, at maximum binding capacity, $2 * 10^{10}$ E3Leiden-VLDL particles were bound to HSPG-LPL complexes coated to the surface of one well *versus* $3.5 * 10^{10}$ Enull-VLDL particles. This is in accordance with the observation that E3-Leiden VLDL was about 2-fold larger as compared to Enull-VLDL (Table 1).

There are several reports suggesting that in the arterial wall, LPL is involved in the atherosclerotic process by enhancing the uptake of lipoproteins via macrophages and smooth muscle cells, probably in a process mediated by HSPG [15,16,38]. The facts that macrophages in atherosclerotic plaques synthesize both LPL [39] and HSPG [40,41] further support the pro-atherogenic role of LPL in the intima of the vessel wall. It has also been shown that cholesterol-loading of macrophages enhances the apoE-secretion by these cells [42], indicating that apoE is abundantly present in atherosclerotic lesions. These findings, together with the present finding that apoE further enhances the binding affinity of ß-VLDL for HSPG-LPL complexes, indicate the importance of the combined roles of LPL, apoE and HSPG in the atherosclerotic process.

ACKNOWLEDGEMENTS

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Chapter 5

Uptake by J774 Macrophages of Very Low Density Lipoproteins Isolated from ApoE-Deficient Mice is Mediated by a Distinct Receptor and Stimulated by Lipoprotein Lipase

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Uptake by J774 Macrophages of Very Low Density Lipoproteins Isolated from ApoE-deficient Mice is Mediated by a Distinct Receptor and Stimulated by Lipoprotein Lipase

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ABSTRACT

Apolipoprotein E (ApoE) deficient mice display marked accumulation in the plasma of VLDL lipoproteins which are deficient in both apoE and apoB100, but contain apoB48, apoAI, apoCs and apoAIV. Since apoE-deficient mice develop severe atherosclerotic lesions with lipid laden macrophages, we reasoned that the uptake of lipoproteins by intimal macrophages can take place in the absence of both apoE and apoB100. To get more insight into the mechanism of foam cell formation in apoE-deficient mice, we measured the interaction of VLDL from apoE-deficient mice (apoE^{null} VLDL) with the murine macrophage cell line J774. Scatchard analysis revealed that apoE^{null} VLDL is bound to J774 cells with a K_d value comparable to that of control VLDL (8.1 versus 4.7 µg/ml), and with a B_{max} value about half that of control VLDL (40 versus 70 ng/mg cell protein, respectively). ApoE^{null} VLDL is also taken up and degraded by J774 macrophages via a high affinity process, less efficiently than control mouse VLDL (6-fold and 50-fold less efficiently, respecively). In line with this observation, incubation of J774 cells with 50 µg/ml of apoE^{null} VLDL for 24 hours resulted in an increase in intracellular cholesteryl ester (CE) content; 5-fold less pronounced however, than after incubation with 50 µg/ml of control mouse VLDL. Under the conditions applied, simultaneous addition of 5 µg/ml of LPL stimulated the cellular uptake and degradation of apoE^{null} VLDL about 10-fold, and resulted in a 5-fold stimulation of the intracellular CE accumulation from 9 ± 2 to 46 ± 5 µg CE per mg cell protein.
In contrast to control mouse VLDL, apoE^{null} VLDL could not compete with ^{125}I-labelled LDL for binding to the LDL receptor of J774 cells. Furthermore, both LDL and acetylated LDL could not compete with ^{125}I-labelled apoE^{null} VLDL for binding to these cells, whereas control mouse VLDL, VLDL from a hypertriglyceridemic patient (HTG-VLDL) and apoE^{null} VLDL itself were efficient competitors. Thus, VLDL from apoE-deficient mice is taken up by J774 macrophages through recognition by a distinct receptor, which could be the triglyceride-rich lipoprotein (TGRLP) receptor.

We conclude that in apoE-deficient mice foam cell formation occurs via a receptor-mediated uptake of apoE^{null} VLDL, which can be stimulated by the presence of LPL.

Keywords: ApoE-deficient mice, macrophages, foam cells, receptor-mediated uptake, lipoprotein lipase

Abbreviations used in this paper: AcLDL, Acetylated Low Density Lipoprotein; Apo, Apolipoprotein; ApoE^{null} VLDL, Very Low Density Lipoprotein from apoE-deficient mice; CE, Cholesteryl Ester; DMEM, Dulbecco's Modified Eagle's Medium; FCS, Foetal Calf Serum; HSA, Human Serum Albumin; HTG-VLDL, Very Low Density Lipoprotein from Hypertriglyceridemic patients; LPL, Lipoprotein Lipase; TGRLP, Triglyceride Rich Lipoprotein.

INTRODUCTION

Apolipoprotein E (apoE) is a major protein constituent of chylomicrons, very low density lipoproteins (VLDL), chylomicron and VLDL remnants and of high density lipoproteins (HDL). One of the major functions of apoE in lipoprotein metabolism is that it serves as a ligand for hepatic receptors mediating the removal of apoE-containing lipoproteins from the circulation.

By using gene targeting in murine embryonic stem (ES) cells, several groups have generated mice lacking apoE [1-3]. These mice exhibit extreme hypercholesterolemia which is much more severe than that observed in apoE-deficient humans [4-6]. The reason for this difference might be the fact that in mice, particles containing both apoB100 and apoB48 are produced by the liver, whereas in humans, the liver produces only particles containing apoB100 [7,8]. In contrast to apoB100, apoB48 does not function as a ligand for hepatic lipoprotein receptors, and therefore, apoE-deficient mice display a marked accumulation of chylomicrons and VLDL lipoproteins which are deficient in both apoE and apoB100 but which contain apoB48 [1]. Since apolipoprotein E-deficient mice develop extensive atherosclerotic lesions with lipid laden macrophages, even on a regular chow diet [9], we suggest that uptake of lipoprotein particles into macrophages present in the intima, can occur in the absence of both apoB100 and apoE. Hence, additional uptake mechanisms may play a role in the process of foam cell formation in apoE-deficient mice.

To provide more insight into the formation of foam cells in mice lacking apoE, we studied the processing of VLDL isolated from apoE-deficient mice (ApoE^{null} VLDL) by J774 macrophages, a murine macrophage cell line commonly used as a model for foam cell formation, and which does not produce apoE [10].

We also investigated the role of lipoprotein lipase (LPL) in binding and uptake of apoE^{null} VLDL by J774 cells, since LPL is synthesized by macrophages present in the intima [11] and known to enhance the binding and uptake of LDL and VLDL by different cell types, including macrophages [12-17].

Our results show that VLDL deficient in both apoE and apoB100 is taken up by J774 macrophages via a receptor-mediated pathway, leading to the accumulation of cholesteryl esters in these cells. In addition, like normal VLDL, this apoE^{null} VLDL can also be taken up via an LPL-mediated process.

MATERIALS AND METHODS

Cells

Murine macrophage-like J774 cells were cultured in 75 cm^2 flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 0.85 g/l $NaHCO_3$, 4.76 g/l Hepes, 100 i.u./ml penicillin, 100 µg/ml streptomycin and 2 mM glutamin. The cells were incubated at 37°C in an atmosphere containing 5% CO_2 in air. For each experiment, cells were plated in 12- or 24-well plates. The cells were fed every three days, and used for experiments within seven days of plating.

Animals and lipoproteins

Mice were allowed access to food and water ad libitum. ApoE-deficient mice were generated as described [1] and fed a regular chow diet (SRM-A). The control mice were of a genetic background similar to that of the apoE-deficient mice (129 Sv x C57BL/6J). To obtain lipoprotein samples from control mice with a lipid composition comparable to that of apoE-deficient mice, control mice were fed a semi-synthetic severe high fat/cholesterol (HFC 0.5%) diet [18] for a period of 4 weeks, which is composed essentially according to Nishina *et al* [19] and purchased from Hope Farms, Woerden, The Netherlands. This diet contains 15% cocoa butter, 1% cholesterol, 0.5% cholate, 40.5% sucrose, 10% cornstarch, 1% corn oil and 4.7% cellulose (all percentages are by weight) and all the required nutrients, minerals and vitamins. After a 4-h fasting period, mice were anaesthetized using diethylether. Blood was collected from apoE-deficient (n = 11) and control mice (n = 62) by orbital punction. The d < 1.006 g/ml lipoproteins (VLDL lipoproteins) were isolated from the mouse serum by density gradient ultracentrifugation. Human serum was prepared from freshly collected blood either from healthy volunteers or from a type IV hyperlipidemic patient. Human LDL and hypertriglyceridemic VLDL (HTG-VLDL) were isolated from the respective sera by density gradient ultracentrifugation as described by Redgrave *et al* [20]. A portion of the LDL sample was acetylated by repeated addition of acetic anhydride as described by Basu *et al* [21]. The conversion of LDL into acetylated LDL (AcLDL) was confirmed by agarose gel electrophoresis (100 V, 30 min, Paragon Lipoprotein Electrophoresis Kit, Beckman Instruments Inc., Fullerton, CA). The protein content of the lipoprotein samples was determined according to Lowry *et al* [22].

Total and free cholesterol, triglyceride (without free glycerol) and phospholipid contents of the lipoproteins were measured enzymatically, using commercially available kits (236691 and 310328: Boehringer Mannheim GmbH, Mannheim, Germany; 337-B: Sigma Diagnostics, St. Louis, MO; and 990-54009: Wako Chemicals GmbH, Neuss, Germany, respectively).
To determine the relative apolipoprotein composition of the lipoproteins, some 12.5 µg of lipoprotein protein was applied on SDS polyacrylamide gradient gels (4-20%). After electrophoresis, the protein was stained with Coomassie Brilliant blue, followed by densitometric scanning of the bands using a Hewlett Packard ScanJet Plus.
Mouse VLDL and human LDL were radio-iodinated by using the ^{125}I-monochloride method described by Bilheimer *et al* [23]. The specific activity ranged from 90 to 400 cpm per ng of protein. After iodination, the lipoprotein samples were dialysed extensively against PBS (pH 7.4), stabilized with 1% (w/v) of human serum albumin (HSA), stored at 4°C and used within two weeks.
Whenever unlabelled lipoproteins were used, immediately after isolation, dialysis against PBS containing 10mM of EDTA and, subsequently, DMEM was performed at 4°C.

Lipoprotein lipase
LPL was partly purified from fresh bovine milk by using heparin-Sepharose chromatography as described previously [24]

Binding and uptake of lipoproteins by J774 macrophages
The J774 cells were cultured in 24-well plates as described above. Twenty-four hours prior to each experiment, the cells were washed with DMEM containing 1% (w/v) bovine serum albumin (BSA) and further incubated with DMEM containing 5% (v/v) of lipoprotein deficient serum (LPDS d < 1.21 g/ml) instead of FCS. The binding and uptake studies were subsequently conducted in medium containing no serum components other than the respective lipoproteins and 1% (w/v) of bovine serum albumin.
The binding of ^{125}I-labelled lipoproteins to the cells in the absence or in the presence of 5 µg/ml of LPL, was determined after a 3 hour incubation at 4°C with different concentrations of ^{125}I-labelled lipoprotein, either in the presence or in the absence of a 20-fold excess of the respective unlabelled lipoprotein. The concentration of the labelled lipoproteins in the respective incubations are described in the text and the figure legends. The receptor-mediated (specific) cell-binding was calculated by subtracting the amount of labelled lipoproteins that was cell-bound after incubation in the presence of the excess of unlabelled lipoprotein (non-specific) from the amount of labelled lipoprotein that was cell-bound after incubation in the absence of unlabelled lipoprotein (total cell binding). After removal of the medium, the cells were washed four times with ice-cold PBS containing 0.1% (w/v) of BSA, and subsequently, once with PBS without BSA. Cells were then dissolved in 1 ml of 0.2 M NaOH. Protein content was measured by the method of Lowry *et al* [22]. In an aliquot, the radioactivity represented the amount of cell-bound lipoprotein.
The association and degradation of ^{125}I-labelled lipoproteins in the absence or in the presence of 5 µg/ml of LPL, was determined after a 3 hour incubation at 37°C with 10 µg/ml ^{125}I-labelled lipoprotein either in the absence or in the presence of 200 µg/ml of the respective unlabelled lipoprotein. At the end of the incubation period, a fraction of the medium was removed to determine the amount of lipoprotein degraded as described previously [25,26]. After removing the remaining portion of the medium, the cells were washed four times with ice-cold PBS containing 0.1% (w/v) of BSA, and subsequently, with PBS without BSA. To measure the cell-associated (bound plus internalized) lipoprotein fraction, the washed cells were dissolved in 1 ml 0.2 M NaOH, and an aliquot of the cell lysate was counted for

radioactivity [25]. Another aliquot was used for protein determination according to Lowry *et al* [22].
To identify the nature of the receptor which mediates the binding of apoE^{null} VLDL to J774 macrophages, competition experiments were performed. Therefore, J774 cells were incubated for 3 hours at 4°C with 10 µg/ml ^{125}I-labelled human LDL, with 10 µg/ml ^{125}I-labelled apoE^{null} VLDL, or with 10 µg/ml ^{125}I-labelled HTG-VLDL in the presence of different concentrations of the indicated unlabelled lipoprotein samples. Thereafter, cells were washed and binding at 4°C was measured as described above.

Cellular accumulation of lipid
J774 cells were cultured in 12-well plates as described above. Twenty-four hours before the start of the experiment, DMEM supplemented with 5% (v/v) of LPDS instead of FCS was added to the cells. At the start of the experiment, fresh DMEM media containing 1% HSA (w/v) and 50 µg/ml of lipoprotein protein either in the presence or in the absence of 5 µg/ml of LPL, were added in triplicate dishes of cells and incubated for 24 hours at 37°C. Control incubations were performed with DMEM-1% HSA without any further additions or with DMEM-1% HSA with 5 µg/ml of LPL. At the end of the incubation period, the cells were washed four times with 1 ml of PBS containing 0.1% (w/v) of BSA, followed by one wash with PBS alone. Intracellular lipid content was determined as described by Havekes *et al* [27]. Briefly, the cells were harvested by scraping with a rubber policeman and resuspended by three successive slow passages through a syringe needle (G25). Samples (100 µl) were taken for measurement of protein. Lipids were extracted from the cell suspension using methanol/chloroform (2:1, v/v) as described by Bligh and Dyer [28], after addition of cholesteryl acetate (2 µg) as an internal standard. The lipids were separated using high performance thin layer chromatography (HPTLC). Subsequently, the lipid bands were quantified densitometrically on a Shimadzu (Kyoto, Japan) CS910 chromatograph scanner at 380 nm and areas under the curve were integrated by using a data processor.

RESULTS

Lipid and apolipoprotein composition of apoE^{null} VLDL and control VLDL

As expected, the cholesterol, triglyceride and phospholipid levels are similar in VLDL isolated from control mice fed a high fat, high cholesterol diet (HFC 0.5%) and in VLDL from apoE-deficient mice fed a regular chow diet (Table 1).
In apoE^{null} VLDL, apoB48, apoAIV and apoCs are the major protein constituents, whereas small amounts of apoAI are also found (Figure 1). No apoB100 and apoE could be detected in this lipoprotein sample. In control mouse VLDL apoE is the major apolipoprotein, whereas equal amounts of apoB100 and apoB48 are found in addition to apoAIV and apoCs.

Table 1. Lipid composition of mouse VLDL

	TC	FC	CE	TG	PL	
	(μmol/mg lipoprotein protein)					Shell/Core ratio
Control VLDL	14.5	3.1	11.4	1.5	2.1	0.4
ApoE^{null} VLDL	12.5	3.7	8.8	1.3	1.5	0.5

TC, total cholesterol (free plus esterified); FC, free cholesterol; CE, cholesteryl ester; TG, triglyceride; PL, phospholipids. Total and free cholesterol, triglycerides (without glycerol) and phospholipids were determined in the VLDL fraction from pooled blood samples from control mice (control VLDL) and from apoE-deficient mice (apoE^{null} VLDL). Values are expressed as μmol of lipid per mg of lipoprotein protein. The cholesteryl ester content was calculated by subtracting the amount of free cholesterol from the amount of total cholesterol, the shell/core ratio was calculated using the formula (PL + FC)/(TG + CE).

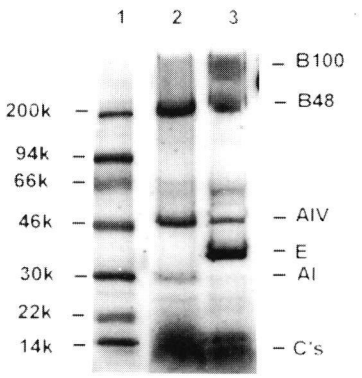

Figure 1 SDS-PAGE of apoE^{null} VLDL and control VLDL
Some 12.5 μg of lipoprotein protein was applied on a 4-20% SDS polyacrylamide gradient gel. After electrophoresis and staining with Coomassie Brilliant Blue the relative apolipoprotein composition of the lipoproteins was determined by densitometric scanning. Lane 1, Marker; Lane 2, apoE^{null} VLDL; and Lane 3, control VLDL.

Binding and uptake of lipoproteins by J774 macrophages

J774 macrophages were incubated with increasing concentrations of ^{125}I-labelled apoE^{null} VLDL or control VLDL in the absence or presence of a 20-fold excess of unlabelled lipoprotein for 3 h at 4°C to measure the total and non-specific binding, respectively. In Figures 2A and 2B, the upper curve represents total binding (specific plus non-specific) of apoE^{null} VLDL and control VLDL, respectively, whereas the lower curve represents non-specific binding, which occurs in the presence of excess

unlabelled iipoprotein. The calculated difference between the two curves represents specific binding (middle curve). J774 cells express a saturable binding site for both apoE^{null} VLDL and control VLDL. Scatchard plot analysis, shown in the insets of the graphs, reveals that the dissociation constants (K_d) are 8.1 and 4.7 µg/ml for apoE^{null} VLDL and control VLDL, respectively; the maximal binding (B_{max}) of apoE^{null} VLDL and control VLDL is 40 and 70 ng/mg cell protein, respectively.

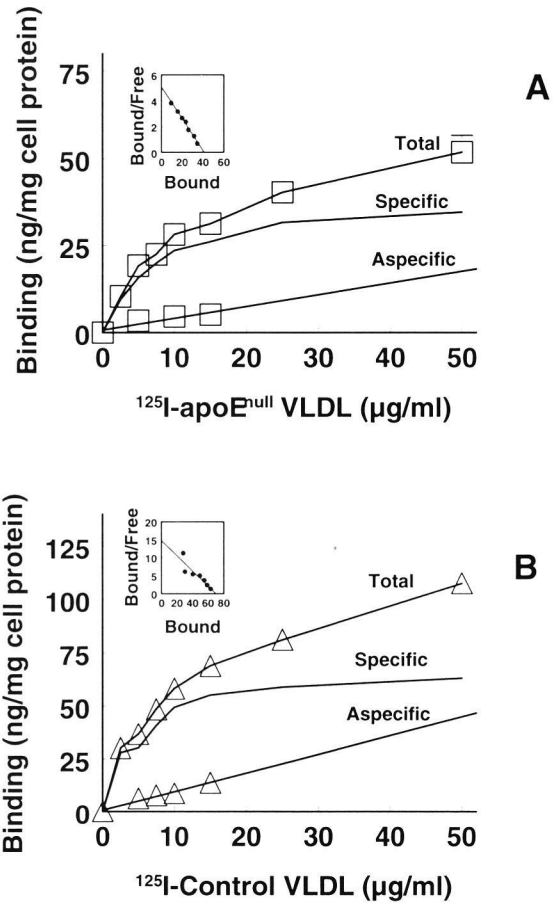

Figure 2 Binding curves of ^{125}I-labelled apoE^{null} VLDL and control VLDL to J774 macrophages

The J774 macrophages were incubated for 3 hours at 4°C with increasing concentrations (from 2.5 to 50 µg/ml) of ^{125}I-labelled apoE^{null} VLDL (A) or control VLDL (B) in the absence or in the presence of a 20-fold excess of unlabelled lipoprotein to measure total (upper curve) and non-specific (lower curve) binding, respectively, as described in 'Materials and Methods'. The specific binding (middle curve) was calculated by substracting the non-specific binding from the total binding. Values represent the mean ± S.D. of four measurements. Insets represent the respective Scatchard analysis.

As presented in Figure 3A, at 10 µg labelled lipoprotein per ml, the high affinity (receptor-mediated) binding of ^{125}I-labelled apoE^{null} VLDL by J774 macrophages is low as compared to ^{125}I-labelled control VLDL (13 ± 6 versus 57 ± 2 ng/mg cell protein, respectively). Addition of 5 µg/ml of LPL resulted in a 20-fold increase of the binding of control VLDL to J774 macrophages, whereas the binding of apoE^{null} VLDL was stimulated about 100-fold upon addition of LPL (compare figure 3A with figure 3B, note the difference in scale).

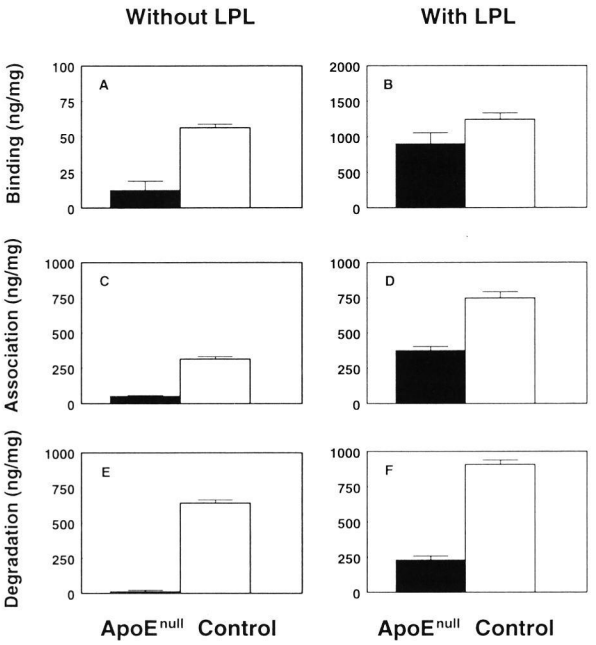

Figure 3 Binding, association and degradation of apoE^{null} VLDL and control VLDL in the absence or in the presence of LPL

The binding (A,B), association (C,D) and degradation (E,F) of apoE^{null} VLDL and control VLDL were measured upon incubation of the cells with 10 µg/ml of labelled lipoprotein at 4°C and 37°C for a period of 3 hours, in the absence (A,C,E) or in the presence (B,D,F) of 5 µg/ml of LPL. Binding, association and degradation are expressed as ng labelled lipoprotein per mg cell protein and were determined as described in 'Materials and Methods'. Values represent the mean ± S.D. of three measurements.

ApoE^{null} VLDL was also associated (bound plus internalized) and degraded by J774 cells via a high affinity process, however to a much lesser extent than control VLDL (55 ± 5 and 15 ± 10 versus 315 ± 19 and 642 ± 22 ng/mg of cell protein, respectively;

figures 3C and 3E). The association and degradation of control VLDL by J774 cells were stimulated approximately 2- and 1.5-fold, respectively, upon addition of 5 µg/ml of LPL. The association and degradation of apoE^{null} VLDL was enhanced by a factor 8 and 16, respectively, in the presence of 5 µg/ml of LPL (compare figures 3C with 3D and 3E with 3F, respectively).

Our data indicate that ^{125}I-labelled apoE^{null} VLDL is bound and internalized by J774 cells via a specific saturable receptor, since its binding, association and degradation is reduced upon addition of an excess amount of unlabelled apoE^{null} VLDL. To identify the nature of the receptor involved in the binding and uptake of apoE^{null} VLDL, competition experiments were performed. Upon addition of 20 µg/ml of unlabelled LDL, the binding of ^{125}I-labelled human LDL by J774 cells was reduced to about 60% of the control binding (Figure 4). Unlabelled control VLDL was much more efficient in competing with ^{125}I-labelled LDL (reduction up to 30% of the control binding). This high efficiency of control VLDL in competing for the binding of LDL can be explained by the fact that control mouse VLDL is relatively rich in apoE, whereas human LDL consists of apoB100 only. ApoE^{null} VLDL was not able to compete with ^{125}I-labelled LDL for binding to the J774 cells.

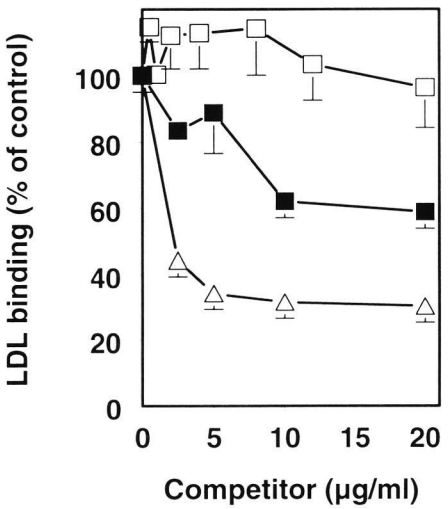

Figure 4 Competition for binding of ^{125}I-labelled human LDL to J774 macrophages by various unlabelled lipoprotein samples

Competition studies were performed by incubating J774 cells with 10 µg/ml of ^{125}I-labelled LDL for 3 hours at 4°C in the presence of the indicated amounts of unlabelled LDL (■), apoE^{null} VLDL (□), and control VLDL (Δ). The binding is expressed as a percentage of the value in the absence of competitor, and is determined as described in 'Materials and Methods'. Values represent the mean ± S.D. of four measurements

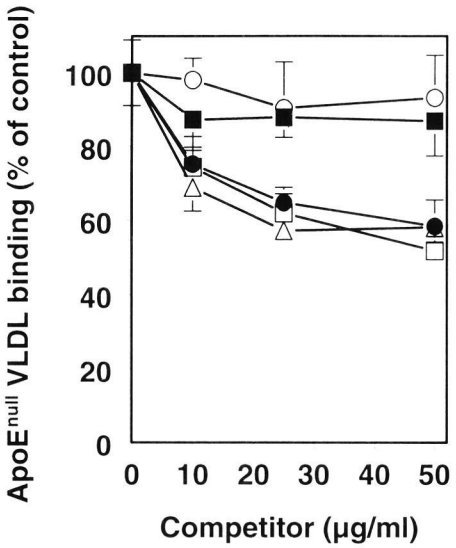

Figure 5 Competition for binding of ^{125}I-labelled apoE^{null} VLDL to J774 macrophages by various unlabelled lipoprotein samples

Competition studies were performed by incubating J774 cells with 10 µg/ml of ^{125}I-labelled apoE^{null} VLDL for 3 hours at 4°C in the presence of the indicated amounts of unlabelled apoE^{null} VLDL (□), control VLDL (Δ), LDL (■), AcLDL (○) or HTG-VLDL (●). The binding is expressed as a percentage of the value in the absence of competitor, and is determined as described in 'Materials and Methods'. Values represent the mean ± S.D. of four measurements.

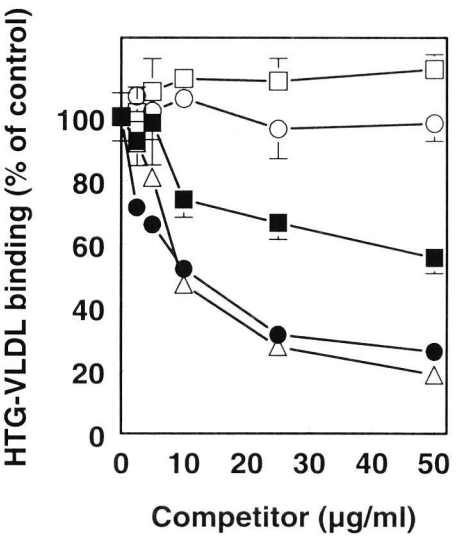

Figure 6 Competition for binding of ^{125}I-labelled HTG-VLDL to J774 macrophages by various unlabelled lipoprotein samples

Competition studies were performed by incubating J774 cells with 10 µg/ml of ^{125}I-labelled HTG-VLDL for 3 hours at 4°C in the presence of the indicated amounts of unlabelled HTG-VLDL (●), apoE^{null} VLDL (□), control VLDL (Δ), LDL (■) or AcLDL (○). The binding is expressed as a percentage of the value in the absence of competitor, and is determined as described in 'Materials and Methods'. Values represent the mean ± S.D. of four measurements.

As shown in Figure 5, the binding of ^{125}I-labelled apoE^{null} VLDL by J774 cells was inhibited to a similar extent (60% of control value) upon addition of either unlabelled apoE^{null} VLDL, control VLDL or human HTG-VLDL. On the contrary, addition of unlabelled LDL or AcLDL did not significantly reduce the binding of ^{125}I-apoE^{null} VLDL. Figure 6 shows that the binding of ^{125}I-labelled HTG-VLDL to the cells was inhibited to approximately 20% of control value by both unlabelled HTG-VLDL and control VLDL

and to 55% of control value by LDL. ApoE^{null} VLDL and AcLDL were not able to compete with ^{125}I-labelled HTG-VLDL for binding to J774 macrophages. From these experiments we conclude that the binding of apoE^{null} VLDL does not occur via the LDL receptor or the scavenger receptor, but that a receptor also recognizing control mouse VLDL and HTG-VLDL is involved in the binding of apoE^{null} VLDL to J774 macrophages.

Accumulation of cholesteryl esters in J774 macrophages after incubation with different lipoproteins

As apoE-deficient mice develop atherosclerotic plaques containing lipid laden macrophages (foam cells), we wondered whether apoE^{null} VLDL, which is deficient in both apoE and apoB100, can cause accumulation of cholesteryl esters (CE) in J774 macrophages. The data presented in Table 2 demonstrate that incubation of J774 cells with apoE^{null} VLDL resulted in a 3-fold increase in cellular CE content. Simultaneous incubation with LPL stimulated the cellular CE content by an additional factor of 5, reaching similar levels as obtained after incubation with control VLDL. However, simultaneous addition of LPL did not stimulate the cellular CE level in J774 cells after incubation with control VLDL.

Table 2 **CE content in J774 cells after incubation with apoE^{null} VLDL and control VLDL**

	CE content *(µg/mg cell protein)*	
	Without LPL	With LPL
No addition	3.0 ± 0.7	3.4 ± 0.4
ApoE^{null} VLDL	9.3 ± 2.0	45.8 ± 5.2
Control VLDL	44.2 ± 8.3	47.1 ± 12.6

Cellular cholesteryl ester (CE) content was measured after a 24 h incubation at 37°C with apoE^{null} VLDL and control VLDL (50 µg lipoprotein protein/ml medium) in the absence or in the presence of 5 µg/ml of LPL. Control incubations were performed in DMEM/1% HSA in the presence or in the absence of 5 µg/ml of LPL without any further additions. The values are expressed as the mean ± S.D. of three measurements.

DISCUSSION

ApoE-deficient mice have been shown to display a marked accumulation in the plasma of chylomicron and VLDL remnant lipoproteins, which are deficient in both apoE and apoB100. ApoE and apoB100 are commonly assumed to be responsible for the receptor-mediated uptake of lipoproteins, also by macrophages. Thus, since apoE-deficient mice develop extensive atherosclerotic lesions with lipid laden macrophages, uptake of lipoproteins by macrophages can take place in the absence of both apoB100 and apoE. In the present paper we studied the mechanisms by which VLDL isolated from apoE-deficient mice ($apoE^{null}$ VLDL) can be taken up by macrophages, by using the murine macrophage cell line J774, which does not produce apoE itself [10].

We found that $apoE^{null}$ VLDL is bound by J774 macrophages via a high affinity, saturable receptor, with a dissociation constant (K_d) of the same order of magnitude than that of control VLDL (Figure 2). Furthermore, uptake of $apoE^{null}$ VLDL resulted in the accumulation of cholesteryl esters in these cells. The LDL receptor was not expected to be a candidate for the uptake of $apoE^{null}$ VLDL, since apoE and apoB100, which are both absent on the surface of this VLDL, are known to be the only ligands for this receptor. We found that the scavenger receptor was also not involved in the uptake by J774 macrophages of $apoE^{null}$ VLDL. However, since the scavenger receptor is involved in the uptake of oxidized lipoproteins by macrophages [29] and lipoprotein oxidation occurs in the intima of apoE-deficient mice [30-32], it is plausible that *in vivo*, the scavenger receptor does indeed contribute to foam cell formation and subsequent development of atherosclerosis in apoE-deficient mice.

Gianturco *et al* [33,34] and Ramprasad *et al* [35,36] described a distinct receptor which is able to mediate the uptake of VLDL from hypertriglyceridemic patients (HTG-VLDL), and which is denominated as the triglyceride-rich lipoprotein (TGRLP) receptor. They reported that this receptor plays a major role in the uptake of HTG-VLDL by human monocyte-macrophages [33,35,36] and murine macrophages [34]. Recently, Gianturco *et al* [37] reported that neither apoB100 nor apoE is necessary to mediate the binding of HTG-VLDL to this receptor and that apoB48 is sufficient for this purpose. The fact that apoB48 and not apoE or apoB100 is present on the surface of $apoE^{null}$ VLDL, supports the hypothesis that the binding and uptake of $apoE^{null}$ VLDL by macrophages occurs via this TGRLP-receptor. This hypothesis is strongly sustained by our observation that HTG-VLDL does indeed compete with VLDL from apoE-deficient mice for binding to the J774 macrophages (Figure 5). The observation that an excess concentration of $apoE^{null}$ VLDL does not compete for the binding of ^{125}I-labelled HTG-VLDL, is not in disaccordance with our hypothesis, since

HTG-VLDL also binds to the cells via apoB100 and apoE, as shown by the finding that both control mouse VLDL and LDL can compete for the binding of ^{125}I-labelled HTG-VLDL (Figure 6). Obviously, this part of the HTG-VLDL binding can not be competed by apoE^{null} VLDL.

It has previously been shown by several groups that LPL stimulates the binding and uptake of LDL and VLDL by different cell types, including macrophages, via bridging between the lipoprotein and the heparan sulphate proteoglycans present on the plasma membrane [12-17]. In the present paper we present data that the binding and uptake of apoE^{null} VLDL by J774 macrophages is also stimulated upon addition of LPL. The finding that the binding of VLDL is stimulated by LPL to a greater extent than both the association and the degradation (Figure 3), suggests that a large part of the LPL-mediated VLDL-binding at 37°C is reversible. This would mean that not all the VLDL which is bound via LPL to the J774 cells, is taken up and, subsequently, degraded. Another explanation could be that under the conditions applied (J774 cells, 3 hours of incubation at 37°C), the LPL-bound VLDL is not internalized and/or degraded as rapidly as VLDL bound to the receptor directly. At present we cannot discriminate between both possibilities. The increased LPL-mediated uptake of apoE^{null} VLDL resulted in an increased accumulation of cholesteryl esters in these cells (Table 2). *In vivo*, LPL is synthesized by macrophages, positively correlated with the amount of intracellular cholesterol [11]. Furthermore, in atherosclerotic lesions high amounts of LPL protein are formed mainly in macrophage-rich intimal regions [38]. Thus, in apoE-deficient mice, the LPL-mediated uptake of VLDL by macrophages may play an important role in the development of atherosclerosis.

The role of apoE in the development of atherosclerotic lesions may be dual. On one hand, the apoE-mediated uptake of lipoproteins by cells of the arterial wall will lead to lipid accumulation and foam cell formation. On the other hand, apoE plays a role in the cholesterol efflux from cholesterol-laden macrophages to extracellular cholesterol acceptors [39]. Thus, the absence in apoE-deficient mice of apoE-mediated cholesterol efflux, may also contribute to the marked atherosclerosis observed in these mice. This is sustained by the finding of Bellosta *et al* [40] that macrophage-specific expression of human apoE is able to prevent or delay the development of atherosclerotic lesions in apoE-deficient mice, even in the presence of high levels of atherogenic lipoproteins.

In summary, in addition to the absence of an apoE-mediated cholesterol efflux from macrophages in apoE-deficient mice, we present evidence that there may be at least two additional processes which contribute to the development of atherosclerosis in apoE-deficient mice. Firstly, VLDL is taken up by macrophages via a specific saturable receptor, possibly the TGRLP receptor, which leads to the accumulation of

cholesteryl esters in these cells. Secondly, this accumulation of cholesteryl esters by macrophages in the intima may be enhanced by production of LPL by macrophages in the intima.

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Chapter 6

Not the Mature 56 kDa Lipoprotein Lipase Protein but a 37 kDa Protein Copurifying with the Lipase Mediates the Binding of Low Density Lipoproteins to J774 Macrophages

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Not the Mature 56 kDa Lipoprotein Lipase Protein but a 37 kDa Protein Copurifying with the Lipase Mediates the Binding of Low Density Lipoproteins to J774 Macrophages

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ABSTRACT

Lipoprotein lipase (LPL) purified from bovine milk showed variable abilities to stimulate the binding of LDL to J774 macrophages. The presence of a 37 kDa protein in the LPL sample seemed to be of importance for its stimulatory capacity. In order to investigate this, we isolated LPL from bovine milk via heparin sepharose chromatography using a continuous salt gradient. Fractions containing the 37 kDa protein (as shown by SDS PAGE under reducing conditions) eluted first from the column, followed by the 56 kDa LPL protein. The LPL enzymatic activity co-eluted with the 56 kDa protein, whereas the amount of 37 kDa protein fully paralleled the stimulatory effect on the binding of LDL to J774 cells. Samples not containing the 37 kDa protein were far less effective in stimulating the binding. Western blotting using a monoclonal antibody 5D2 against amino acids 396-405 in the carboxy-terminal domain of LPL, showed that the 37 kDa protein may be the C-terminal domain of LPL, presumably generated by proteolytic degradation of the mature LPL protein by milk proteases during its isolation. Furthermore, the functional mass of LPL for stimulation of the binding of LDL, as determined by radiation inactivation, was shown to be 30.9 ± 1.8 kDa. We therefore suggest that cleavage of LPL at protease-sensitive sites causes a conformational change, generating an LPL protein which is more effective in mediating the binding and uptake of lipoproteins by cells.

Keywords: Lipoprotein lipase, LPL-mediated uptake, lipoproteins, macrophages, C-terminal domain

Abbreviations used in this paper: LPL, Lipoprotein Lipase; VLDL, Very Low Density Lipoprotein; LDL, Low Density Lipoprotein; LRP, Low Density Lipoprotein Receptor-related Protein; DMEM, Dulbecco's modified Eagle's Medium; FCS, Foetal Calf Serum; BSA, Bovine Serum Albumin; LPDS, Lipoprotein Deficient Serum; PBS, Phosphate Buffered Saline.

INTRODUCTION

Lipoprotein lipase (LPL) is the major enzyme responsible for the hydrolysis of triglycerides in circulating lipoproteins. It also mediates nonenzymatic interactions between lipoproteins and heparan sulphate proteoglycans [1, 2, 3], thereby stimulating the binding and uptake of VLDL and LDL by different cell types [4, 5, 6, 7]. LPL is also a ligand for the low density lipoprotein receptor-related protein (LRP) [8, 9, 10, 11], the LDL receptor [12, 13] and the VLDL receptor [14]. The carboxy-terminal, non-catalytic domain of LPL is thought to be important for LPL binding to either heparan sulphate proteoglycans or receptors [11, 15, 16], indicating that lipase activity is not required for proteoglycan- or receptor-mediated lipoprotein binding. This is also confirmed by Salinelli et al [12], who reported that there is no correlation between bridge function and lipolytic activity.

Whether LPL should have the monomeric or dimeric form for LPL-mediated uptake of lipoproteins is still controversial. Krapp et al [17] and Nykjær et al [10, 15] found that for this function LPL should be in the dimeric state, whereas Williams et al [11] and Chappell et al [16] proposed that LPL monomers are also able to mediate uptake of lipoproteins into cells.

The source of the LPL protein varies among different studies. In many reports bovine milk LPL was used [9, 10, 11, 15, 16, 17], but human LPL [12, 15, 16, 17], and bacterial lipase [10] have also been studied. It was found that the latter does not bind to either α_2MR/LRP or to ß-VLDL [10].

In our laboratory, we make use of LPL purified from bovine milk as described previously [7]. The various LPL samples showed different abilities to stimulate the uptake of LDL by J774 macrophages, varying from less than 2-fold to as high as 50-fold at an LPL protein concentration of 5 µg/ml. It seemed that the stimulatory effect of LPL strongly correlated with the presence of a protein band of approximately 37 kDa, which variably copurifies with LPL (56 kDa).

From our results we suggest that the 37 kDa protein represents a C-terminal part of the LPL molecule, having a conformation more suitable for bridging between lipoproteins and receptors or heparan sulphate proteoglycans than the whole LPL molecule.

MATERIALS AND METHODS

Cells

Murine macrophage-like J774 cells were cultured in 75 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS), 0.85 g/l $NaHCO_3$, 4.76 g/l Hepes, 100 i.u./ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine. The cells were incubated at 37 °C in an atmosphere containing 5% CO_2 in air. For each experiment, cells were plated in 24-well plates. The cells were fed every 3 days, and used for experiments within 7 days of plating. The cells were washed, 24 hours before the experiment, with DMEM supplemented with 1% (w/v) bovine serum albumin (BSA) and further incubated with DMEM containing 5% (v/v) of lipoprotein deficient serum (LPDS) instead of FCS.

Lipoproteins

Blood was obtained from healthy volunteers. Serum was separated from the cells by centrifugation at 1200 g for 10 minutes. LDL was isolated from the serum by density gradient ultracentrifugation by the method of Redgrave et al [18]. The protein content of the LDL fraction was determined by the method of Lowry et al [19], after extensive dialysis against phosphate buffered saline (PBS) containing 10 µM of EDTA. LDL samples were iodinated by using the method of Bilheimer et al [20]. The specific activity ranged from 200 to 300 cpm/ng of protein. After iodination the LDL samples were dialysed extensively against PBS, pH 7.4, stored at 4°C and used within 2 weeks. Whenever unlabelled LDL was used, dialysis against DMEM was performed at 4°C.

Lipoprotein lipase

LPL was isolated from fresh bovine milk as described previously [7], with some minor modifications. In short, Heparin-Sepharose (CL-6B, Pharmacia) was equilibrated with 0.4 M NaCl and added to the skimmed milk. The mixture was shaken gently for 3 hours at 4°C, washed, and transferred to a column. After washing, 1.5 M NaCl, 10 mM KH_2PO_4, pH 6.8 was used as an eluent buffer at a flow rate of 1.5 ml/min. Protein-containing fractions were pooled and diluted with 10 mM KH_2PO_4, pH 6.8 to a final salt concentration of 1 M NaCl, 10 mM KH_2PO_4, pH 6.8. The pooled fractions were applied to a HiTrap Heparin affinity column (Pharmacia) equilibrated with 1 M NaCl, and eluted with a linear gradient of 1-1.7 M NaCl, 10 mM KH_2PO_4, pH 6.8; 1 ml fractions were collected at a flow rate of 1 ml/min. To determine the protein constituents, every fraction was submitted to SDS polyacrylamide gel electrophoresis (SDS-PAGE) using 4-20% gels. Thereafter, as judged by the Coomassie Brilliant Blue stained gels, approximately every third fraction of the elution profile was pooled, and the protein was precipitated and collected as described [7]. After resuspension of the protein in 20 mM NaH_2PO_4, 50% glycerol, the samples were again applied to SDS-PAGE. Proteins were either stained using Coomassie Brilliant Blue or transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) followed by incubation with either a polyclonal antibody against bovine LPL (dilution 1:500) or a monoclonal antibody 5D2 (prepared by Dr. J. Brunzell et al, University of Washington, Seattle [21] at a dilution of 1:250) to detect the protein bands corresponding to lipoprotein lipase. The 5D2 antibody is reported to react within residues 396-405 of the C-terminal folding domain of lipoprotein lipase [21]. As a secondary antibody either swine anti-rabbit or rabbit anti-mouse IgG conjugated to peroxidase (DAKO, Denmark) was used at a dilution of 1:1000. The bands were visualised by using 4-chloro-1-naphtol staining (Merck, Darmstadt, Germany).

Samples were reduced before being applied to SDS-PAGE, using a solution containing a final concentration of 1% SDS, 2.5% ß-Mercaptoethanol, 5% glycerol and 0.0025 % Bromophenol Blue.

Lipoprotein lipase enzymatic activity in the different fractions was measured using a glycerol-stabilised glycerol [9,10 (n-^{3}H)]trioleate suspension as described by Nilsson-Ehle and Schotz [22].

Radiation Inactivation of LPL

The functional molecular mass of LPL with respect to its bridging function was determined by radiation inactivation, a method for determining the functional size of a protein without the need for prior purification. LPL samples were irradiated at different doses as described by Schoonderwoerd et al [23] and subsequently, the effect of the irradiation dose on the stimulatory effect of the LPL sample on the binding of LDL was tested in a binding experiment as described below. The fractional activity (A/A0) is the stimulatory capacity of the irradiated LPL samples (A), divided by the stimulatory capacity of the non-irradiated LPL samples (A0). For calculating the functional molecular mass (M), the ln(A/A0) is plotted against the irradiation dose. The relationship between the molecular mass (M) and the dose is given by the equation M=c·K·St where c=6400 kGy/kDa, St=2.88 (correction factor for the irradiation temperature of -135 °C) and K=slope of the ln (fractional activity A/A0) versus dose plot [23].

Binding of ^{125}I-LDL to J774 macrophages

J774 macrophages were cultured in 24-well plates as described above. Twenty-four hours before the start of the experiment, DMEM supplemented with 5% (vol/vol) of LPDS instead of FCS was added to the cells. J774 cells were incubated for 3 hours at 4°C with 10 µg/ml of ^{125}I-labelled LDL, either in the absence or in the presence of 5 µg/ml of the different LPL fractions and either in the presence or in the absence of a 20-fold excess of unlabelled LDL. The receptor-mediated (specific) cell-binding was calculated by subtracting the amount of labelled lipoproteins that was cell-bound after incubation in the presence of a 20-fold excess of unlabelled lipoprotein (aspecific) from the amount of labelled lipoprotein that was cell-bound after incubation in the absence of unlabelled lipoprotein (total cell binding). After removing the medium, the cells were washed 4 times with ice-cold PBS containing 0.1% (w/v) of bovine serum albumin (BSA), and subsequently with PBS without BSA. Cells were then dissolved in 1 ml of 0.2 N NaOH. Protein content was measured according to Lowry et al [19]. In an aliquot, the radioactivity represented the amount of cell-bound lipoprotein.

RESULTS

In Figure 1, we compared different LPL samples with regard to their pattern on SDS-PAGE and their stimulatory effect on the binding of LDL to J774 macrophages. As shown in Figure 1A, on SDS-PAGE gel some LPL samples contain almost exclusively the 56 kDa band (samples 4 and 5), whereas others display a major protein band of approximately 37 kDa in addition (samples 1,2,3 and 6). LPL samples containing the 56 kDa band only, stimulated the binding of ^{125}I-LDL to J774 cells to a very small extent (compare lanes 4 and 5 in Figure 1A and 1B). The ability of LPL to stimulate the binding of LDL to J774 cells seemed to depend on the presence of a protein of approximately 37 kDa copurifying with the lipase (lanes 1,2,3 and 6).

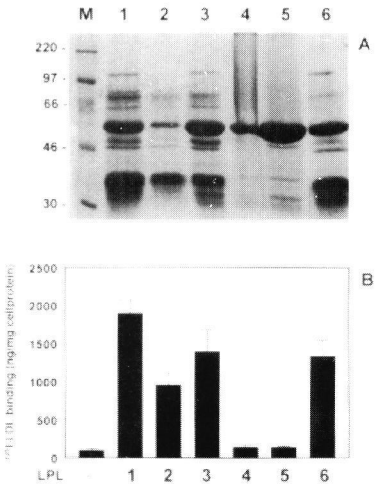

Figure 1 Effect of LPL samples on binding of ^{125}I-LDL to J774 macrophages
About 35 µg of protein was applied to 4% to 20% SDS-polyacrylamide gradient gels. After electrophoresis the gels were stained using Coomassie brilliant blue (Figure 1A). M: Molecular weight marker; lane 1-6: different LPL samples.
The effect of the respective LPL samples on the binding of ^{125}I-LDL was measured after a 3-hour incubation of the cells with 10 µg/ml of ^{125}I-labelled LDL at 4°C in the absence or in the presence of 5 µg/ml of LPL (Figure 1B). Binding is expressed as ng ^{125}I-LDL per mg cell protein and was determined as described in the 'Materials and methods' section. Values represent the mean ± SD of three measurements.

In order to investigate the effect of the presence or the absence of this 37 kDa protein in LPL samples on the binding of LDL to J774 cells, we isolated LPL from bovine milk via heparin sepharose chromatography. The heparin binding fraction was subsequently applied to a HiTrap Heparin affinity column and eluted using a continuous salt gradient. A representative LPL isolation is shown in Figure 2A. SDS-PAGE of fractions across the elution profile indicated several protein constituents (Figure 2B). In the fractions eluting first from the HiTrap column, a protein with an apparent molecular weight of about 37 kDa was present. Fractions eluting from the column at higher salt concentrations contained a major protein band of approximately 56 kDa. A minor 46 kDa protein and some low molecular weight proteins of about 15 to 20 kDa were also found. In Figure 2C

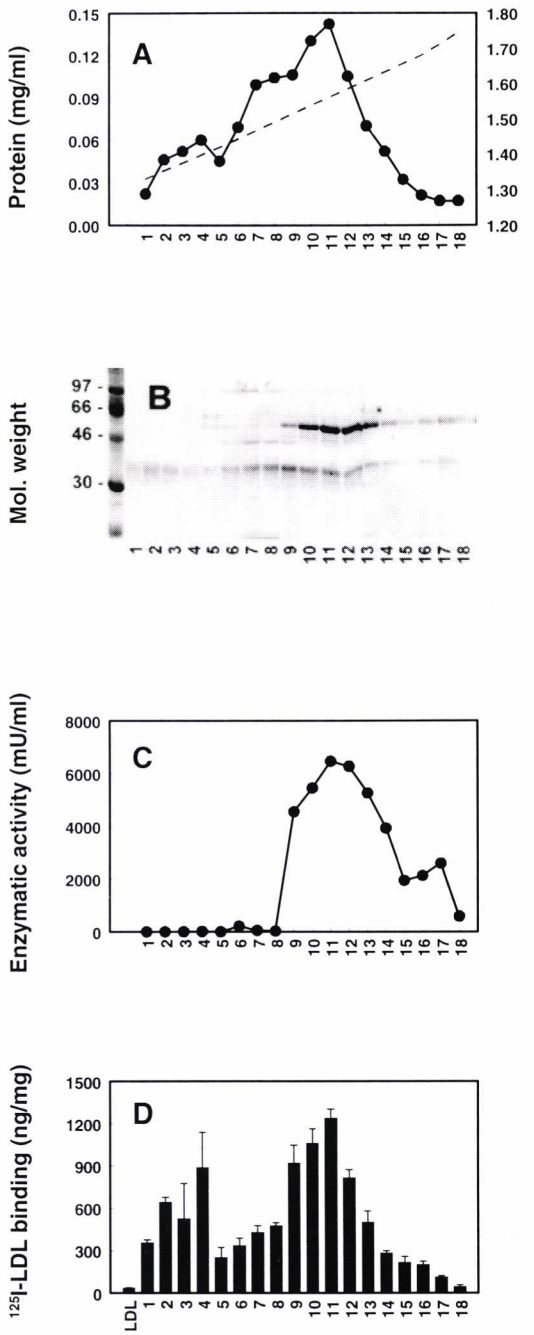

Figure 2 Chromatography on heparin-Sepharose, SDS-PAGE, enzymatic activity and effect on cellular ^{125}I-LDL binding of bovine lipoprotein lipase

Bovine skimmed milk was adjusted to 0.4 M NaCl and added to heparin-Sepharose. The mixture was shaken gently for 3 hours at 4°C, washed, and transferred to a column. After washing, protein was eluted, pooled and applied to a Hi-Trap Heparin affinity column as described in 'Materials and methods'. Fractions were eluted with a linear gradient of 1.0 M to 1.7 M NaCl, 10 mM KH_2PO_4, pH 6.8. Approximately every third fraction of the elution profile was pooled, and protein was precipitated and subsequently resuspended in 20 mM NaH_2PO_4, 50% glycerol. The protein content of the pooled fractions was measured as described in 'Materials and methods' (Figure 2A). Thirty microliters of each LPL fraction was applied to 4% to 20% SDS-polyacrylamide gradient gels. After electrophoresis the gels were stained using Coomassie brilliant blue (Figure 2B). Lipoprotein lipase activity was measured with a glycerol-stabilised glycerol [9,10 (n-^{3}H)] trioleate suspension as described in 'Materials and methods' (Figure 2C). The effect of the respective LPL samples (30 µl/well) on the binding of ^{125}I-LDL (Figure 2D) was measured as described in the legends for Figure 1. Binding is expressed as ng ^{125}I-LDL per mg cell protein. Values represent the mean ± SD of three measurements.

it is shown that fractions containing the 56 kDa band displayed the highest LPL activity. The effect of the different LPL fractions on the binding of ^{125}I-LDL to J774 cells is shown in Figure 2D. Fractions containing only the 37 kDa protein (fractions 1-4) stimulated the binding of LDL to the cells up to about 25-fold. Furthermore, the stimulatory effect of the LPL sample corresponded roughly to the amount of 37 kDa protein in the sample, rather than to the 56 kDa protein.

To find out whether the 37 kDa protein is (a part of) LPL, Western blotting of fractions from another LPL-isolation was performed, using a polyclonal antibody against bovine milk LPL (Figure 3A). The 37 kDa protein bound to this antibody and it is shown that the stimulatory effect of an LPL fraction on the binding of ^{125}I-LDL to J774 cells, corresponded dose-dependently to the 37 kDa protein and not to the 56 kDa protein (Figure 3B).

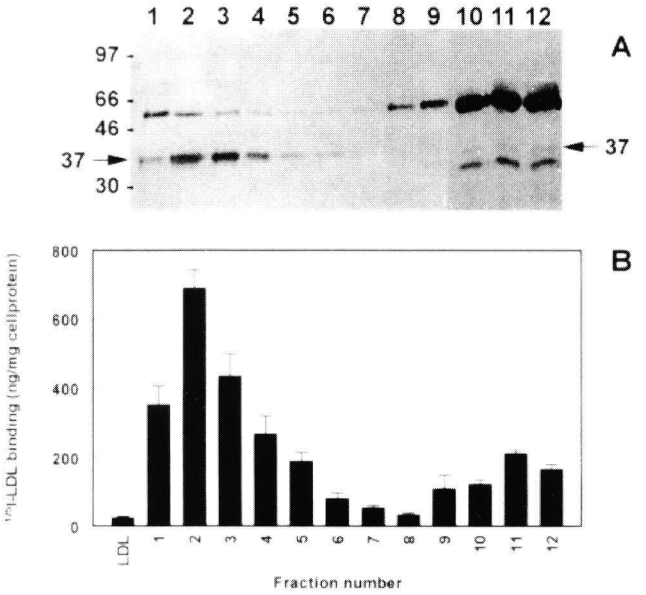

Figure 3 Western blot analysis using a polyclonal antibody and effect on cellular ^{125}I-LDL binding of bovine lipoprotein lipase

About 1 µg of protein was subjected to 4%-20% SDS-polyacrylamide gradient gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated with a polyclonal antibody against bovine LPL at a dilution of 1:500. As a secondary antibody swine anti-rabbit IgG conjugated to peroxidase was used at a dilution of 1:1000. The bands were visualised by using the 4-chloro-1-naphtol staining (Figure 3A). The effect of the respective LPL samples (at concentration of 5 µg/ml) on the binding of ^{125}I-LDL (Figure 3B) was measured as described in the legends for Figure 1. Binding is expressed as ng ^{125}I-LDL per mg cell protein and was determined as described in the 'Materials and methods' section. Values represent the mean ± SD of three measurements.

Since it is known that binding sites for lipoproteins and receptors are within the carboxy-terminal domain of LPL [10, 11, 13, 16], we hypothesised that the 37 kDa protein may be the C-terminal part of LPL. To test this hypothesis, we performed Western blotting using the monoclonal antibody 5D2, which is reported to react within residues 396-405 of the C-terminal domain of LPL [21]. We found that the 5D2 antibody binds to both the 56 kDa protein and the 37 kDa protein, in a similar pattern as shown in lanes 10-12 of Figure 3A.

To prove that mainly the 37 kDa protein rather than the 56 kDa LPL protein mediates the binding of lipoproteins to cells, we subjected an LPL fraction which contained both the 37 and the 56 kDa protein, to radiation inactivation. With this technique, the biological activity of a sample is decreased by exposure to increasing doses of radiation, whereby relatively large molecules are inactivated at relatively low radiation doses as compared to small molecules. Thus, by using an LPL sample that has been irradiated with different doses, the size of the smallest unit required to stimulate the binding of LDL to cells (functional molecular weight) can be determined without the need for prior purification [24]. The effect of various doses of irradiation on the stimulatory effect of an LPL sample on the binding of ^{125}I-LDL to J774 cells is shown in Figure 4. The fractional activity A/A0 decreased with increasing irradiation doses. The functional molecular mass was calculated to be 30.9 ± 1.8 kDa, which is in the same order of magnitude as 37 kDa. We therefore conclude that in an LPL sample, the 37 kDa protein presumably representing the C-terminal 300 amino acids of lipoprotein lipase, is the major component that stimulates the binding of ^{125}I-LDL to cells.

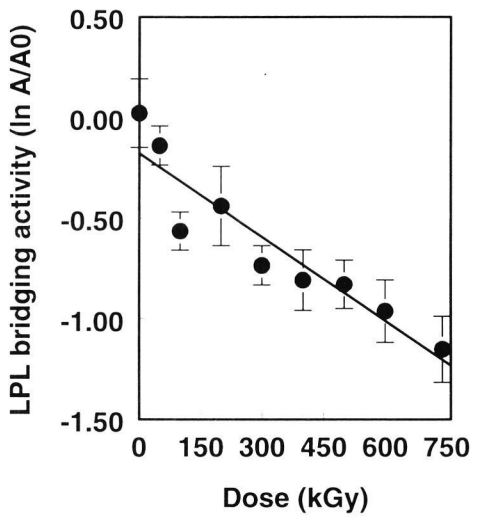

Figure 4 Radiation inactivation of LPL
The effect of irradiated LPL samples on the binding of ^{125}I-LDL to J774 macrophages was determined as described in the 'Materials and methods' section. Data are expressed as ln [stimulation factor of irradiated samples (A) divided by the stimulation factor of non-irradiated samples (A0)]. The fitted line is from least-square analysis and the functional molecular mass (M) is calculated according to the formula M=c·K·St as described in 'Materials and Methods'.

DISCUSSION

The present study was conducted to elucidate the role of a 37 kDa protein, copurifying with bovine milk lipoprotein lipase, in the binding of LDL by J774 macrophages. LPL samples containing this protein (as shown in Figure 1A by SDS-PAGE under reducing conditions) were much more potent in stimulating the binding and uptake of ^{125}I-labelled LDL by J774 macrophages than LPL samples without the 37 kDa protein band. In this figure, there is no correlation between the amount of 37 kDa protein present in the sample and the extent of stimulation of LDL-binding. This can be explained by the fact that the LPL samples shown in Figure 1A are obtained from different, independent LPL isolations over a two year time period. Since the activity of a freshly isolated LPL sample, with respect to its bridging function, varies between the different isolations, the LPL samples shown in Figure 1 can not be compared with regard to the amount of stimulatory effect on the binding of LDL.

We also found that the presence of the 37 kDa protein in LPL samples resulted in an increased stimulatory effect on the association and degradation of LDL to J774 cells, in a similar way as for the binding (data not shown). This indicates that the 37 kDa protein can affect the intracellular lipid accumulation in macrophages and may indeed influence the atherogenic process.

Several lines of evidence indicated that the 37 kDa protein may be the C-terminal domain of lipoprotein lipase. First, Western blotting using a monoclonal antibody 5D2 against amino acid residues 396-405 of LPL (originally prepared by Brunzell et al, University of Washington, Seattle [21]), showed that the 37 kDa protein contained the carboxy-terminal region of LPL. Furthermore, by using the method of radiation inactivation, we showed that the functional molecular mass of lipoprotein lipase with respect to its bridging function was about 31 kDa, which is of the same order of magnitude as 37 kDa. However, it may be argued that the graph shown in Figure 4 can also be drawn in a bi-phasic manner, indicating that there may be two sizes of the LPL molecule that can mediate the binding of lipoproteins to cells; one being approximately 100 kDa, presumably representing the LPL dimer. The second, in accordance with our previous findings, is approximately 30 kDa. This is in accordance with our finding that the LPL protein itself can stimulate the binding of LDL to cells (Figure 3), however to a much lesser extent than the 37 kDa protein.

If the 37 kDa protein indeed represents the C-terminal part of bovine LPL, it would approximately comprise residues 150-450 of the LPL molecule. Although an attempt was made to establish definite proof, sequencing the N-terminal part of the 37 kDa protein was not entirely successful. However, the first two residues were analysed as lysines, and in bovine LPL, a Lys-Lys sequence only occurs at residues 149-150 and

414-415. Thus, the finding that the N-terminal sequence starts with two lysine residues, sustains our hypothesis that the 37 kDa protein may represent the C-terminal part of the LPL molecule. However, we cannot definitely rule out the possibility that the 37 kDa band represents (an)other protein(s), which are able to stimulate the binding of LDL to cells.

It has been shown by several groups that the carboxy-terminal domain of LPL binds to several receptors and stimulates the receptor-mediated binding and catabolism of lipoproteins by different cell types, in a process facilitated by cell surface proteoglycans [10, 11, 13, 16]. Furthermore, Socorro and Jackson have shown that milk proteases, co-purifying with the LPL, cause degradation of the lipase [25, 26]. They reported LPL samples containing additional proteins with molecular weights of 36 kDa and 18-22 kDa, which represented proteolytic degradation products of LPL. Bengtsson-Olivecrona et al [27] also reported on the presence of protease-sensitive regions within the LPL molecule. Cleavage of the LPL protein at these sites by trypsin or plasmin resulted in the production of three fragments of apparent sizes of about 32-38 kDa; one of these (the T1 fragment) appeared to be the carboxy-terminal domain of LPL. The fragments are held together by disulphide bonds, and they are only shown on SDS-PAGE gels under reducing conditions. Furthermore, it is known that plasmin is present in milk [28].

From these previous reports and the results presented in this study, we hypothesise that LPL can be degraded by milk proteases during its isolation, resulting in the appearance of a 37 kDa protein band on SDS-PAGE gels under reducing conditions. Furthermore, we hypothesise that the proteolytic cleavage of LPL results in such a conformational change of the LPL molecule, that its ability to bridge between lipoproteins and receptors and/or proteoglycans is dramatically increased. This hypothesis is sustained by the current thought that binding sites for lipoproteins and lipoprotein receptors are in close proximity within the carboxy-terminal domain of LPL [10]. Cleavage of the LPL subunit may facilitate binding of both the lipoprotein and the receptor to the LPL monomer at the same time.

In Figure 3 we showed that the 56 kDa protein itself can also stimulate the binding of LDL to cells, although this stimulation is much less effective as compared to the stimulatory effect of the 37 kDa protein. The finding that the 56 kDa LPL protein can stimulate the binding of lipoproteins, is in accordance with the results of Salinelli et al [12], who performed binding experiments by using transfected COS cells producing the wild type human LPL and several mutant LPLs. They found that the binding and catabolism of VLDL was stimulated in the cells producing the wild type LPL, whereas the non-transfected cells did not bind or degrade VLDL. However, from their experiments the specific activity of LPL in this respect cannot be deduced, since the amount of LPL

produced by the transfected COS cells was not reported. Furthermore, in their system the LPL molecule may also have been cleaved.

It has been reported that macrophages in atherosclerotic plaques synthesise LPL and that the concentration of LPL in the vessel wall is related to the concentration of cholesterol in the vessel wall [29]. Thus, in the intima, an LPL-mediated increase of lipoprotein uptake by macrophages most probably occurs and may be sufficient to enhance the formation of foam cells and, eventually, atherosclerotic plaques. However, if LPL cleavage does indeed occur *in vivo* in the intima, the LPL-mediated stimulation of the uptake of lipoproteins by macrophages would be even more relevant with respect to the development of atherosclerosis.

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Chapter 7

GENERAL DISCUSSION

One of the objectives of this thesis was to study the role of lipoprotein lipase (LPL) in the atherogenic process. It was reported that LPL enhances the binding and uptake of different lipoproteins by various cell types. This process involves bridging of LPL between the lipoproteins and heparan sulphate proteoglycans (HSPG) on the cell surface, which, in turn, facilitate the uptake of the lipoproteins by their receptors [1,2,3,4,5,6,7,8,9,10]. It was suggested that, if this process takes place in the intima of the vessel wall, LPL may play an important role in atherogenesis. This hypothesis was further supported by the observation that cells of the arterial wall, including macrophages, secrete LPL, most probably dose-dependently with the amount of cholesterol which is present [11]. It was also reported that oxidatively modified LDL, which is taken up at an advanced rate by macrophages, is present in atherosclerotic lesions *in vivo* [12]. At that time, it was not known whether LPL also stimulates the uptake of oxidatively modified LDL, and in Chapter 2 we present data on this subject. We found that LPL stimulates the uptake of moderately oxidized LDL by the murine macrophage cell line J774, resulting in an increased accumulation of cholesteryl esters in these cells. In addition to the finding that oxidative stress enhances the production of LPL by macrophages [13], our results provide further evidence for the hypothesis that LPL may play an important role in foam cell formation *in vivo*, by enhancing the uptake of atherogenic lipoproteins by macrophages in the intima.

Recently, another pro-atherogenic role for LPL has been reported by Mamputu *et al* [14]. They showed that LPL facilitates monocyte-adhesion to endothelial cells, in a process involving HSPG. The adherence of monocytes to vascular endothelial cells is one of the earliest events observed in atherogenesis [15]. Presumably, the adherent monocytes migrate into the intima under the influence of various chemotactic proteins as described below, leading to further development of the lesion. Hence, the LPL-induced monocyte adhesion to the endothelium can be considered as a pro-atherogenic factor.

In Chapter 2 we furthermore report that the uptake of severely oxidized LDL was not enhanced upon addition of LPL, and we proposed that this was caused by a somewhat decreased binding of LPL to severely oxidized LDL on one hand, and,

more importantly, by a decreased binding of the LPL-lipoprotein-complex to HSPG, on the other hand. We suggested that the latter may be due to electrostatic repulsion forces, caused by the negative charges of both the severely oxidized LDL particle and the proteoglycans. There is some controversy in literature about the binding affinity of oxidized LDL for proteoglycans, either in the presence or absence of LPL. In contrast to our results, Auerbach *et al* [16] reported that the affinity of matrix-bound LPL for oxidized LDL was increased when compared with that for native LDL. Also Kaplan and Aviram [17] showed that oxidation of LDL increases its affinity for proteoglycans, which could be further stimulated by addition of LPL. However, in accordance with our findings, Öörni *et al* [18] and Chait *et al* [19] reported that oxidative modification of LDL decreases its affinity for proteoglycans, due to a modification of the lysine residues of apoB100. These discrepancies implicate that, in order to further elucidate the contribution of LPL and oxidized LDL to the atherogenic process, further research into the LPL-mediated uptake of oxidized LDL by macrophages is needed. As to the implications for the *in vivo* situation in the vessel wall, one should keep in mind that the presence of a severely oxidized LDL particle, having an electrophoretic mobility relative to that of native LDL of more than 3, is highly unlikely [12,20,21].

Recently, new aspects about the roles of oxidized LDL and proteoglycans in atherogenesis have been reported. Pillarisetti *et al* [22] found that oxidized LDL stimulates the production of heparanase by endothelial cells, resulting in the reduction of HSPG. The removal of HSPG gives rise to the exposition of fibronectin, a binding site for atherogenic lipoproteins, such as Lp(a). In addition, Chait *et al* [19], reported that rather than to proteoglycans, oxidized LDL binds to other molecules secreted by arterial cells, possibly fibronectin. It is therefore suggested that loss of HSPG, which occurs during atherogenesis [23], is a pro-atherogenic process. These findings open new perspectives about the role of oxidized LDL in atherosclerosis, and may stimulate further research into the mechanisms of action of this particle.

Many questions remain unanswered regarding oxidized LDL and its effects on the atherogenic process *in vivo*. First, it is quite certain that LDL oxidation occurs in the intima of the vessel wall, but it may very well also occur at any site of inflammation where LDL is exposed to activated macrophages. The consequences of the occurrence of oxidized LDL on sites other than in the intima, remain to be elucidated. Second, whether the *ex vivo* susceptibility of LDL to oxidation is a good predictor for the formation of fatty streaks *in vivo,* remains to be seen. Finally, a lot of intervention studies have been performed in experimental animals, showing a inhibitory effect of anti-oxidant drugs on the progression of atherosclerosis. To establish the protective effect of anti-oxidants such as vitamin E against LDL-oxidation and atherosclerosis in humans, large-scale intervention trials in humans will

have to be performed. One such trial has been reported [24], showing that high doses of vitamin E prevent non-fatal myocardial infarctions in patients with angina and coronary atherosclerosis, although there was no beneficial effect on cardiovascular deaths. However, the patients involved in this study had very advanced disease, and a trial including subjects in a more early stage of atherosclerosis would be more informative about the direct effect of anti-oxidants on fatty streak formation.

In general, as described in Chapter 1.5.2., oxidized LDL in the intima of the vessel wall is suggested to play a role in atherogenesis in various ways. First, it stimulates the expression of MCP-1 by macrophages [25], smooth muscle cells, and endothelial cells [26], resulting in the recruitment of monocytes into the intima. Furthermore, oxidized LDL itself is a chemotactic agent for monocytes [27]. The monocytes in the intima subsequently differentiate into resident macrophages. Second, it inhibits the motility of resident macrophages, thereby preventing them from leaving the intima to re-enter the lumen [28]. Third, it can be taken up at an enhanced rate via scavenger receptors by macrophages present in the intima, leading to cholesterol-loading of the cell and subsequent foam cell formation [29]. Finally, oxidized LDL is cytotoxic to the endothelium of the vessel wall, leading to loss of the endothelial integrity, and generation of the necrotic core of the lesion [30,31]. By intervening in one or more of these processes, it may in the future be possible to prevent or delay the atherosclerotic process. Anti-oxidant therapy to prevent LDL-oxidation at an early stage has already been mentioned. Furthermore, regulation of the expression of MCP-1 or other chemotactic proteins such as monocyte colony stimulating factor (MCSF), and proteins to which monocytes adhere at the endothelium (vascular cell adhesion molecules, VCAM; endothelial leukocyte adhesion molecules, ELAM), may slow the progression of the disease. Naturally, these possible treatments against the oxidation of LDL and its effects on the atherogenic process in the intima, should be in combination with cholesterol-lowering therapy.

At the time the work described in Chapter 3 was initiated, little was known about the oxidation of VLDL. We hypothesised that oxidation of VLDL, like oxidation of LDL, may occur *in vivo,* and we therefore studied the processing of oxidized VLDL by J774 macrophages. We found that oxidized VLDL causes less accumulation of triglycerides in J774 macrophages when compared with native VLDL, as a consequence of being a poor substrate for LPL-mediated hydrolysis. Our results emphasize the significant role of extracellular LPL activity in the uptake of triglyceride-rich lipoproteins by cells and subsequent intracellular lipid accumulation. On one hand, enzymatic lipase activity is needed for hydrolysis of trigycerides into free fatty acids, which are subsequently taken up by the cell and re-esterified into triglycerides.

The remaining cholesterol-rich remnant is taken up via a receptor-mediated process. On the other hand, LPL can mediate the uptake of lipoproteins via bridging between the lipoproteins and the cell surface proteoglycans on the cell surface, as described above. At present, we have no data on the latter pathway, *i.e.* the LPL-mediated uptake of oxidized VLDL particles. It would be of importance to know whether the uptake of oxidized VLDL is stimulated by LPL, in a similar way as moderately oxidized LDL [10], leading to an enhanced intracellular lipid accumulation.

Up to now, there are no reports providing evidence that oxidized VLDL, like oxidized LDL, is present in atherosclerotic lesions *in vivo*. As mentioned above, the degree of oxidation of LDL in the atherosclerotic plaque is usually very modest, with an electrophoretic mobility on agarose gel of about twice that of native LDL [12,20,21]. Since it has been shown that VLDL can undergo oxidation *in vitro* [32,33], we suggest that VLDL oxidation may occur in the intima *in vivo*, probably to a similar modest degree as that of LDL. This should be taken into consideration if one speculates about the *in vivo* atherogenicity of oxidized VLDL as compared to that of native VLDL. Our findings that OxVLDL is not a good substrate for LPL, and therefore causes less lipid accumulation in macrophages, imply that OxVLDL is less atherogenic than native VLDL. However, if the VLDL particle is only minimally oxidized, the efficiency of LPL activity may still be considerable. Furthermore, oxidized lipoproteins are suggested to play a role in the atherogenic process in various other ways. Recently, it has been found that oxidized VLDL, similar to oxidized LDL, enhances the expression of monocyte chemoattractant protein-1 (MCP-1) by macrophages *in vitro* [25]. It is suggested that oxidized LDL and VLDL play a role in atherogenesis by stimulating the recruitment of monocytes into the subendothelial space, followed by their differentiation into macrophages. Furthermore, it may be that OxVLDL, like OxLDL [30,31] is cytotoxic to the endothelium of the vessel wall, leading to loss of the endothelial integrity and facilitating the entering of even more monocytes into the intima.

One of the roles of apolipoprotein E (apoE) in lipid metabolism is to mediate the clearance of remnant lipoproteins from the circulation. This process is thought to be mediated via a so-called secretion-recapture process, in which the remnants are enriched in liver-derived apoE in the space of Disse. This results in an increased binding of the remnants to the heparan sulphate proteoglycans (HSPG) present on the hepatic cells. Subsequently, the remnants are taken up by the liver via either the LDL receptor or the LRP, as described in Chapter 1.2.1. It is also postulated that LPL plays a role in remnant clearance, by enhancing the uptake of the remnants by the

receptor, in a process which is initiated by the bridging of LPL between the proteoglycans and the lipoproteins. It was reported by Ji *et al* [34] and Mann *et al* [35], that ß-VLDL enriched in various apoE variants, displayed different binding affinities for proteoglycans. They suggested a correlation between the expression of type III hyperlipidemia and the binding affinity of ApoE mutations for proteoglycans: ß-VLDL enriched in apoE mutations causing a dominant form of the disease (apoE3 Leiden, apoE(Arg^{142}→Cys)) bound less efficiently to HSPG as compared to particles enriched in apoE mutations causing a recessive form (apoE2(Arg^{158}→Cys)). ß-VLDL enriched in ApoE3 showed the highest binding affinity for HSPG. However, in Chapter 4 we showed that both the binding affinity and maximal binding capacity of different ß-VLDL samples, including that of ß-VLDL containing high amounts of apoE2, to HSPG coated to microtiter plates is very low. As discussed in this chapter, differences in the method of apoE-enrichment of the ß-VLDL particles, and whether or not free apoE is present during the incubation, may explain the discrepancies between our results and those of Ji *et al* [34] and Mann *et al* [35].

In accordance with Lookene *et al* [36], we found that addition of LPL markedly enhanced the K_a and the B_{max} values for the binding to HSPG of Enull-VLDL, apoE-VLDL, and apoE3-Leiden-VLDL. We showed that for the binding of ß-VLDL to HSPG-LPL complexes, the presence of apoE is not a prerequisite, but results in an increased binding affinity of ß-VLDL for these complexes. Considering the facts that macrophages present in atherosclerotic lesions, secrete LPL as well as HSPG, and that in these lesions apoE is abundantly present [37], our results further emphasize the important roles of LPL, HSPG and apoE in the atherosclerotic process.

ApoE-deficient mice display a marked accumulation in the plasma of chylomicron and VLDL remnant lipoproteins, which are deficient in both apoE and apoB100. Since apoE and apoB100 are the common ligands for the receptor-mediated uptake of lipoproteins, and since apoE-deficient mice develop severe atherosclerosis with lipid laden macrophages, we reasoned that uptake of lipoproteins by macrophages can take place in the absence of these apolipoproteins. In Chapter 5 we investigated the mechanisms by which VLDL isolated from apoE-deficient mice can be taken up by J774 macrophages. We found that apoE-deficient VLDL is taken up via a high affinity receptor, which may be the triglyceride-rich lipoprotein (TGRLP) receptor present on macrophages, described by Gianturco *et al* [38,39] and Ramprasad *et al* [40,41]. This receptor recognises apoB48, which is present on VLDL isolated from apoE-deficient mice, as a ligand to mediate the binding of VLDL, and does not need apoE or apoB100 for this purpose [42]. It would be of interest to determine the effect of ectopic

expression of the TGRLP receptor in the liver of apoE-deficient mice, via adenovirus-mediated gene transfer. If the apoE-deficient VLDL particles are indeed taken up via this receptor, one would expect a reduction in plasma lipid levels in apoE-deficient mice transfected with the TGRLP receptor virus. Since adenovirus-mediated gene transfer only results in short-term expression of the receptor, and since apoE also functions as a mediator of cholesterol-efflux from macrophages in the vessel wall [43,44,45], the effect on atherosclerosis in these mice will most probalby be nihil.

Our observation that the presence of a protein band of approximately 37 kDa in an LPL sample was essential for the stimulatory effect of this sample on the binding of LDL to J774 cells, led to the investigations described in Chapter 6. We presented several lines of evidence that the 37 kDa protein may be the carboxy-terminal domain of LPL, generated by proteolytic cleavage of the mature LPL molecule by proteases during its isolation from bovine milk. Since the carboxy-terminal domain of LPL contains binding sites for heparan sulphate proteoglycans, lipoprotein receptors, and lipoproteins, we hypothesized from our findings that a conformational change of the LPL protein, due to cleavage by proteases during the isolation of LPL from bovine milk, results in an enhanced ability of LPL to bridge between lipoproteins and receptors and/or proteoglycans.

Our results do not rule out the possibility that the 37 kDa protein represents (an)other protein(s) which can stimulate the binding and uptake of lipoproteins by cells. Sequencing of the 37 kDa band would provide definite proof about the identity of this protein, and it would clarify the implications of our findings for the exact role of LPL in the uptake of lipopoteins. If the 37 kDa protein indeed turns out to be a protein other than LPL, the interpretation of reports, including our own, regarding the stimulatory effect of lipoprotein lipase on uptake of lipoproteins by cells should be reconsidered.

Usually the purity of the used LPL samples is not shown. Thus, it may be that proteins other than LPL play a role in the effects reported. On the other hand, studies by Kawamura *et al* [46] and Salinelli *et al* [47], showed that expression of wild type human LPL by transiently transfected COS cells resulted in an enhanced binding, uptake and degradation of VLDL as compared to non-transfected cells, indicating that LPL itself is capable of bridging. Although the amount of stimulation in these papers was only 2- to 4-fold, no direct comparison with our results can be made, since the specific activity of the LPL secreted by the transfected cells was not reported. In agreement with the latter two reports, in Chapter 6 we also show that the 56 kDa LPL protein can enhance the binding of LDL to J774 cells, although to a much lesser

extent than the 37 kDa protein. Our current thought is that in the intima of the vessel wall, LPL acts as a pro-atherogenic component by stimulating the uptake of lipoproteins by macrophages. It may be that during the atherosclerotic process, various proteolytic enzymes are activated. Cleavage of the LPL molecule by these enzymes at protease-sensitive sites, results in a further enhancement of the stimulatory capacity of LPL, and progression of the disease. Further investigations however, are needed to sustain our hypothesis.

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Summary

Lipoprotein lipase (LPL) enhances the binding and uptake of lipoproteins by various cell types, including macrophages, through bridging of LPL between the lipoproteins and the heparan sulphate proteoglycans (HSPG) present on the cell membrane. These findings suggested that LPL may play an important role in the formation of foam cells, and thus in atherosclerosis. The atherogenicity of oxidized LDL *in vitro*, and its presence in atherosclerotic plaques *in vivo*, had also already been established. Since it was not known whether LPL also stimulates the binding and uptake of oxidized LDL, we decided to investigate the effect of LPL on the binding and uptake of LDL which was chemically oxidized to different degrees, by the murine macrophage cell line J774 (Chapter 2). We found that LPL dose-dependently stimulated the binding and uptake of both native LDL and moderately oxidized LDL by the macrophages, leading to an increased intracellular cholesteryl ester content. Addition of heparinase inhibited the LPL-mediated stimulation, indicating that it was due to bridging of LPL between the lipoproteins and the HSPG. The binding and uptake of both severely oxidized LDL and acetylated LDL was not stimulated by LPL, probably due to electrostatic repulsion forces of the LPL-lipoprotein complex and the HSPG, which are both negatively charged. These data further establish the pro-atherogenic role of LPL in the intima of the vessel wall.

At the time the work described in Chapter 3 was initiated, little was known about the oxidation of VLDL. Since we thought it likely that oxidized VLDL, like oxidized LDL, may occur *in vivo*, we examined the interactions of oxidized VLDL (OxVLDL) with J774 macrophages (Chapter 3). We found that the uptake of ^{125}I-labelled VLDL by the J774 cells increased with oxidation time. Despite this increased uptake of OxVLDL protein, the cell association of triglyceride-derived fatty acids by the J774 macrophages was less pronounced after incubation with OxVLDL, than with native VLDL. Also the *de novo* synthesis of triglycerides was approximately 3-fold less efficient after incubation with OxVLDL than after incubation with VLDL. In the presence of polyinosinic acid (PolyI), the receptor-mediated uptake of OxVLDL is completely inhibited, indicating that OxVLDL is taken up by the cells via the scavenger receptor. Furthermore, the OxVLDL-induced *de novo* synthesis of triglycerides is reduced to control levels in the presence of PolyI. These results indicated that OxVLDL stimulates the synthesis of triglycerides by J774 cells only through uptake of OxVLDL via the scavenger receptor, and not via extracellular lipolysis of OxVLDL-triglycerides, as is the case for native VLDL. We presented evidence that oxidation decreases the suitability of VLDL as a substrate for LPL, leading to an impaired LPL-mediated hydrolysis of triglycerides. Therefore, we conclude that

oxidized VLDL is a less efficient inducer of triglyceride accumulation in J774 cells when compared with native VLDL, as a consequence of an impaired extracellular lipolysis.

The role of apoE in lipoprotein metabolism is further addressed in this thesis. In Chapter 4 we investigated the effect of both the isoform and the amount of apoE per particle, as well as the role of LPL on the binding of ß-VLDL to HSPG. Therefore, we isolated ß-VLDL from transgenic mice, expressing either APOE*2(Arg158→Cys) or APOE*3-Leiden (E2-VLDL and E3Leiden-VLDL, respectively), as well as from apoE-deficient mice (Enull-VLDL). In the absence of LPL, the binding affinity and maximal binding capacity of all ß-VLDL samples for HSPG coated to microtiter plates, are very low. Addition of LPL to this cell-free system, resulted in a marked increase in both the K_a and the B_{max} of all three ß-VLDL particles tested. In the presence of LPL, the K_a increased in the order Enull-VLDL < E2-VLDL < E3Leiden-VLDL, whereas the B_{max} increased in reversed order. Similarly, in the J774 cells, addition of LPL resulted also in an increase in the maximal binding capacity of the three ß-VLDL samples. In addition, both the K_a and the B_{max} for the binding of ß-VLDL to J774 cells in the presence of LPL, increase in the same order as found in the cell-free system. From these results we conclude that for the binding of ß-VLDL to HSPG-LPL complexes, the presence of apoE is not a prerequisite, but results in an increased binding affinity.

ApoE-deficient mice display a marked accumulation in the plasma of VLDL lipoproteins which are deficient in both apoE and apoB100. Since apoE-deficient mice develop severe atherosclerotic lesions with lipid-laden macrophages, we reasoned (Chapter 5) that the uptake of lipoproteins by intimal macrophages can take place in the absence of both apoB100 and apoE, which are the common ligands for receptor-mediated uptake of lipoproteins. To obtain more insight into the mechanism of foam cell formation in apoE-deficient mice, we measured the interaction of VLDL isolated from these mice with J774 macrophages. We found that apoE-deficient VLDL is bound and taken up by these cells, although less efficiently than control VLDL, via a high affinity process. Furthermore, incubation of the cells with apoE-deficient VLDL, resulted in an increased intracellular cholesteryl ester content. Addition of LPL further enhanced the binding, uptake and degradation of apoE-deficient VLDL by the J774 macrophages, as well as the accumulation of cholesteryl esters in these cells after incubation with apoE-deficient VLDL. Competition experiments showed that the receptor mediating the uptake of apoE-deficient VLDL by J774 cells, may be the triglyceride-rich lipoprotein receptor, which recognises apoB48 as a ligand. Thus, in addition to other proposed mechanisms mediating atherosclerosis in apoE-deficient mice (*i.e.* absence of apoE-mediated cholesterol-efflux from macrophages, and oxidation of apoE-deficient VLDL followed by its uptake via the scavenger receptor), we presented evidence that in apoE-deficient mice, VLDL is taken up by macrophages via a distinct receptor, leading to the

accumulation of cholesteryl esters in these cells. Furthermore, the accumulation of cholesteryl esters is stimulated by the presence of LPL.

Finally, in Chapter 6 we examined the role of a 37 kDa protein co-purifying with LPL during its isolation from bovine milk. This protein seemed to be obligatory for the stimulatory effect of LPL on the binding of lipoproteins by J774 cells. Fractions containing this 37 kDa protein eluted first from the heparin-sepharose column, followed by the 56 kDa LPL protein. We found that lipase activity co-eluted with the 56 kDa protein, whereas the amount of 37 kDa protein corresponded with the stimulatory effect of the sample on the binding of LDL to J774 macrophages. Western blotting using the monoclonal antibody 5D2 against the carboxy-terminal domain of LPL, showed that the 37 kDa protein may be C-terminal domain of LPL. Furthermore, the functional molecular mass of LPL for the stimulation of the binding of LDL, as determined by radiation-inactivation, was shown to be about 31 kDa. From these results we concluded that the 37 kDa protein, mediating the binding of LDL to cells, may be the carboxy-terminal domain of LPL, presumably generated by proteolytic degradation of the mature LPL protein by milk proteases. We hypothesise that cleavage of LPL at protease-sensitive sites, results in a conformational change, generating an LPL protein which is more effective in mediating the binding and uptake of lipoproteins by cells.

Samenvatting

Lipoproteïne lipase (LPL) stimuleert de binding en opname van lipoproteïnen door verschillende celtypen, waaronder macrofagen, door een brug te vormen tussen deze lipoproteïnen en de heparan sulfaat proteoglycanen (HSPG) op de cel membraan. Deze bevindingen suggereerden dat LPL een belangrijke rol zou kunnen spelen bij de vorming van schuimcellen in de intima, en dus bij het ontstaan van atherosclerose (aderverkalking). Inmiddels was de atherogeniciteit van geoxideerd LDL ook al vastgesteld in *in vitro* experimenten, evenals de aanwezigheid ervan in atherosclerotische plaques *in vivo.* Omdat het nog niet bekend was of LPL ook de opname van geoxideerd LDL door cellen kan stimuleren, besloten wij het effect van LPL op de binding en opname van LDL dat in verschillende mate was geoxideerd, te onderzoeken in J774 macrofagen (Hoofdstuk 2). We vonden dat LPL op een dosis-afhankelijke wijze, de binding en opname stimuleert van natief LDL en matig geoxideerd LDL door J774 cellen. Deze stimulatie resulteerde in een toename van de intracellulaire cholesterol hoeveelheid in de cellen. Het stimulatoire effect van LPL kon geremd worden met heparinase, hetgeen aantoonde dat de stimulatie een gevolg was van het vormen van een brug door LPL, tussen de lipoproteïnen en de HSPG. De binding en opname van zwaar geoxideerd LDL en geacetyleerd LDL werden niet gestimuleerd door toevoeging van LPL, waarschijnlijk ten gevolge van electrostatische afstoting tussen het LPL-lipoproteïne complex en de HSPG, die beiden zeer negatief geladen zijn. Deze resultaten versterken de hypothese dat LPL, in de intima van de vaatwand, een pro-atherogene factor is.

Aan het begin van het onderzoek, beschreven in Hoofdstuk 3, was nog zeer weinig bekend over de oxidatie van VLDL. Omdat we het waarschijnlijk achtten dat geoxideerd VLDL, evenals geoxideerd LDL, *in vivo* voorkomt, hebben we de interacties bestudeerd van geoxideerd VLDL (OxVLDL) met J774 macrofagen. We vonden dat de opname door de J774 cellen van ^{125}I-gelabelde VLDL deeltjes toenam met de mate van VLDL-oxidatie. Ondanks de verhoogde opname van OxVLDL eiwit, was de hoeveelheid cel-geassocieerde vrije vetzuren, verkregen uit triglyceriden, lager na incubatie van de cellen met OxVLDL dan met natief VLDL. Ook de *de novo* synthese van triglyceriden door de cellen was minder efficient na incubatie met OxVLDL dan met ongeoxideerd VLDL. In de aanwezigheid van polyinosinezuur (PolyI), werd de receptor-gemedieerde opname van OxVLDL volledig geremd, hetgeen aantoont dat OxVLDL wordt opgenomen door de cellen via de scavenger receptor. De *de novo* synthese van triglyceriden werd gereduceerd tot controle waarden na gelijktijdige incubatie van de J774 cellen met OxVLDL en PolyI. Dit betekent dat OxVLDL alleen via opname door de scavenger receptor de synthese van

triglyceriden door cellen kan induceren, en niet, zoals natief VLDL, ook door extracellulaire lipolyse van OxVLDL-triglyceriden. Onze resultaten toonden aan dat oxidatie van VLDL, leidt tot een verlaagde affiniteit van LPL voor VLDL-triglyceriden. Dit resulteert in een gestoorde LPL-gemedieerde hydrolyse van triglyceriden uit geoxideerd VLDL. We concludeerden daarom dat de opname van lipid door cellen, na incubatie met geoxideerd VLDL, plaatsvindt via receptor-gemedieerde opname van het gehele VLDL deeltje, en niet via extracellulaire lipolyse. Dit resulteert in een verminderde hoeveelheid cel-geassocieerd lipid na incubatie met geoxideerd VLDL, in vergelijking tot ongeoxideerd VLDL.

In dit proefschrift werd de rol van apoE in het atherosclerotische proces nader onderzocht. In Hoofdstuk 4 hebben we het effect bekeken van zowel de apoE-isovorm, als de apoE hoeveelheid op de binding van ß-VLDL aan HSPG. Ook hebben we gekeken naar de rol van LPL bij dit proces. Hiervoor hebben we ß-VLDL geïsoleerd uit APOE*2(Arg158→Cys) en APOE*3-Leiden transgene muizen (E2-VLDL en E3Leiden-VLDL, respectievelijk), en uit apoE-deficiënte muizen (Enull-VLDL). We vonden dat in afwezigheid van LPL, de affiniteit (K_a) en de maximale binding (B_{max}) van deze drie ß-VLDL deeltjes aan plastic-gecoate HSPG, zeer laag zijn. Toevoegen van LPL resulteerde in een toename van zowel de K_a als de B_{max} voor alle drie de deeltjes, waarbij de K_a toenam in de volgorde Enull-VLDL < E2-VLDL < E3Leiden-VLDL, en de B_{max} in de omgekeerde volgorde. Ook in de J774 macrofagen resulteerde additie van LPL in een verhoging van de B_{max} voor alle drie de ß-VLDL deeltjes. Bovendien vonden we in de J774 cellen, in aanwezigheid van LPL, dezelfde volgorde van toename van de K_a en de B_{max} voor de binding van ß-VLDL als aan HSPG-LPL complexen. Uit onze resultaten blijkt dat de aanwezigheid van apoE, geen voorwaarde is voor de binding van ß-VLDL aan HSPG-LPL complexen. Echter, in aanwezigheid van apoE, neemt de affiniteit van het ß-VLDL deeltje hiervoor wel toe.

De rol van apoE in de atherogenese hebben we ook bestudeerd door gebruik te maken van apoE-deficiënte muizen. Deze muizen vertonen een sterke accumulatie in het bloed van VLDL deeltjes die deficiënt zijn in zowel apoE als apoB100. Deze muizen ontwikkelen ernstige atherosclerose, hetgeen impliceert dat opname van lipoproteïnen door macrofagen in de intima kan optreden in afwezigheid van zowel apoE als apoB100, de liganden voor receptor-gemedieerde opname van lipoproteïnen. Om meer inzicht te verkrijgen in het proces van schuimcelvorming in apoE-deficiënte muizen, hebben we de interactie bekeken van VLDL geïsoleerd uit deze muizen met J774 macrofagen (Hoofdstuk 5). Hoewel minder efficiënt dan controle VLDL, werd apoE-deficiënt VLDL gebonden aan en opgenomen door deze macrofagen, via een receptor-gemedieerd proces. Daarnaast resulteerde incubatie

van de J774 cellen met apoE-deficiënt VLDL, in een toename van de intracellulaire cholesterol hoeveelheid. Door additie van LPL werden de binding en de opname van apoE-deficiënt VLDL, alsmede de accumulatie van cholesterol in de cellen, sterk gestimuleerd. Competitie-experimenten toonden aan, dat de receptor die de binding en opname van apoE-deficiënt VLDL aan J774 macrofagen medieert, de 'triglyceride-rich lipoprotein receptor' zou kunnen zijn. Deze receptor herkent apoB48, dat aanwezig is op VLDL geïsoleerd uit apoE-deficiënte muizen, als ligand, en is de aanwezigheid van apoE of apoB100 daarvoor niet noodzakelijk. Er zijn verschillende processen beschreven voor het ontstaan van atherosclerose in apoE-deficiënte muizen. Naast de afwezigheid van een apoE-gemedieerde cholesterol efflux uit macrofagen in de vaatwand, is ook de scavenger receptor-gemedieerde opname van geoxideerde lipoproteïnen genoemd als mogelijke aanleiding voor het ontstaan van atherosclerose in apoE-deficiënte muizen. Onze resultaten tonen aan dat er nog een ander mechanisme een rol speelt in dit proces. In apoE-deficiënte muizen kan VLDL namelijk worden opgenomen door macrofagen in de intima via een specifieke receptor. Dit leidt tot de stapeling van cholesterol esters in deze macrofagen. Bovendien kan deze ophoping van cholesterol in macrofagen worden gestimuleerd door de aanwezigheid van LPL.

Tenslotte hebben we de rol onderzocht van een 37 kDa eiwit, dat samen met LPL uit koeienmelk wordt geïsoleerd (Hoofdstuk 6). De aanwezigheid van dit eiwit in een LPL-fractie, leek noodzakelijk voor het stimulatoire effect van LPL op de binding van LDL aan J774 macrofagen. Tijdens het isolatie-proces met behulp van een heparine-sepharose kolom en een continue zoutgradiënt, elueerde dit 37 kDa eiwit eerst van de kolom, gevolgd door het 56 kDa LPL eiwit. We vonden dat de lipase-acitiveit in de fracties samengaat met de aanwezigheid van het 56 kDa bandje, terwijl het stimulatoire effect van een fractie op de binding van LDL aan J774 cellen, volledig overeenkomt met de aanwezigheid van het 37 kDa bandje in de fractie. Western blotting met behulp van het 5D2 monoclonale antilichaam tegen het carboxy-terminale deel van LPL, suggereerde dat het 37 kDa eiwit het carboxy-terminale domein van LPL zou kunnen zijn. Bovendien hebben we, met behulp van de 'radiation-inactivation' techniek, aangetoond dat de functionele massa van LPL voor de stimulatie van de binding van LDL aan cellen ongeveer 31 kDa is. Hieruit concludeerden we dat het 37 kDa eiwit inderdaad de binding van LDL aan cellen medieert. Vermoedelijk is dit 37 kDa eiwit het carboxy-terminale deel van LPL, ontstaan ten gevolge van proteolytische degradatie van het mature LPL eiwit door proteases die in de melk aanwezig zijn. We veronderstellen dat knippen van LPL op protease-gevoelige plaatsen, resulteert in een zodanige conformatieverandering van het eiwit, dat het zijn brugfunctie beter kan vervullen. Dit leidt tot een betere stimulatie van de binding en opname van lipoproteïnen aan cellen.

Publications

Full papers

Hendriks WL, van der Boom H, van Vark LC, and Havekes LM. (1996). Lipoprotein lipase stimulates the binding and uptake of moderately oxidized low density lipoprotein by J774 macrophages. *Biochemical Journal* 314: 563-568

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Abbreviations

AcLDL	acetylated low density lipoprotein
Apo	apolipoprotein
B_{max}	maximum binding
BSA	bovine serum albumin
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
DMEM	Dulbecco's modified Eagle's medium
FCS	foetal calf serum
FFA	free fatty acid
HDL	high density lipoprotein
HL	hepatic lipase
HSA	human serum albumin
HSPG	heparan sulphate proteoglycans
IDL	intermediate density lipoprotein
K_a	association constant
K_d	dissociation constant
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
LPDS	lipoprotein deficient serum
LPL	lipoprotein lipase
LRP	low density lipoprotein receptor-related protein
OxLDL	oxidized low density lipoprotein
OxVLDL	oxidized very low density lipoprotein
PBS	phosphate buffered saline
PL	phospholipid
REM	relative electrophoretic mobility
TBARS	thiobarbituric acid reactive substances
TG	triglyceride
TGRLP	triglyceride rich lipoprotein
VLDL	very low density lipoprotein

Curriculum Vitae

Wilhelmina Leonie Hendriks werd geboren op 20 februari 1970 te Weert. In 1988 behaalde zij haar Gymnasium-ß diploma aan het Bisschoppelijk College te Weert. In augustus van datzelfde jaar ving zij aan met de studie Biomedische Wetenschappen (toen Gezondheidswetenschappen), aan de Rijksuniversiteit van Leiden. De propaedeuse werd behaald in augustus 1989. Vervolgens werd het doctoraalexamen afgelegd in september 1993, met als onderwerp: 'The effect of caloric restriction on the age-related increase in glucagon-stimulated gluconeogenesis' onder begeleiding van dr. H.F.J. Hendriks, bij het toenmalige IVVO-TNO, Gaubius Laboratorium, Leiden.
Van oktober 1993 tot en met oktober 1997 was zij werkzaam als assistent in opleiding (AIO) op een door de Nederlandse Hartstichting gesubsidieerd project (# 92.337), bij TNO Preventie en Gezondheid, Gaubius Laboratorium, Leiden, onder begeleiding van prof. dr. ir. L.M. Havekes. De resultaten van het promotieonderzoek staan beschreven in dit proefschrift. Sinds 1 februari 1998 is zij werkzaam als Clinical Research Associate (CRA) bij Kendle (voorheen U-Gene) te Utrecht, gedetacheerd bij Astra Pharmaceutica te Zoetermeer.