STUDIES ON THE CATABOLISM OF VLDL AND LDL

Monique Mulder

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Promotor:	Prof.dr. Th.J.C. van Berkel
Copromotoren:	Dr.Ir. L.M. Havekes (IVVO-TNO, Gaubius Laboratorium)
	Dr. R.R. Frants
Referent:	Dr. A.F.H. Stalenhoef (Katholieke Universiteit Nijmegen)
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Aan mijn ouders
Voor Arjen

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Chapter 1

GENERAL INTRODUCTION

1.1 Introduction

Cholesterol and triglycerides have an essential role in the human body. Cholesterol has important functions in membranes where it modulates fluidity and maintains the barrier between cell and environment. Cholesterol also serves as a precursor for the synthesis of steroid hormones, bile acids and vitamins. This complex molecule which requires 25 or more enzymes for its creation, can be synthesized in every mammalian cell, with the exception of mature red blood cells.

The metabolism of cholesterol is a dynamic process. A normal person weighing 70 kg contains approximately 145 g of cholesterol. Of this total amount of cholesterol about 8 g (5.5%) is present in plasma. Humans increase their cholesterol content each day by producing about 1.0 g of cholesterol themselves, and by consuming about 0.5 g. The maximal amount of cholesterol that can be absorbed daily in the intestine from diet and bile acids is 0.3 g. The amount absorbed can be affected by the dietary intake of fat. Daily the total metabolic requirement of cholesterol is no more than 350 mg (one egg contains 220 mg). Therefore, to prevent accumulation, the excess of cholesterol is secreted from the body in the form of bile acids, as free cholesterol or in the form of steroid hormones (in faeces, through the skin, in milk and urine) [Myant, 1981]. Most of the bile acids are reabsorbed, giving rise to the enterohepatic circulation.

Triglycerides supply cells with fatty acids, which are used as an energy source in muscle, or for storage in adipose tissue. It has been suggested that the amount and the type of triglycerides affect the absorption of cholesterol. Disturbances in the balance of both cholesterol and triglyceride may be harmful to the human body.

In 1913 Anitschkow first recognized that a high blood level of cholesterol, as a result of a high cholesterol diet, could produce atherosclerosis in rabbits. Atherosclerosis is a disease in which cholesterol accumulates in the wall of arteries, and forms bulky plaques that inhibit the flow of blood, and, eventually, may even close down the artery itself. More often, however, the atherosclerotic plaque predisposes the artery to occlusion by a thrombus, which may lead to a heart attack or a stroke. As early as 1733, it had been observed by Vallisniere that gallstones were soluble in alcohol. In 1789 De Fourcroy isolated a substance from gallstones which he called "adipocire" (fatty wax). Later, in 1816, Chevreul designated this substance, which is now known as cholesterol, *cholesterine* which comes from the Greek:

chole, meaning bile and *stereos*, meaning solid. Since these early studies, Nobel Prizes have been awarded on thirteen occasions to scientists who have devoted the major part of their careers to cholesterol, giving an indication of the interest in this molecule.

Numerous studies have indicated serum cholesterol as the major risk factor for atherosclerosis. The role of triglycerides in the development of atherosclerosis is much less clear.

1.2 Cholesterol and triglyceride metabolism

Since cholesterol and triglycerides are insoluble in water, for transport they are packaged into lipoprotein particles in which they form a hydrophobic core surrounded by a surface monolayer of polar phospholipids (Fig. 1). The surface coat also contains unesterified cholesterol in relatively small amounts, together with proteins called apolipoproteins. Through interactions with enzymes and cell surface receptors the apolipoproteins direct each lipoprotein to its site of metabolism.

The plasma lipoproteins consist of five major classes (chylomicrons, VLDL, IDL, LDL, HDL) and several subclasses (Table 1). The major classes are most often separated using ultracentrifugation on account of their different densities. Each class is highly heterogeneous because of the constant modification of the composition and size of the lipoproteins. As shown in Fig. 2, the lipoprotein metabolism can be divided conceptually into exogenous and endogenous pathways which transport lipids of dietary and hepatic origin, respectively.

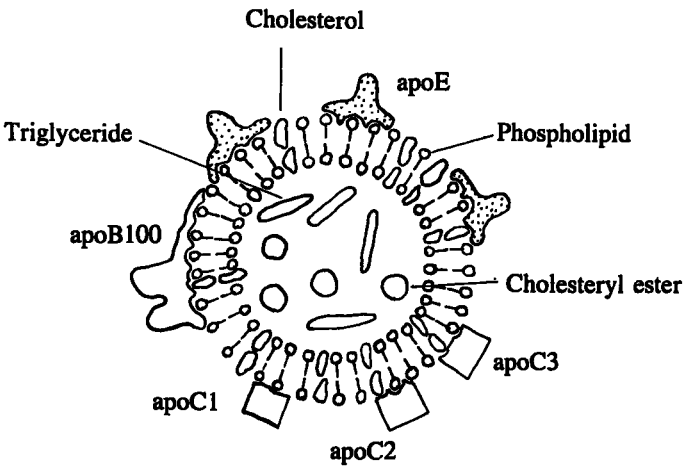

Figure 1. A model of Very Low Density Lipoprotein. In 1929 Macheboeuf observed for the first time that lipids circulating in blood were bound to proteins. He designated this as "complexe lipido-proteidique". The term "lipoprotein" was introduced in 1937.

Figure 2. Next page: Exogenous and endogenous fat-transport pathways are outlined. For reasons of clarity only those apolipoproteins that play a role in receptor-mediated transport are shown. ○, Cholesterol; □, Proteins; ∫, Fatty acids.

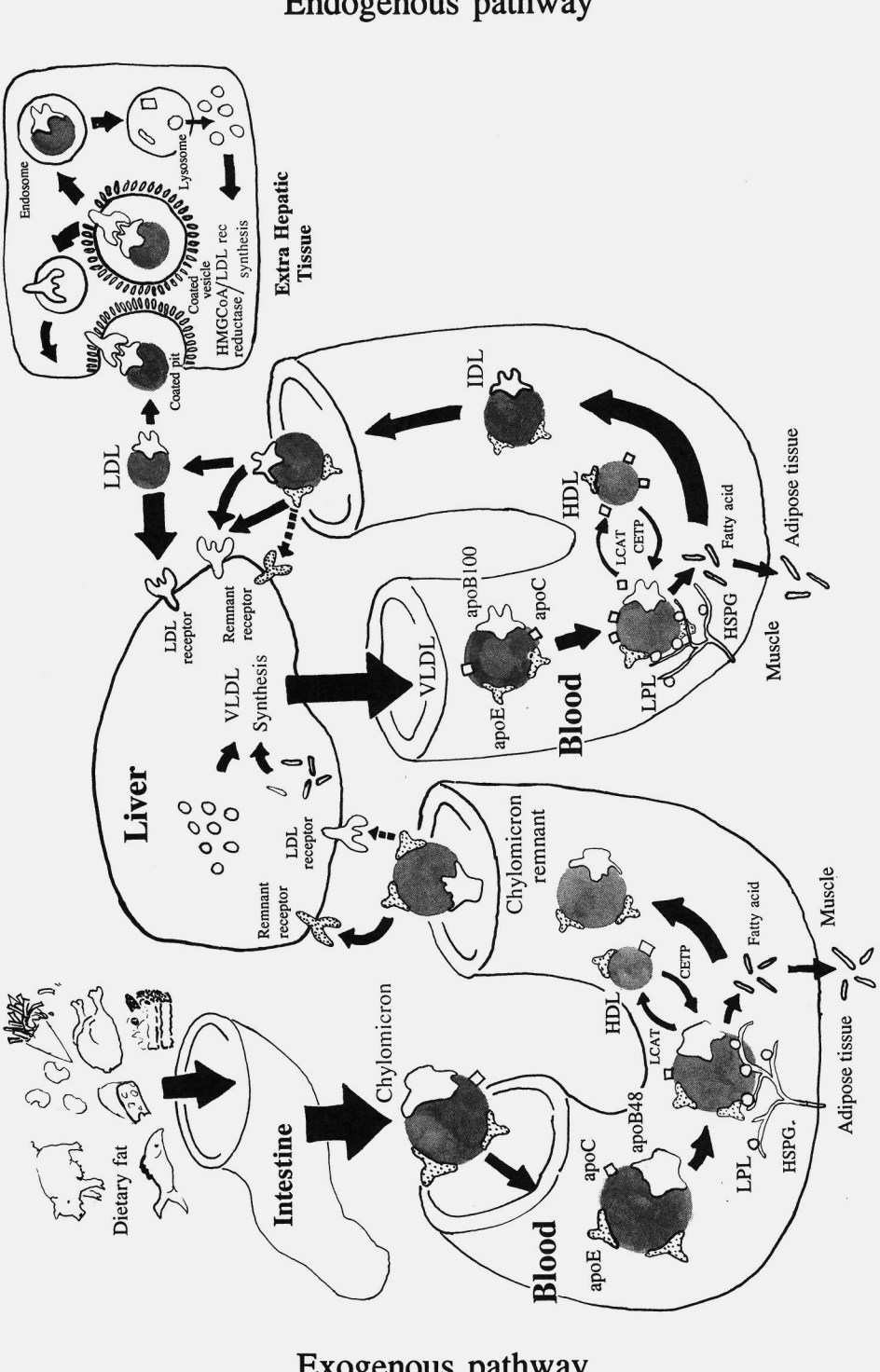
Endogenous pathway
Endosome
Lysosome
Coated pit
Coated vesicle
HMGCoA/LDL rec reductase/ synthesis
Extra Hepatic Tissue
LDL
LDL receptor
Remnant receptor
Liver
VLDL Synthesis
IDL
VLDL
apoB100
apoC
apoE
HDL
LCAT
CETP
LPL
HSPG
Fatty acid
Muscle
Adipose tissue
Blood
Chylomicron remnant
Chylomicron
apoB48
Dietary fat
Intestine
Exogenous pathway

Table 1. Physical properties and composition of human plasma lipoproteins [Gotto, 1986].

	Chylomicron	VLDL	IDL	LDL	HDL
diameter (nm)	75-1200	30-80	25-35	19-25	5-12
mobility[1]	remain at origin	pre-β	pre-β/β	β	α
density (g/ml)	< 0.96	0.96-1.006	1.006-1.019	1.019-1.063	1.063-1.210
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
major apolipoproteins	A1,A4,B48, C1,C3,E	B100,C1,C2, C3,E	B100,E	B100	A1,A2,E

[1] According to the mobility of plasma α- and β-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 Exogenous pathway

The exogenous pathway begins with the formation of triglyceride-rich particles in the intestinal epithelium during the absorption of dietary fat [Attie, 1982; Mahley 1991]. These particles, called chylomicrons, carry dietary triglycerides as their main constituent together with cholesterol. On their surface the chylomicrons bear apolipoproteins, primarily apoB48, apoA1 and apoA4. After synthesis the chylomicrons enter the blood circulation via the lymph. Once in the circulation they release their apoA1 and part of apoA4 and they acquire apoC1, C2, C3, and apoE. Additionally, their triglycerides are rapidly hydrolysed by the enzyme lipoprotein lipase (LPL). This enzyme is attached to the endothelial cells lining the blood capillaries, and, for its activity, uses apoC2 as co-factor [Olivecrona, 1990]. As a consequence of lipolysis, fatty acids are generated and transported to muscle tissue for energy and to adipose tissue for storage. Because of the reduction of the amount of triglycerides in the core of the lipoprotein, part of the surface components become superfluous. The excess surface components, phospholipids together with small amounts of cholesterol, apoC3, apoC2 apoA1 and apoA4, are transferred to existing HDL particles [Tall, 1978], or may form nascent HDL-like particles. The chylomicrons which have lost part of their triglycerides through the action of LPL, and have become relatively enriched in cholesterol and apoE, are referred to as chylomicron remnants.

These remnants are rapidly removed from the circulation by the liver through receptor-mediated endocytosis [Sherill, 1978; Windler 1980]. The ligand responsible for the binding of the remnants to the receptor is apoE [Mahley, 1989a; Sherill, 1980]. The mechanism of uptake is not yet fully understood. It is believed to be a two-step mechanism; the chylomicron remnants first pass through the fenestrae of hepatic endothelial cells and

accumulate in the space of Disse [Stein, 1969]. The remnants are then internalized via the LDL receptor and/or a specific remnant- or apoE receptor.

It has long been assumed that only the remnant receptor was involved in the liver uptake of chylomicron remnants. One likely candidate for the remnant receptor appeared to be the recently-discovered LDL receptor-related protein (LRP) [Herz, 1988]. However, the long-stated assumption that the LDL receptor is not essential to the uptake of the chylomicron remnant by the liver is being increasingly challenged [Kita, 1982; Floren, 1981; Koo, 1988; Windler, 1988; Choi, 1991]. The relative importance of the LDL receptor in the clearance of chylomicrons *versus* other receptors remains to be determined.

The chylomicron remnants carry cholesterol to the liver. The cholesterol is then either reused by the liver for the production of nascent lipoproteins, such as VLDL, or secreted from the body in the form of bile acids and free cholesterol. Recently, it has been demonstrated that in certain animal species, and possibly in humans, chylomicrons are also catabolized by the bone marrow, where they could be involved in the maintenance, proliferation, differentiation, and maturation of bone marrow stem cells, or in the delivery of fat-soluble vitamins [Hussain, 1989a; Hussain, 1989b].

1.2.2 Endogenous pathway

The endogenous pathway begins with the secretion by the liver into the plasma, of triglycerides together with cholesterol, packaged in the form of VLDL. Cholesterol can be derived from chylomicrons or can be synthesized by the liver itself. The triglycerides which are incorporated in VLDL are composed of fatty acids derived either from de novo synthesis within the liver, or as a result of hydrolysis of lipids transported to the liver mainly in the form of chylomicron remnants or attached to albumin. Cholesterol and triglycerides associate intracellularly with apoB100, thereby creating VLDL particles. Each VLDL particle contains one apoB100 protein which remains associated with the lipoprotein throughout its lifetime. Upon secretion into the plasma, the VLDL acquire apoC1, C2, C3 and apoE. VLDL are secreted by the liver in order to transport triglycerides to adipose tissue for storage and to muscle as an energy source. In the circulation, the triglycerides of VLDL are hydrolysed by the action of LPL, as described for chylomicrons. This results in the formation of smaller relatively cholesterol-enriched lipoproteins, called VLDL remnants or intermediate density lipoproteins (IDL). It has been proposed that the interaction of VLDL with LPL is less efficient than the interaction of chylomicrons with the enzyme due to their smaller size [Olivecrona, 1983], this may result in a longer circulation time for VLDL. In humans, the half-time for the clearance of chylomicrons and their remnants from the plasma is less than an hour, and can be as short as 5 minutes. For VLDL it is 2 to 4 hours. During the process of lipolysis the major surface components of the VLDL particles are transferred to HDL or may give rise to new HDL-like particles as has been described for chylomicrons.

In normal human subjects 10 to 60% of the VLDL are supposed to be removed directly from the plasma [Berman, 1978; Sigurdsson, 1975; Reardon, 1978]. The remainder is converted into LDL, which is the end product of lipolysis [Eisenberg, 1973; Sigurdsson, 1975; Havel, 1984]. The removal of most of the VLDL remnants is mediated by LDL

receptors which are mainly present in the liver. This is one of the reasons why the liver is the most important organ in lipoprotein metabolism [Kita, 1982].

The mechanism and the site of conversion of IDL into LDL are still unknown, but there is speculation that it occurs in the liver sinusoids possibly by the action of hepatic lipase [Rubinstein, 1985]. It has been suggested that apoE present on the VLDL remnants, facilitates the activity of hepatic lipase [Thuren, 1991]. The eventual formation of LDL from IDL is accompanied by a further loss of triglycerides, phospholipids, apolipoprotein C's and E [Gotto, 1987; Marzetta, 1990]. ApoB100, the only remaining protein component of LDL, serves as the ligand for binding to the so-called LDL-receptor.

About two-thirds of the amount of cholesterol that is present in the human plasma circulates in the form of LDL. The average lifespan of the LDL particles is approximately 3 days. The hepatic LDL receptors are responsible for the removal of approximately 70% of the circulating LDL [Pittman, 1979; Kita, 1982] and the rest is removed by extrahepatic LDL receptors. The fraction of the LDL which is left is taken up by other, less efficient, LDL-receptor-independent mechanisms [Attie, 1982] including possibly the scavenger receptor.

Although VLDL also contain apoB100, their major constituent for cellular recognition is apoE. The uptake and degradation of large human plasma VLDL is, however, considerably lower than expected from their apoE content [Krul, 1988]. The capacity of apoE, which is present on the surface of the large VLDL, to serve as a ligand for LDL receptor binding appears to be somewhat depressed [Demant, 1988; Gianturco, 1982]. ApoCs have been shown to be able to inhibit the interaction of apoE with the LDL receptor, with apoC1 being the most effective [Sehayek, 1991a]. As a consequence of lipolysis the lipoproteins lose their apoC [Sehayek, 1991b] and, consequently, the accessibility of the apoE present on the surface of the VLDL remnants increases. This results in an enhancement of the ability of these particles to bind to the LDL receptor [Krul, 1988].

1.3 Several Aspects of Very Low Density Lipoprotein catabolism

1.3.1 The role of lipoprotein lipase

Lipoprotein lipase (LPL) was first noted by Hahn in 1943 as a "clearing factor" present in lipemic dogs after heparin injection. Later on this was recognized as the result of the activity of a lipolytic enzyme [Anfinsen, 1952], which was named *lipoprotein lipase* by Korn [1955]. LPL is involved in the catabolism of triglyceride-rich lipoproteins. It catalyses the first and rate-limiting step in the removal of triglyceride from these lipoproteins. The enzyme is located on the luminal surface of the capillary endothelial cells, where it is anchored via ionic interactions to glycan chains of heparan sulphate proteoglycans [Saxena, 1991; Cryer, 1987]. Saxena et al. [1991] have identified this endothelial receptor as a 220 kDa proteoglycan. Heparan sulphate proteoglycans, which are present on most cell types, are heterogenous. Differences in chemical composition and core proteins can affect the release, recycling and translocation of LPL [Cassaro, 1977; Hoogewerf, 1991]. As well as these structures, a

variety of other binding-sites, including dermatan sulphate and chondroitin sulphate proteoglycans, also exist, which bind LPL albeit with a lower affinity.

LPL hydrolyses triglycerides from chylomicrons and VLDL and possibly LDL, using apoC2 as a co-factor. It has been observed that several lipase molecules are capable of interacting with a single lipoprotein at the same time [Olivecrona, 1989]. LPL hydrolyses triglycerides sequentially into diglycerides and further into monoglycerides, or directly into monoglycerides [Wang, 1992]. The fatty acids are subsequently taken up by the underlying tissues. If the removal is not fast enough, the accumulation of fatty acids, may result in the formation of LPL-fatty acid complexes. It has been suggested that the formation of these complexes may lead to the inhibition of the LPL activity, and the release of LPL into the circulation [Wang, 1985; Scow, 1977; Olivecrona, 1990]. This phenomenon was observed in endothelial cells but not in either cardiac myocytes or perfused rat hearts [Rodrigues, 1992]. Since LPL regulates the delivery of fatty acids to tissues for storage in adipose tissue or oxidation in muscle tissue, its activity is tightly controlled. Regulation of LPL occurs via a change in total tissue activity or by redistribution of the enzyme towards the vascular endothelium. A variety of factors, other than the removal of the fatty acids are known to affect LPL activity. One of these is the nutritional status of an individual. During fasting, the LPL activity in adipose tissue decreases, and after a meal it rapidly increases [Semb, 1989]. This regulation may at least in part be mediated by insulin [Kiens, 1989]. Other hormones are also known to affect the LPL activity. A regulating effect of tumour necrosis factor has been reported [Feingold, 1992]. Furthermore the LPL activity has been found to be influenced by apolipoproteins. High amounts of apoC3 have been shown to inhibit LPL activity [Wang, 1985; McConnathy, 1992]. In addition, apoE has been reported to exhibit inhibitory activity [Wang, 1981; McConnathy, 1989], although others have suggested that apoE plays a role in the conversion of VLDL-remnants into LDL [Chung, 1983; Ehnholm, 1984].

Individuals who are genetically deficient in LPL activity exhibit extreme postprandial hypertriglyceridemia [Santamarina-Fojo, 1992]. This usually results in hyperchylomicronemia, a disorder inherited as an autosomal recessive trait. The variability of expression of hyperlipidemia in LPL heterozygotes, indicates that unidentified genetic and/or environmental factors may modulate the expression of the heterozygous state for LPL deficiency.

In the case of low LPL activity, as in inherited LPL deficiency, HDL levels are decreased and patients display hyperchylomicronemia, indicating a relationship between LPL and both triglyceride-rich lipoproteins and HDL-cholesterol levels [Hayden, 1991].

Structure and function of LPL

Together with hepatic lipase and pancreatic lipase, LPL belongs to a conserved lipase gene family [Datta, 1988]. The sequence of lipases is also homologous to Drosophila yolk proteins [Hayden, 1991]. Characteristics which differentiate LPL from other lipases are its pH optimum (8.4), its activation *in vitro* by serum apoC2, and its inhibition by high concentrations of sodium chloride. An important characteristic shared by all three lipases is that they become activated at the lipid-water interface [Desnualle, 1960; Sarda, 1958].

The human LPL gene includes 10 exons spanning 30 kb, and is localized on chromosome 8 [Wang, 1992; Lalouel, 1992; Yang, 1989]. Its primary structure has recently been established [Kirchgessner, 1989; Yang, 1989]. The hepatic lipase gene which is very similar to the LPL gene in its organisation, is localized on chromosome 15 [Sparkes, 1987].

LPL is a glycoprotein that is synthesized as a 49 kd polypeptide, and becomes a mature protein of 55 kd after glycosylation [Kinnunen, 1976]. For human LPL, two potential N-glycosylation sites, Asn-43 and Asn-359, have been reported [Semankovich, 1990].

Insight into the structure of LPL has been provided by the three-dimensional crystallographic structure of human pancreatic lipase. LPL is thought to consist of two structural domains. The N-terminal domain of human lipase includes the catalytic triad, Ser^{132}-Asp^{156}-His^{241}, which is buried in a hydrophobic pocket [Winkler, 1990]. LPL, and also pancreas lipase and hepatic lipase, contain a lid structure located between the disulphide-bridged residues 216 and 239, which covers the active site [Winkler, 1990; Lawson, 1992]. It has been proposed that interfacial activation occurs through repositioning of the lid to allow access to the catalytic site by the substrate.

LPL needs apoC2 as a plasma activator protein to express its full activity [Sparkes, 1987, 58]. The site of interaction of LPL with apoC2 has been located at the N-terminal domain (lysine at residues 147 and 148) [Bruin, 1992]. This is also the domain responsible for inhibition by salt. The heparin-binding site has been located at the C-terminal domain, and consists of a high concentration of positively-charged amino acids between residue 402 and 446 [Davis, 1992]. Parenchymal cells of a variety of tissues have been shown to synthesize LPL [Nilsson-Ehle, 1980]. The highest amounts of LPL mRNA were found in adipose tissue, heart and some red muscles [Kirchgessner, 1989; Semenkovich, 1989]. It was also detected in the mammary gland, brain, diaphragm, lung, aorta, kidney, uterus, intestine and spleen. Furthermore, LPL is synthesized by monocyte/macrophages or macrophage-derived foam cells and by smooth muscle cells present in plaques, hereby suggesting a role for LPL in the development of atherosclerosis [Hamosh, 1983; O'Brien, 1992; Yla-Herttuala, 1991].

After its synthesis, part of the mature lipase is rapidly intracellularly degraded. Only a fraction of the lipase is secreted into the medium [Semb, 1989; Masuno, 1990] and is transported across the endothelial cells to binding sites at the cell surface of adjacent capillaries [Saxena, 1991], which is the major site of its activity. From its binding site the enzyme can dissociate into the circulation, or be recycled into the cells and degraded. In the circulation, low concentrations of LPL are usually present. Goldberg et al. [1986] found that the majority of the circulating enzyme is associated with lipoproteins. Lateron Villela et al. [1991] reported that it is associated mainly with LDL and HDL. The amount of circulating LPL is generally kept low because of a rapid removal by the liver [Olivecrona, 1989a].

1.3.2 LDL receptor

The LDL receptor was first identified in 1973 by Goldstein and Brown (Fig. 3) [Brown, 1974]. Evidence has been obtained that domains of the LDL receptor have been conserved for over 350 million years [Mehta, 1991a; Mehta, 1991b].

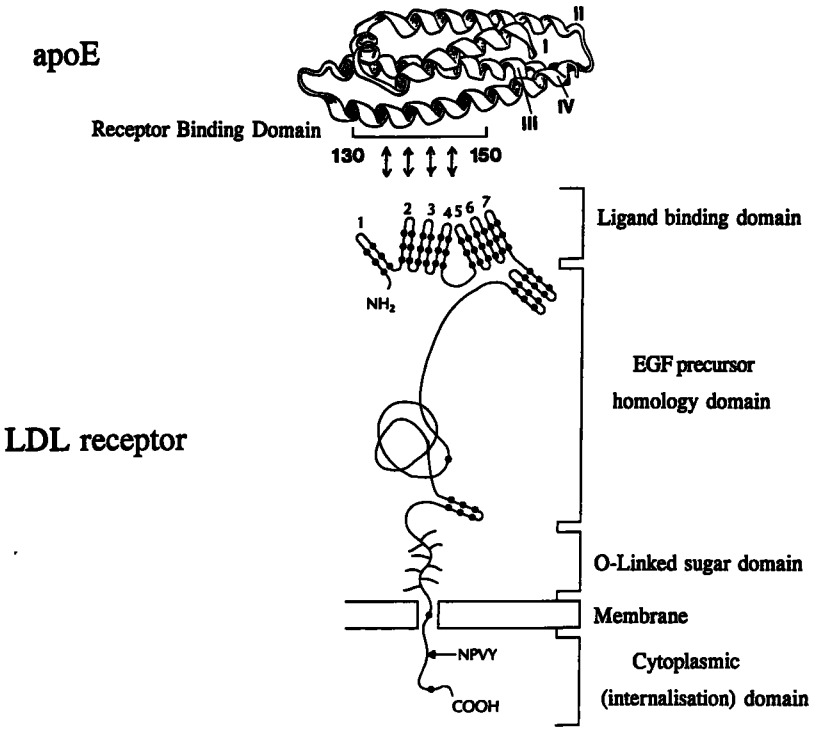

Figure 3. Schematic illustration of the interaction of the LDL receptor (a single protein with five domains), and the putative receptor-binding domain of apoE. NPVY, a short tyrosine-containing sequence that is recognized by coated pit proteins. •, cysteine. The three dimensional structure of the major part of the N-terminal domain of apoE (residues 23-166) determined by x-ray crystallography [Aggerbeck, 1988] is shown. The four helices (numbered I to IV) are faced antiparallel. The putative LDL receptor-binding domain is indicated. The figures of apoE and of the LDL receptor were kindly supplied by Dr. Weisgraber and by Dr. Hobbs, and are reproduced here with their permission.

The LDL receptor has been found to play a central role in lipoprotein metabolism and cholesterol homeostasis [Innerarity 1991]. The ligands for the LDL receptor are apoB100 and apoE. ApoB100 is responsible for the interaction of LDL with the receptor. The single copy of apoB100 present on LDL interacts with a single LDL receptor [Van Driel, 1989]. ApoE is responsible for the interaction of IDL, VLDL remnants, and several other lipoproteins including β-VLDL with the receptor [Innerarity, 1978]. ApoE has a higher affinity for the LDL receptor than apoB100. In addition, multiple copies of apoE present on VLDL- or chylomicron remnants are able to interact with a single LDL receptor, and a single lipoprotein, containing multiple copies of apoE, can also bind to more than one LDL receptor simultaneously [Van Driel, 1987].

The number of LDL receptors present on the cell surface is regulated by the amount of unesterified cholesterol available within the cell [Goldstein, 1977]. Whenever the demand is high, cells will increase their production of LDL receptors. On the other hand, if the demand is low, the number of LDL receptors decreases [Brown, 1985; Russell, 1983; Brown, 1986].

The receptor will allow as much cholesterol to enter the cell as is required, and thus prevent cholesterol-overloading of the cell. The majority of LDL receptors are located within the liver, and only a small number of receptors exist extrahepatically. The LDL receptors that are found in the liver are assumed to be immunologically indistinguishable from the LDL receptors present in extrahepatic tissues. At the cellular level, the hepatic LDL receptors are distributed uniformly over the sinusoidal surface, especially on the microvilli and intermicrovillus membranes, while in most extrahepatic tissues the LDL receptors are concentrated in coated pits [Pathak, 1990].

The mature LDL receptor is a single transmembrane glycoprotein containing 839 amino acids [Goldstein, 1985]. The receptor is synthesized as a precursor with an apparent molecular weight of 120 kD, and is converted in the Golgi to a mature form with an apparent molecular weight of 160 kD by the addition of sialic acid and two N-linked and eighteen O-linked oligosaccharides, co- and post-translationally, respectively [Cummings, 1983; Brown, 1983]. The receptor is organized into five functional domains, each contributing to the functional activity of the receptor (Fig. 3). The five domains are: 1) the ligand-binding domain, 2) the epidermal growth factor (EGF) precursor homology domain, 3) the O-linked sugar domain, 4) the membrane-spanning domain and 5) the cytoplasmic domain [Innerarity, 1991; Brown, 1986].

The amino-terminal 292 amino acids of the LDL receptor contain seven copies of a 40-residue repeat, which constitute the ligand-binding domain. Each repeat contains a negatively-charged cluster of residues, which interacts with the positively-charged receptor binding domains of apoB and apoE [Brown, 1978; Weisgraber, 1978]. The epidermal growth factor (EGF) precursor homologous domain consists of about 400 amino acids, located next to the ligand-binding domain. This domain is strongly homologous (35% of the aminoacids are identical) with the epidermal growth factor precursor. The precise function of this domain is still unknown [Russell, 1984]. So is the function of the O-linked polysaccharide domain, which consists of 58 amino acids just outside the plasma membrane. It has been suggested that it may be possible to influence the three-dimensional structure of the ligand-binding domain of the protein in order to facilitate its interaction with lipoproteins.

The membrane-spanning domain consists of 25 hydrophobic amino acids. Its function is to anchor the LDL receptor protein to the cell surface. Should the exon encoding for this domain be deleted, the mutant LDL receptor dissociates from the cell membrane [Lehrmann, 1985].

The cytoplasmic domain or the carboxy-terminal part of the LDL receptor consists of 50 amino acids which are located inside the cell. This domain is involved in directing the receptor into coated pits. Naturally-occurring mutations in this domain resulted in a disturbed clustering of the LDL receptor into coated pits and a disturbed subsequent internalisation of the LDL receptor-lipoprotein complex [Davis, 1987].

1.3.3 Remnant receptor

The remnant- or apoE-receptor has long been a puzzle to researchers. Suspicions about a special receptor for chylomicron remnants were aroused when their apparently normal

clearance from the bloodstream was observed in Watanabe-heritable-hyperlipidemic (WHHL) rabbits and in patients with homozygous familial hypercholesterolemia (FH), who have severe deficiencies of LDL receptors [Kita, 1982; Kita, 1981]. Hui et al. [1981] found that a binding site specific for apoE, and distinct from the LDL receptor was present in dog livers. The postulated remnant- or apoE receptor is thought to differ from the LDL receptor with respect to its regulation and its Ca^{2+} requirement. In contrast to the LDL receptor, this receptor is thought to require only moderate amounts of Ca^{2+} for the binding of lipoproteins, and not to be regulated by cellular cholesterol levels or drugs or other factors.

In contrast to these early observations, it has recently been reported that a delay in the clearance of chylomicron remnants from the plasma of WHHL rabbits has been observed. Demacker et al. [1992] have suggested that this discrepancy could be due to the marker used to trace the chylomicron remnants.

Several proteins have been proposed as a candidate for the remnant receptor [Kinoshita, 1985; Hui, 1986] and have subsequently been rejected [Beisiegel, 1988]. In 1988, Herz et al., while searching for clones homologous to the LDL receptor, also isolated cDNA for low density lipoprotein receptor related protein (LRP) from a cDNA library prepared from human cytotoxic T lymphocyte RNA.

The LRP was found to be roughly equivalent to four LDL receptors and was considered to be a likely candidate for the remnant receptor. It turned out to be the largest plasma membrane protein (4525 amino acids) ever described with messenger RNA of 15 kb. LRP is synthesized as a precursor with an apparent molecular weight of ~ 600 kD. On its way to the cell surface LRP is cleaved into two subunits of 515 kD and 85 kD. The larger subunit, which contains the ligand-binding domain remains attached to the membrane through non-covalent association with the smaller subunit, which contains the membrane spanning domain and cytoplasmic tail. It has been shown that the LRP binds apoE and calcium [Beisiegel 1989; Herz, 1988]. It also binds apoE-enriched β-VLDL, which is known to stimulate the cholesterol esterification more than 40-fold in LDL receptor-defective fibroblasts [Kowal, 1989; Kowal, 1990]. It has been shown that the binding of ligands to the LRP can be blocked by a 39-kDa protein [Herz, 1991]. However, just as the hypothesis of the LRP as the remnant receptor was becoming convincing, the α_2-macroglobulin receptor was found to be identical to the LRP [Strickland, 1990; Kristensen, 1990]. Van Dijk et al. [1992] and Huettinger et al. [1992] have shown that, *in vivo* in the rat, lactoferrin specifically inhibits endocytosis of β-VLDL and chylomicron remnants but not of α_2-Macroglobulin, suggesting that different binding sites are involved. However, Willnow et al. [1992] reported that lactoferrin inhibited the binding of the LPL/β-VLDL-complex to the LRP on nitrocellulose blots. Recently Jäckle et al. [1993] have reported that *in vivo* in the rat the LRP is not involved in the removal of either chylomicron remnants or β-VLDL from the circulation. In conclusion, the LRP is a multifunctional receptor, which does not appear to have a major role in the catabolism of triglyceride-rich lipoproteins.

1.3.4 Apolipoprotein E

ApoE, initially referred to as "arginine-rich" apoprotein, was first identified in 1973 as a

protein constituent of VLDL (Fig. 3) [Shore 1973]. It is a single polypeptide with a molecular weight of 34,200 kD. ApoE plays a key role in cholesterol and triglyceride metabolism. It is one of the major protein constituents of several plasma lipoproteins, including chylomicron- and VLDL remnants, and a subclass of HDL. It is involved in the maintenance of the structure of these lipoprotein particles and the regulation of their catabolism [Mahley, 1984; Breslow, 1985]. An important role of apoE is to mediate the interaction of these lipoproteins with the LDL receptor, a role apoE shares with apoB100, and with the postulated remnant- or apoE receptor [Mahley, 1984; Brown, 1986; 1983].

The apolipoprotein is synthesized by a large variety of cells throughout the body. The main sites of synthesis and secretion are the liver parenchymal cells where it is secreted in association with VLDL [Elshourbagy, 1985; Lin, 1986]. It has also been suggested that a large percentage of nascent apoE is secreted in a lipid-poor form which can associate extracelluarly with preformed lipoprotein particles [Dolphin, 1986; Hussain, 1989]. The organ with the second highest level of apoE mRNA is the brain, where the major source of apoE is the astrocyte [Elshourbagy, 1985]. ApoE is not able to cross the blood-brain barrier, and thus a separate pool of apoE is present in the brain [Kraft, 1989; Linton, 1991]. In addition, macrophages in many tissues synthesize large quantities of apoE, especially as a response to injury. A role has therefore been suggested for apoE in the repair response to tissue injury, and specifically nerve injury [Mahley, 1988]. It may also be involved in other processes unrelated to lipid transport, such as immunoregulation and modulation of smooth muscle cell growth and differentiation [Mahley, 1990; Davignon, 1988].

Structure and function of apoE

The APOE gene is 3.7 kb long and consists of four exons and three introns [Das, 1985; Paik, 1985], and is linked to the APOC1 gene, the APOC1 pseudogene, and the APOC2 gene in a 50 kb cluster on the long arm of chromosome 19 [Tata, 1984]. ApoE is synthesized as a prepeptide of 317 amino acids. Post-translational cleavage of a signal peptide of 18 amino acids results in a major apoE protein of 299 animo acids. The polymorphic nature of apoE, which appeared to be unique to human beings [Chan, 1991], was established in 1980 by Utermann and his associates using isoelectric focusing, and was further elucidated by Zannis and Breslow [1981]. The three major isoforms of apoE, referred to as apoE2, E3 and E4 are products of the major alleles, E*2, E*3, and E*4 at a single gene locus. Three homozygous phenotypes (E2E2, E3E3 and E4E4) and three heterozygous phenotypes (E3E2, E3E4, E2E4) arise from the expression of any two of the three alleles. Determination of the primary structure of apoE revealed that the isoforms E4, E3 and E2 differed from one another by single amino acid substitutions at two sites in the protein [Rall, 1982].

The most common isoform, E3, contains a cysteine residue at position 112 and an arginine residue at position 158. The isoform E4 is identical to E3 but has an arginine residue at position 112. This introduces an extra single positive charge unit as compared with apoE3. ApoE2 is also identical to E3, except for the loss of a single positive charge as a result of the substitution of an arginine for a cysteine at position 158.

The predicted secondary structure of apoE is shown in Fig. 3. The mature apoE protein

is made up of two independently-folded domains, that differ in stability and function [Wetterau, 1988]. Digestion with thrombin produces a 22 kD fragment (residues 1-191) corresponding to the N-terminal domain and a 10 kD fragment (residues 216 to 299) corresponding to the C-terminal domain. The regions with amino acids 1 to 20 and 165 to 191 are probably unstructured.

The carboxy-terminal domain contains a region of strong amphiphatic α-helical character between residue 230 and 265. It has been postulated that these structures are involved in lipid binding [Weisgraber, 1990]. The carboxy-terminal domain also contains at least one heparin-binding domain, probably between residues 214-236 [Loof, 1986], which may mediate the binding of the lipoproteins to heparan sulphate like structures. The N-terminal domain associates *in vitro* with phospholipid to form discoidal particles. The N-terminal domain contains five helices and the region of apoE that binds to the LDL receptor [Innerarity, 1983; Wilson, 1991]. Several lines of independent data have indicated the basic arginine and lysine (and histidine) residues in the vicinity of residues 136-150 as the region mediating the binding of apoE to the LDL receptor via an ionic interaction. The crystal structure of this region shows that residues 131-150 form an extended α-helix.

Much has been learned about the nature of the interaction of apoE with the LDL receptor by studying the various naturally-occurring mutants clustered near residues 140-160. At present more than 24 different isoforms of apoE have been unequivocally identified by DNA and/or amino acid sequencing analyses [de Knijff, 1992].

The three most common apoE variants (E2, E3, and E4) were found to have an impact on lipid and apolipoprotein concentrations in the plasma. The E*2 allele appeared to be associated with decreased levels of total cholesterol and LDL cholesterol and somewhat higher levels of triglycerides [Utermann, 1979], while the E*4 allele was found to be associated with increased cholesterol and lower triglyceride levels [Bouthillier, 1983; Davignon, 1984]. Furthermore, E*2 correlated with increased apoE and decreased apoB plasma levels, while E*4 had precisely the opposite effect [Davignon, 1988; Smit, 1988]. Gregg and co-workers [1981] have shown that apoE2 is catabolized *in vivo* more slowly than apoE3, due to a defective binding of the apoE2 variant to lipoprotein receptors. It has been suggested that in apoE2(arg158 → cys) homozygous subjects the lipolytic conversion of VLDL into their remnants is retarded [Byung Hong Chung, 1983; Ehnholm, 1984]. However, Demant et al. [1991] have reported that it is not the conversion of VLDL into their remnants, but the conversion of IDL into LDL which is retarded. The mechanism and the site of the conversion of IDL into LDL is still unknown. In contrast to apoE2, apoE4 is catabolized *in vivo* faster than apoE3. People with apoE4 absorb more, and synthesize less, cholesterol than those with apoE2 [Kesaniemi, 1987]. ApoE4 does not differ from apoE3 in its binding efficiency, but it does differ in its lipoprotein distribution: apoE4 is predominantly associated with VLDL, while apoE3 appears to be preferably associated with HDL [Weisgraber, 1990]. This might be due to the fact that, in contrast to apoE4, apoE3 exists largely as an E-A2 complex in HDL [Weisgraber, 1990; Weisgraber, 1991; Borghini, 1991]. This has important functional implications for this plasma source of apoE. The apoE-A2 complex is more stable than free apoE to ultracentrifugal manipulations. If this is the case,

it suggests that the principal role of plasma HDL, with respect to apoE metabolism would be to accept apoE shed during lipolysis of triacylglycerol-rich lipoproteins. ApoE might remain associated with HDL as a result of the formation of E-A2 complexes. These findings already imply that some major differences in the regulation of lipoprotein metabolism are associated with the apoE polymorphism.

1.3.5 ApoE and Familial Dysbetalipoproteinemia

The key role that apoE plays in normal lipoprotein metabolism is highlighted by the association between the abnormal apoE2 and Familial Dysbetalipoproteinemia (FD). The disease has also been seen in several kindreds who have complete apoE deficiency [Ghiselli, 1981; Schaefer, 1986; Mabuchi, 1989]. Recently, it has been reported that apoE-deficient mice created by homologous recombination, display severe hypercholesterolemia and atherosclerosis [Plump, 1992].

The lipoprotein disorder now known as familial dysbetalipoproteinemia or as type III hyperlipoproteinemia, was first reported by Gofman et al. in 1954. It was originally termed xanthoma tuberosum, based on the occurrence of tuberous xanthomas, and it has also been called "broad-beta disease", "floating-beta disease" or "remnant removal disease". FD is usually inherited as a recessive trait and is most commonly associated with the E2E2 phenotype. The most serious consequence of FD is the development of premature atherosclerosis involving both the coronary and peripheral arteries [Mahley, 1989].

Patients with FD have elevated concentrations of both plasma cholesterol and triglyceride and of apoE [Havel, 1973]. These patients usually also display reduced concentrations of LDL [Utermann, 1979]. A biochemical characteristic of the disorder is the occurrence of β-VLDL (β-migrating VLDL). These are remnants of both intestinal and hepatic origin, as demonstrated by the presence of both apoB100 and apoB48. The β-VLDL are considerably more cholesterol-enriched (mostly as cholesteryl esters) and are relatively depleted in triglycerides. Their apolipoprotein composition is characterized by increased amounts of apolipoprotein E and decreased amounts of the apolipoproteins C compared with normal VLDL. A fairly unique property of β-VLDL is that they are able to induce foam cell formation in macrophages [Mahley, 1980; Goldstein, 1980; Bersot, 1983].

Most FD patients are E2E2 homozygous. In *in vitro* experiments it has been shown that after complexation with phospholipid vesicles, apoE2 displays less than 2% of the binding to the LDL receptor when compared with binding of the most common apoE3, irrespective of whether apoE2 is isolated from normo- or from hyperlipidemic E2E2 homozygous subjects [Schneider, 1981; Weisgraber, 1982; Rall, 1983]. The defective interaction of apoE2 with lipoprotein receptors has therefore been suggested as the primary defect in FD, leading to the accumulation of chylomicron- and VLDL-remnants in the plasma [Weisgraber, 1982]. However, Stalenhoef et al. [1986] have found that also the removal from the plasma of triglyceride-rich lipoproteins, obtained from an LPL-deficient subject heterozygous E4E2, was also less efficient in FD subjects carrying the apoE2(arg145 → cys) or the apoE2(arg158 → cys) mutation, than the removal of these lipoproteins in normal subjects.

Approximately one percent of the North European and North American populations is

homozygous for apoE2. However, only about 4% of all subjects homozygous for APOE*2 will develop the disease at older age [Utermann, 1985]. All have detectable β-VLDL in their plasma. Many are normolipidemic or even hypolipidemic, owing to reduced levels of LDL. This low penetrance of FD indicates that the disorder is modulated by other genetic or environmental factors such as gender, age, weight, hormones, diabetes, hypothyroidism, familial combined hyperlipidemia, or variation in the expression of LDL receptors. The latter is particularly sensitive to regulation by diet, drugs, and hormones. FD has been found to come to expression in families with a tendency towards hypertriglyceridemia. Other mechanisms that have been mentioned are overproduction of specific lipoproteins [Stuyt, 1982], or impaired processing [Mahley, 1989; Utermann, 1985].

It has been reported that the defective binding of apoE2 is reversible. The binding activity of the aberrant apoE2 can be fully restored by treating the apoE2 with cysteamine, which converts the cysteine into a lysine analogue and adds an extra positive charge, together with the removal of the carboxyl-terminal part of the molecule by cleavage with thrombin [Innerarity, 1984]. Evidence has been obtained that the composition and/or structure of the apoE2-containing lipoproteins synthesized by E2E2 homozygous FD patients, can also alter the conformation of the apoE2 on the surface of the particle, and modulate its receptor-binding activity. The possibility of restoring the binding of apoE2 derives from the fact that the 158arg → cys mutation does not occur inside the LDL receptor binding domain, but is located near the COOH-terminal end of helix-4. Here the guanidinium group of Arg158 does not contribute directly to the large positive electrostatic potential surrounding the receptor-binding helix. Instead, it forms salt bridges with the acidic side chains of Glu96 and Asp154 and as such may help to stabilize the pairing of helices 3 and 4 [Wilson, 1991].

Besides the apoE2 variant, at least seven of the other naturally-occurring rare variants of apoE have been found in association with FD (Table 2).

Table 2. ApoE variants found in association with FD.

IEF position[1]	Parental allele	Responsible abnormality	Trivial name
E1	E2	gly127 → asp; arg158 → cys	
E1	E3	lys146 → glu	Harrisburg
E2	E3	arg145 → cys	
E2	E3	lys146 → gln	
E2	E3	arg136 → ser	Christchurch
E3	E4	cys112 → arg; arg142 → cys	
E3	E4	cys112 → arg: 7 aa insertion	Leiden
E4	E3	gly13 → lys; arg145 → cys	Philadelphia

[1] IEF, isoelectric focusing position.

References: Weisgraber, 1984; Gabelli, 1989; Steinmetz, 1990; Mann, 1988, 1989; Rall, 1982; Emi, 1988; Rall, 1983; Smit, 1987, 1990; Emi, 1988; Wardell, 1987; Havel, 1983, Rall, 1989; Havekes, 1986; Wardell, 1989; van den Maagdenberg, 1991; de Knijff, 1991; Lohse, 1991.

All of these known substitutions in apoE disrupt the binding to some extent. For example, normal apoE, purified and reconstituted into phospholipid complexes, has a 5, 4, 2.5, or 2-fold higher affinity for lipoprotein receptors, than does apoE with a substitution at residue, 142, 121-127, 146, or 145 (Table 3). Many of these apoE mutants, which are defective in their binding, are assumed to be associated with a dominant rather than a recessive expression of FD, as many individuals who are heterozygous for these functionally-defective mutants develop FD. Rall et al. [1990] hypothesized that any substitution of a neutral amino acid for a basic amino acid within the putative α-helix segment 131-150 affects the binding activity of apoE by reducing the strength of the ionic interaction with the LDL receptor. They proposed that the binding defect of the other apoE variants, in contrast to the binding of apoE2, might not be reversible, thereby causing the dominant expression of FD [Mahley, 1990]. However, the severity of the binding defect, when associated with phospholipid vesicles did not correlate with the severity of the hyperlipidemia. Although the binding efficiency of the rare apoE variants was defective when complexed with phospholipid vesicles (Table 3), the d < 1.006 lipoproteins from FD subjects carrying the rare mutants E2(lys146 → gln), E3-Leiden, E3(arg142 → cys), E2(arg145 → cys) displayed a paradoxically high affinity for the LDL receptor [Chappell, 1989]. The mutation in apoE3-Leiden is located outside the binding domain between residue 121 and 127. It has been suggested that this mutation does not directly affect the binding, but results in a change of the conformation of the receptor binding region of apoE. The accumulation of d < 1.006 lipoproteins in these FD subjects cannot be ascribed simply to low affinity for the LDL receptor.

It remains to be determined how the occurrence of a single defective allele can disrupt normal clearance of plasma lipoproteins, which possess several apoE molecules per particle.

Table 3. The binding efficiency of naturally occurring apolipoprotein E variants associated with FD.

Abnormal apoE variant	Percentage of normal receptor-binding activity	Mode of FD inheritance
E3(arg142 → cys)	< 4	dominant
E3-Leiden (7 amino-acid insertion 121-127)	25	dominant
E2(lys146 → gln)	40	dominant
E2(arg145 → cys)	45	unknown
E2(arg158 → cys)	< 2	recessive

1.4 Outline of this thesis

The aim of this study was to explore several aspects of the metabolism of VLDL and LDL. After being secreted into the blood circulation, a major part of the VLDL are transformed into VLDL remnants mainly by the action of LPL. Subsequently, the remnants are rapidly removed by the liver. The remainder of the VLDL are converted into LDL, which are taken

up mainly by the liver LDL receptors. No general agreement, however, exists concerning the hepatic receptor that mediates the uptake of VLDL and VLDL-remnants. In order to clarify the nature of the receptor involved, the binding of LPL-treated VLDL, taken as representative for VLDL-remnants, to Hep G2 cells was studied (Chapter 2).

ApoE plays a major role in the interaction of VLDL and VLDL-remnants with lipoprotein receptors. The defective interaction of apoE2 with lipoprotein receptors has been suggested as the primary defect in FD, leading to the accumulation of chylomicron- and VLDL-remnants in the plasma. It has been reported that the defective binding of apoE2 in patients with FD could be restored by treating the patients with a low calorie diet. We investigated whether treatment of six E2E2 homozygous hyperlipidemic FD patients with gemfibrozil, also improved the binding efficiency of lipoproteins with a density of less than 1.019 g/ml to the LDL receptor (Chapter 4).

FD is not always associated with E2E2 homozygosity. Heterozygosity for the rare E2(lys146 → gln) variant cosegregates with FD with a high penetrance. This indicates that E2(lys146 → gln) behaves like a dominant trait in the expression of the disease. Hence, subjects heterozygous for this variant develop FD, despite the presence of a normal apoE. Chapter 5 reports studies on the possible mechanism behind the behaviour of E2(lys146 → gln) as a dominant trait.

It is commonly assumed that lipolysis of chylomicrons and VLDL by LPL, turns these lipoprotein particles into better ligands for both hepatic lipoprotein receptors. However it has also been reported that LPL, independent of its lipolytic activity, enhances the binding of apoE containing lipoproteins to the LRP. Chapters 6 and 7 describe studies of the effect of LPL on the binding and subsequent processing of VLDL and LDL in cultured Hep G2 cells and fibroblasts. Studies of the effect of LPL on the metabolism of LDL and VLDL in the rat *in vivo* are described in Chapter 8.

1.5 References

Anfinsen, C.B., Boyle, E., and Brown, R.K. (1952) The role of heparin in lipoprotein metabolism. Science 115: 583-586.

Anitschkow, N., and Chalatow, S. (1913) Ueber experimentelle cholesterin steatose und ihre bedeutung für die entstehung einiger pathologische prozesse. Centralbl. Allgem. Pathol. Pathol. Anat. 24: 1-9.

Attie, A.D., Pittman, R.C., and Steinberg, D. (1982) Hepatic catabolism of low density lipoproteins and chylomicrons as regulators of rate of cholesterol synthesis in rat liver in vivo. Hepatology 2: 269-281.

Basu, S.K., Brown, M.S., Ho, Y.K., Havel, R.J., and Goldstein, J.L. (1981) Mouse macrophages synthesize and secrete a protein resembling apolipoprotein E. Proc. Natl. Acad. Sci. USA 78: 7545-7549.

Beisiegel, U., Weber, W., Havinga, J., Ihrke, G., Hui, D.Y., Wernette-Hammond, M.E., Turck, C.W., Innerarity, T.L., and Mahley, R.W. (1988) Apolipoprotein E binding proteins isolated from dog and human liver. Arteriosclerosis 8: 288-297.

Beisiegel, U., Weber, W., Ihrke, G., Harz, J., and Stanley, K.K. (1989) The LDL-receptor-related protein, LRP, is an apolipoprotein E-binding protein. Nature 341: 162-164.

Bengtsson, G., and Olivecrona, T. (1980) Interaction of lipoprotein lipase with native and modified heparin-like polysaccharides. Biochem. J. 189: 625-633.

Berman, M., Hall, M., Levy, R.I., et al. (1978) Metabolism of apoB and apoC lipoproteins in man: kinetic studies in normal and hyperlipoproteinemic subjects. J. Lipid Res. 19: 38-56.

Bersot, T.P., Innerarity, T.L., Mahley, R.W., and Havel, R.J. (1983) Cholesteryl ester accumulation in mouse peritoneal macrophages induced by beta-migrating very low density lipoproteins from patients with atypical dysbetalipoproteinemia. J. Clin. Invest. 72: 1024-1033.

Borghini, I., James, R.W., Blatter, M.-C., and Pometta, D. (1991) Distribution of apolipoprotein E between free and A-II complexed forms in very-low- and high-density lipoproteins: functional implications. Biochim. Biophys. Acta 1083: 139-146.

Bouthillier, D., Sing, C.F., and Davignon, J. (1983) Apolipoprotein phenotyping with a single gel method application to the study of informative matings. J. Lipid Res. 24: 1060-1069.

Breslow, J.L. (1985) Human apolipoprotein molecular biology and genetic variation. Annu. Rev. Biochem. 54: 699-727.

Brown, M.S., and Goldstein, J.L. (1974) Expression of the familial hypercholesterolemia gene in heterozygotes: mechanism for a dominant disorder in man. Science 185: 61-63.

Brown, M.S., Deuel, T.F., Basu, S.K., and Goldstein, J.L. (1978) Inhibition of the binding of low density lipoproteins to its cell surface acceptor in human fibroblasts by positively charged proteins. J. Supramol. Struct. 8: 223-234.

Brown, M.S., Anderson, R.W., and Goldstein, J.L. (1983) Recycling receptors: the round-trip itinerary of migrant membrane proteins. Cell 32: 663-667.

Brown, M.S., and Goldstein, J.L. (1985) Regulation of the activity of the low density lipoprotein receptor in human fibroblasts. Cell 6: 307-316.

Brown, M.S., and Goldstein, J.L. (1986) A receptor-mediated pathway for cholesterol homeostasis. Science 232: 34-47.

Bruin, T., Van der Sluis, B.E.M., and Kastelein, J.J.P. (1992) Identification of the Apo-CII binding site on human lipoprotein lipase. Circulation Suppl. I 86: I-608.

Byung H.C., and J.P. Segrest. (1983) Resistance of a very low density lipoprotein subpopulation from familial dysbetalipoproteinemia to *in vitro* lipolytic conversion to the low density lipoprotein density fraction. J. Lipid Res. 24: 1148-1159.

Cassaro, C.M.F., and Dietrich, C.P. (1977) Distribution of sulfated mucopolysaccharides in invertebrates. J. Biol. Chem. 252: 2254-2261.

Chan, L., and Li W.-H. (1991) Apolipoprotein variation among different species. Curr. Opin. Lipidol. 2: 96-103.

Chappell, D.A. (1989) High receptor binding affinity of lipoproteins in atypical dysbetalipoproteinemia (Type III hyperlipoproteinemia). J. Clin. Invest. 84: 1906-1915.

Chappell, D.A., and Lindgren, F.T. (1989) Increased receptor binding affinity of lipoproteins during *in vivo* lipolysis. Arteriosclerosis 9: 728a.

Chevreul, M.E. (1815) Recherches chimiques sur plusieurs corps gras, et particulièrement sur leurs combinations avec les alcalics. Cinquième mémoire. Des corps qu'on a appelés adipocre, c'est-à-dire, de la substance cristallisée des calculs bilaires humains, du spermacéti de la substance grasse des cadavres. Ann. Chim. 95: 5-50, especially p. 7-10.

Choi, S.Y., Fong, L.G., Kirven, M.J., and Cooper, A.D. (1991) Use of anti-low density lipoprotein receptor antibody to quantify the role of the LDL receptor in the removal of chylomicron remnants in the mouse. J. Clin. Invest. 88: 1173-1181.

Chung, B.H., and Segrest, J.P. (1983) Resistance of a very low density lipoprotein subpopulation from familial dysbetalipoproteinemia to in vitro lipolytic conversion to the low density lipoprotein density fraction. J. Lipid Res. 24: 1148-1159.

Cisar, L.A., Hoogewerf, A.J., Evans, D.C., and Bensadoun, A. (1989) Secretion and degradation of lipoprotein lipase in cultured adipocytes. J. Biol. Chem. 264: 1767-1771.

Cryer, A. (1987) In: Borensztajn, J., ed. Lipoprotein lipase. Chicago, IL: Evener Publishers, pp. 277-327.

Cummings, R.D., Kornfeld, S., Schneider, W.J., Hobgood, K.K., Tolleshaug, H., Brown, M.S., and Goldstein, J.L. (1983) Biosynthesis of N- and O-linked oligosaccharides of the low density lipoprotein receptor. J. Biol. Chem. 258: 15261-15273.

Das, H.K., McPherson, J., Bruns, G.A.P., Karathanasis, S.K., and Breslow, J.L. (1985) Isolation, characterization and mapping to chromosome 19 of the human apolipoprotein E gene. J. Biol. Chem. 260: 6240-6247.

Dashti, N., and Wolfbauer, G. (1986) Studies on the binding and degradation of human very low density lipoproteins by human hepatoma cells. Biochim. Biophys. Acta 875: 473-486.

Datta, S., Luo, C.C., Li, W.H., Van Tuinen, P., Ledbetter, D.H., Brown, M.A., Chen, S.H., Liu, S., and Chan, L. (1988) Human hepatic lipase cloned CDNA sequence, restriction fragment length polymorphism, chromosomal localization, and evolutionary relationships with lipoprotein lipase and pancreatic lipase. J. Biol. Chem. 263: 1107-1110.

Davignon, J., Sing, C.F., Lussier-Cacan, S, and Bouthillier, D. (1984) Xanthelasma, latent dyslipoproteinemia and atherosclerosis: Contribution of apoE polymorphism. In: De Gennes, J.L., Polonovsky, J., and Paoletti, R., eds. Latent dyslipoproteinemia and atherosclerosis. New York: Raven Press, pp. 213-223.

Davignon, J., Gregg, R.E., and Sing, C.F. (1988) Apolipoprotein E polymorphism and atherosclerosis. Arteriosclerosis 8: 1-21.

Davis, C.G., Van Driel, I.R., Russell, D.W., Brown, M.S., and Goldstein, J.L. (1987) The low density lipoprotein receptor. Identification of amino acids in cytoplasmic domain required for rapid endocytosis. J. Biol. Chem. 262: 4075-4079.

Davis, R.C., Wong, H., Nikazy, J., Wang, K., Han, Q., and Schotz, M.C. (1992) Chimeras of HL and LPL. J. Biol. Chem. 267: 21499-21504.

Demacker, P.N.M., Van Heijst, P.J., and Stalenhoef, A.F.H. (1992) A study of the chylomicron metabolism in WHHL rabbits after fat loading. Discrepancy between results based on measurement of apoprotein B48 or retinylpalmitate. Biochem. J. 285: 641-646.

Demant, T.H., Shepherd, J., and Packard, C.J. (1988) Very low density lipoprotein apolipoprotein B metabolism in humans. Klin. Wochenschr. 66: 703-712.

Demant, T., Bedford, D., Packard, C.J., and Shepherd, J. (1991) Influence of apolipoprotein E polymorphism on apolipoprotein B100 metabolism in normolipidemic subjects. J. Clin. Invest. 88: 1490-1501.

Desnuelle, P., Sarda, L., and Ailhard, G. (1960) Inhibition de la lipase pancreatique par le diethyl-p-nitrophenyl phosphate en emulsion. Biochem. Biophys. Acta 37: 570-571.

Van Dijk, M.C.M., Ziere, G.J., and van Berkel, T.J.C. (1992) Characterization of the chylomicron-remnant-recognition sites on parenchymal and Kupffer cells of rat liver. Selective inbition of parenchymal cell recognition by lactoferrin. Eur. J. Biochem. 205: 775-784.

Dolphin, P.J., Forsyt, S.J., and Drul, E.S. (1986) Post-secretory acquisition of apolipoprotein E by nascent rat hepatic very low density lipoproteins in the absence of cholesteryl transfer. Biochim. Biophys. Acta 875: 21-30.

Van Driel, I.R., Davis, C.G., Goldstein, J.L., and Brown, M.S. (1987) Self-association of the low density lipoprotein receptor mediated by the cytoplasmic domain. J. Biol. Chem. 262: 16127-16134.

Van Driel, I.R., Brown, M.S., and Goldstein, J.L. (1989) Stoichiometric binding of low density lipoprotein (LDL) and monoclonal antibodies to LDL receptors in a solid phase assay. J. Biol. Chem. 264: 9533-9538.

Ehnholm, C., Mahley, R.W., Chappell, D.A., Weisgraber, K.H., Ludwig, E., and Witztum, J.L. (1984) Role of apolipoprotein E in the lipolytic conversion of β-very low density lipoproteins to low density lipoproteins in type III hyperlipoproteinemia. Proc. Natl. Acad. Sci. USA 81: 5566-5570.

Eisenberg, S., Bilheimer, D.W., Levy, R.I., and Lindgren, F.T. (1973) On the metabolic conversion of human plasma very low density lipoprotein to low density lipoprotein. Biochim. Biophys. Acta 326: 361-377.

Elshourbagy, N.A., Liao, W.S., Mahley, R.W., and Taylor, J.M. (1985) Apolipoprotein E mRNA is abundant in brain and adrenals as well as in the liver and is present in other peripheral tissues of rats and marmosets. Proc. Natl. Acad. Sci. USA 82: 203-207.

Emi, M., Wu, L.L., Robertson, M.A., Myers, R.L., Hegele, R.A., Williams, R.R., White, R., and Lalouel, J.-M. (1988) Genotyping and sequence analysis of apolipoprotein E isoforms. Genomics 3: 373-379.

Feingold, K.R., Doerrler, W., Dinarello, C.A., Fiers, W., and Grunfeld, C. (1992) Stimulation of lipolysis in cultured fat cells by tumor necrosis factor, interleukin-1 and the interferons is blocked by the inhibition of prostaglandin synthesis. Endocrinology 130: 10-16.

Floren, C.-H., Albers, J.J., Kudchodkar, B.J., and Bierman, E. (1981) Receptor-dependent uptake of human chylomicron remnants by cultured skin fibroblasts. J. Biol. Chem. 236: 425-433.

De Fourcroy (1789) De la substance feuilletée et cristalline continue dans les calcule billaries et de la nature des concrétions cystiques cristallisée. Ann. Chim. Phys. [1] 3: 242-252.

Gabelli, C., Baggio, G., Pagnan, A., Zanetti, G., Barbato, G.M., Martini, S., Bilato, C., Corti, C., and Crepaldi, G. (1989) Identification of an Italian kindred with a variant apolipoprotein E (E1) associated with type III hyperlipoproteinemia. In: Sirtori, C.R., Franceschini, G., Brewer, H.B. Jr., and Assmann, G., eds. Human apolipoprotein mutants 2. From gene structure to phenotypic expression. New York and London: Plenum Press, pp. 181-188.

Ghiselli, G., Schaefer, E.J., Garcon, P., and Brewer, H.B. (1981) Type III hyperlipoproteinemia associated with apolipoprotein E deficiency. Science 214: 1239-1241.

Gianturco, S.H., Brown, F.B., Gotto, A.M., Bradley, W.A. (1982) Receptor-mediated uptake of hypertriglyceridemic very low denstiy lipoproteins by normal human fibroblasts. J. Lipid Res. 23: 984-993.

Gofman, J.M., Dellabela, O., Glazier, F. et al. (1954) The serum lipoprotein transport system in health, metabolic disorders, atherosclerosis and coronary heart disease. Plasma 2: 413-484.

Goldberg, I.J., Kandel, J.J., Blum, C.B., and Ginsberg, H.N. (1986) Association of plasma lipoproteins with postheparin lipase activities. J. Clin. Invest. 78: 1523-1528.

Goldstein, J.L., and Brown, M.S. (1977) The low-density lipoprotein pathway and its relation to atherosclerosis. Ann. Rev. Biochem. 46: 897-930.

Goldstein, J.L., Ho, Y.K., Brown, M.S., Innerarity, T.L., and Mahley, R.W. (1980) Cholesteryl ester accumulation in macrophages resulting from receptor-mediated uptake and degradation of hypercholesterolemic canine β-very low density lipoproteins. J. Biol. Chem. 255: 1839-1848.

Goldstein, J.L., Brown, M.S., Anderson, R.G.W., Russell, D.W., and Schneider, W.J. (1985) Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. Ann. Rev. Cell Biol. 1: 1-39.

Gotto, A.M., Pownall, H., and Havel, R.J. (1986) Introduction to the plasma lipoproteins. Methods Enzymol. 128: 3-40.

Gotto, A.M. (1987) In: Gotto, A.M., ed. Plasma lipoproteins. Elsevier, Vol. 14, chapter I.

Gregg, R.E., Zech, L.A., Schaefer, E.J., and Brewer, H.B. (1981) Type III hyperlipoproteinemia: Defective metabolism of an abnormal apolipoprotein E. Science 211: 584-586.

Gregg, R.E., Zech, L.A., Schaefer, E.J., Stark, D., Wilson, D., and Brewer, H.B. (1986) Abnormal in vivo metabolism of apolipoprotein E4 in humans. J. Clin. Invest. 78: 815-821.

Hahn, P.F. (1943) Abolishment of alimentary lipemia following injection of heparin. Science 98: 19-20.

Hamilton, R.L., Wong, J.S., Gus, L.S.S., Krisans, S., and Havel, R.J. (1990) Apolipoprotein E localization in rat hepatocytes immunogold labeling of cryothin sections. J. Lipid Res. 31: 1589-1603.

Hamosh, M., and Hamosh, P. (1983) In: H. Baum, ed. Molecular aspects of medicine. Oxford: Pergamon Press, Vol. 6, pp. 199-289.

Havekes, L., De Wit, E., Gevers Leuven, J.A., Klasen, E., Utermann, G., Weber, W., and Beisiegel, U. (1986) Apolipoprotein E3-Leiden. A new variant of human apolipoprotein E associated with familial type III hyperlipoproteinemia. Hum. Genet. 73: 157-163.

Havel, R.J. (1984) The formation of LDL mechanism and regulation. J. Lipid Res. 25: 1570-1576.

Havel, R.J., and Kane, J.P. (1973) Primary dysbetalipoproteinemia: Predominance of a specific apoprotein species in triglyceride-rich lipoprotein. Proc. Natl. Acad. Sci. USA 70: 2015-2019.

Havel, R.J., Kotite, L., Kane, J.P., Tun, P., and Bersot, T. (1983) Atypical familial dysbetalipoproteinemia associated with apolipoprotein phenotype E3/3. J. Clin. Invest. 72: 379-387.

Hayden, M.R., Yuanhong, M.A., Brunzell, J., and Henderson, H.E. (1991) Genetic variants affecting human lipoprotein and hepatic lipases. Curr. Opin. Lipidol. 2: 104-109.

Herz, J., Hamann, U., Rogne, S., Myklebost, O., Gausepohl, H., and Stanley, K.K. (1988) Surface location and high affinity for calcium of a 500 kD membrane protein closely related to the LDL receptor suggests a physiological role as lipoprotein receptor. EMBO J. 7: 4119-4127.

Herz, J., Goldstein, J.L., Strickland, D.K., Ho, Y.K., and Brown, M.S. (1991) 39-kDa protein modulates binding of ligands to low density lipoprotein receptor-related protein/α_2-macroglobulin receptor. J. Biol. Chem. 266: 21232-21238.

Huettinger, M., Retzek, H., Hermann, M., and Goldenberg, H. (1992) Lactoferrin specifically inhibits endocytosis of chylomicron remnants but not α-macroglobulin. J. Biol. Chem. 267: 18551-18557.

Hui, D.Y., Innerarity, T.L., and Mahley, R.W. (1981) Lipoprotein binding to canine hepatic membranes. Metabolically distinct apo-E and apo-B,E receptors. J. Biol. Chem. 256: 5646-5655.

Hui, D.Y., Brecht, W.J., Hall, E.A., Friedman, G., Innerarity, T.L., and Mahley, R.W. (1986) Isolation and characterization of the apolipoprotein E receptor from canine and human liver. J. Biol. Chem. 261: 4256-4267.

Hussain, M.M., Zannis, E.E., Kelly, M., and Zannis, V.I. (1989) Synthesis modification and flotation properties of rat hepatocyte apolipoproteins. Biochim. Biophys. Acta 1001: 90-101.

Hussain, M.M., Mahley, R.W., Boyles, J.K., Fainaru, M., Brecht, W.J., and Lindquist, P.A. (1989a) Chylomicron-chylomicron remnant clearance by liver and bone marrow in rabbits: Factors that modify tissue-specific uptake. J. Biol. Chem. 264: 9571-9582.

Hussain, M.M., Mahley, R.W., Boyles, J.K., Lindquist, P.A., Brecht, W.J., and Innerarity, T.L. (1989b) Chylomicron metabolism. Chylomicron uptake by bone marrow in different animal species. J. Biol. Chem. 264: 17931-17938.

Innerarity, T.L. (1991) The low-density lipoprotein receptor. Curr. Opin. Lipidol. 2: 156-161.

Innerarity, T.L., and Mahley, R.W. (1978) Enhanced binding by cultured fibroblasts of apo E-containing lipoproteins as compared with low density lipoproteins. Biochemistry 17: 1440.

Innerarity, T.L., Friedlander, E.J., Rall, S.C., Weisgraber, K.H., and Mahley, R.W. (1983) The receptor-binding domain of human apolipoprotein E: binding of apolipoprotein E fragments. J. Biol. Chem. 258: 12341-12347.

Innerarity, T.L., Weisgraber, K.H., Arnold, K.S., Rall, S.C., and Mahley, R.W. (1984) Normalisation of receptor binding of apolipoprotein E2. J. Biol. Chem. 259: 7261-7267.

Innerarity, T.L., Hui, D.Y., Bersot, T.P., and Mahley, R.W. (1986) Type III hyperlipoproteinemia: A focus on lipoprotein receptor-apolipoprotein E2 interactions. In: Angel, A., and Frohlich. J., eds. Lipoprotein deficiency syndromes. New York: Plenum Publishing, pp. 273-288.

Jäckle, S., Huber, C., Moestrup, S., Gliemann, J., and Beisiegel, U. (1993) In vivo removal of β-VLDL, chylomicron remnants and α_2-macroglobulin in the rat. J. Lipid Res. 34: 309-315.

Kesaniemi, Y.A., Ehnholm, C., and Miettinen, T.A. (1987) Intestinal cholesterol absorption efficiency in man is related to apoprotein E phenotype. J. Clin. Invest. 80: 578-581.

Kiens, B., Lithell, H., Mikines, K.J., and Richter, E.A. (1989) Effects of insulin and exercise on muscle lipoprotein lipase activity in man and its relation to insulin action. J. Clin. Invest. 84: 1124-1129.

Kinnunen, P.K.J., Huttunen, J.K., and Ehnholm, C. (1976) Properties of purified bovine milk lipoprotein lipase. Biochim. Biophys. Acta 450: 342-351.

Kinoshita, M., Teramoto, T., Karo, H., Hashimoto, Y., Naito, C., Toda, G., and Oka, H. (1985) Partial purification of the apolipoprotein E receptor of rat liver membrane. J. Biochem. 97: 1803-1806.

Kirchgessner, T.G., Chuat, J.-C., Heinzmann, C., Etienne, J., Guilhot, S., Svenson, K., Ameis, D., Pilon, C., S'Auriol, L., Andalibi, A., Schotz, M.C., Galibert, F., and Lusis, A.J. (1989) Organization of the human lipoprotein gene and evolution of the lipase family. Proc. Natl. Acad. Sci. USA 86: 9647-9651.

Kirchgessner, T.G, LeBoef, R.C., Langner, C.A., Zollman, S., Chang, C.H., Taylor, B.A., Schotz, M.C., Gordan J.I., and Lusis, A.J. (1989) Genetic and developmental regulation of the lipoprotein lipase gene: Loci both distal and proximal to the lipoprotein lipase structural gene control enzyme expression. J. Biol. Chem. 264: 1473-1482.

Kita, T., Brown, M.S., Watanabe, Y., and Goldstein, J.L. (1981) Deficiency of low density lipoprotein receptors in liver and adrenal gland of the WHHL rabbit, an animal model of familial hypercholesterolemia. Proc. Natl. Acad. Sci. USA 78: 2268-2272.

Kita, T., Brown, M.S., Bilheimer, D.W., and Goldstein, J.L. (1982) Delayed clearance of very low density and intermediate density lipoproteins with enhanced conversion to low density lipoprotein in WHHL rabbits. Proc. Natl. Acad. Sci. USA 79: 5693-5697.

Kita, T., Goldstein, J.L., Brown, M.S., Watanabe, Y., Hornick, C., and Havel, R.J. (1982) Hepatic uptake of chylomicron remnants in WHHL rabbits. A mechanism genetically distinct from the low density lipoprotein receptor. Proc. Natl. Acad. Sci. USA 79: 3623-3627.

De Knijff, P. (1992) Genetic heterogeneity of apolipoprotein E and its influence on lipoprotein metabolism. Leiden: Thesis.

De Knijff, P., Van den Maagdenberg, A.M.J.M., Stalenhoef, A.F.H., Gevers Leuven, J.A., Demacker, P.N.M., Kuyt, L.P., Frants, R.R., and Havekes, L.M. (1991) Familial dysbetalipoproteinemia associated with apolipoprotien E3-Leiden in an extended multigeneration pedigree. J. Clin. Invest. 88: 643-655.

Koo, C., Wernette-Hammond, M.E., Garcia, Z., Malloy, M., Uauy, R., East, C., Bilheimer, D.W., Mahley, R.W., and Innerarity, T.L. (1988) Uptake of cholesterol-rich remnant lipoproteins by human monocyte-derived macrophages is mediated by low density lipoprotein receptors. J. Clin. Invest. 81: 1332-1340.

Korn, E.D. (1955) Clearing factor, a heparin-activated lipoprotein lipase. J. Biol. Chem. 215: 1-14.

Kowal, R.C., Herz, J., Goldstein, J.L., Esser, V., and Brown, M.S. (1989) Low density lipoprotein receptor-related protein mediates uptake of cholesteryl esters derived from apoprotein E-enriched lipoproteins. Proc. Natl. Acad. Sci. USA 86: 5810-5814.

Kowal, R.C., Herz, J., Weisgraber, K.H., Mahley, R.W., Brown, M.S., and Goldstein, J.L. (1990) Low density lipoprotein receptor-related protein mediates uptake of cholesterylesters derived from apoE-enriched lipoproteins. J. Biol. Chem. 265: 10771-10779.

Kraft, H.G., Menzel, H.J., Hoppichler, F., Vogel, W., and Utermann, G. (1989) Changes of genetic apolipoprotein phenotypes caused by liver transplantation. Implications for apolipoprotein synthesis. J. Clin. Invest. 83: 137-142.

Kristensen, T., Bendtsen, L., Sand, O., and Sottrup-Jensen, L. (1990) Evidence that the newly cloned low-density-lipoprotein receptor related protein (LRP) is the alpha-2-macroglobulin receptor. FEBS Lett. 276: 151-155.

Krul, E.S., Tikkanen, M.J., and Schonfeld, G. (1988) Heterogeneity of apolipoprotein E epitope expression on human lipoproteins: importance for apolipoprotein E function. J. Lipid Res. 29: 1309-1325.

Lalouel, J.M., Wilson, D.E., and Iverius, P.H. (1992) Lipoprotein lipase and hepatic triglyceride lipase: molecular and genetic aspects. Curr. Opin. Lipid. 3: 86-95.

Lawson, D.M., Brzozowski, A.M., and Dodson, G.G. (1992) Lifting the lid off lipases. Curr. Biol. 2: 473-475.

Lehrmann, M.A., Schneider, W.J., Sudhof, T., Brown, M.S., Goldstein, J.L., and Russell, D.W. (1985) Mutation in LDL receptor: Alv-Alv recombination deletes exons encoding transmembrane and cytoplasmic domains. Science 227: 140-146.

Lin, C.-T., Xu, Y., Wu, J.Y., and Chan, L. (1986) Immunoreactive apolipoprotein E is a widely distributed cellular protein. Immunohistochemical localization of apolipoprotein E in baboon tissues. J. Clin. Invest. 78: 947-958.

Linton, M.F., Gish, R., Hubl, S.T., Butler, E., Esquivel, C., Bry, W.I., Boyles, J.K., Wardell, M.R., and Young, S.G. (1991) Phenotypes of apolipoprotein B and apolipoprotein E after liver transplantation. J. Clin. Invest. 88: 270-281.

Lohse, P., Mann, W.A., Stein, E.A., and Brewer, H.B. Jr. (1991) Apolipoprotein E-4 Philadelphia (Glu13 → Lys, Arg145 → Cys). Homozygosity for two rare point mutations in the apolipoprotein E gene combined with severe type III hyperlipoproteinemia. J. Biol. Chem. 266: 10479-10484.

De Loof, H., Rosseneu, M., Brasseur, R., and Ruysschaert, J.M. (1986) Use of hydrophobicity profiles to predict receptor binding domains on apolipoprotein E and the low density lipoprotein apolipoprotein B-E receptor. Proc. Natl. Acad. Sci. USA 83: 2295-2299.

Van den Maagddenberg, A.M.J.M., De Knijff, P., Stalenhoef, A.F.H., Gevers Leuven, J.A., Havekes, L.M., and Frants, R.R. (1989) Apolipoprotein E3-Leiden results from a partial gene duplication in exon 4. Biochem. Biophys. Res. Commun. 165: 851-857.

Mabuchi, H., Itoh, H., Takeda, M., Kajmami, K., Wakasugi, T., Koizumi, J., Takeda, R., and Asagami, C. (1989) A young type hyperlipoproteinemic patient associated with apolipoprotein E deficiency. Metabolism 38: 115-119.

Macheboeuf, M.A. (1929) Technique du fractionnement. Bull. Soc. Chim. Biol. 11: 268.

Mahley, R.W., Innerarity, T.L., Brown, M.S., Ho, Y.K., and Goldstein, J.L. (1980) Cholesteryl ester synthesis in macrophages. Stimulation by β-VLDL from cholesterol-fed animals of several species. J. Lipid Res. 21: 970-980.

Mahley, R.W., Innerarity, T.L., Rall, S.C., and Weisgraber, K.H. (1984) Plasma lipoproteins: apolipoprotein structure and function. J. Lipid Res. 25: 1277-1294.

Mahley, R.W., Innerarity, T.L., Rall, S.C., Weisgraber, K.H., and Taylor, M. (1988) Apo E: cholesterol transport protein with expanding role in cell biology. Science 240: 622-630.

Mahley, R.W., and Rall, S.C. (1989) Type III hyperlipoproteinemia (dysbetalipoproteinemia). The role of apolipoprotein E in normal and abnormal lipoprotein metabolism. In: Scriver, C.R., Beaudet, A.L., Sly W.S., and Valle, D., eds. The metabolic basis of inherited disease, 6th Edition, New York: McGraw-Hill, pp. 1195-1213

Mahley, R.W., Hui, D.Y., Innerarity, T.L., and Beisiegel, U. (1989) Chylomicron remnant metabolism: Role of hepatic lipoprotein receptors in mediating uptake. Arteriosclerosis 9 (suppl. I): I14-I18.

Mahley, R.W., Innerarity, T.L., Rall, S.C., Weisgraber, K.H., and Taylor, J.M. (1990) Apolipoprotein E: genetic variants provide insight into its structure and function. Curr. Opin. Lipidol. 1: 87-95.

Mahley, R.W., and Hussain, M.M. (1991) Chylomicron and chylomicron remnant catabolism. Curr. Opin. Lipidol. 2: 170-176.

Mann, W.A., Gregg, R.E., Ronan, R., Thomas, F., Zech, L.A., and Brewer, H.B. Jr. (1988) Apolipoprotein E1-Harrisburg: point mutation resulting in dominant expression of type III hyperlipoproteinemia. Arteriosclerosis 8: 612a.

Mann, W.A., Gregg, R.E., Sprecher, D.L., and Brewer, H.B. Jr. (1989) Apolipoprotein E-1 Harrisburg: a new variant of apolipoprotein E dominantly associated with type III hyperlipoproteinemia. Biochim. Biophys. Acta 1005: 239-244.

Marzetta, C.A., Foster, D.M., and Brunzell, J.D. (1990) Conversion of plasma VLDL and IDL precursors into various LDL subpopulations using density gradient ultracentrifugation. J. Lipid Res. 31: 975-984.

Masuno, H., Blanchette-Mackie, E.J., Chernick, S.S., and Scow, R.O. (1990) Synthesis of inactive nonsecretable high mannose-type lipoprotein lipase by cultured brown adipocytes of combined lipase-deficient cld/cld mice. J. Biol. Chem. 265: 1628-1638.

McAbee, D.D., and Esbensen, K. (1991) Binding and endocytosis of apo- and holo-lactoferrin by isolated rat hepatocytes. J. Biol. Chem. 266: 23624-23631.

McConnathy, W.J., and Wang, C.-S. (1989) Inhibition of lipoprotein lipase by the receptor binding domain of apolipoprotein E. FEBS Lett. 251: 250-252.

McConnathy, W.J., Gesquiere, J.C., Bass, H., Tartar, A., and Fruchart, J.-C. (1992) Inhibition of lipoprotein lipase activity by synthetic peptides of apolipoprotein C-III. J. Lipid Res. 33: 995-1003.

Mehta, K.D., Chen, W.J., Goldstein, J.L., and Brown, M.S. (1991) The low density lipoprotein receptor in Xenopus laevis I. Five domains that resemble the human receptor. J. Biol. Chem. 266: 10406-10414.

Mehta, K.D., Brown, M.S., Bilheimer, D.W., and Goldstein, J.L. (1991) The low density lipoprotein receptor in Xenopus laevis II. Feedback repression mediated by conserved sterol regulatory element. J. Biol. Chem. 266: 10415-10419.

Metz-Boutigue, M.-H., Jolles, J., Mazurier, J., Schoentgen, F., Legrand, D., Spik, G., Montreuil, J., and Jolles, P. (1984) Human lactotransferrin: amino acid sequence and structural comparisons with other transferrins. Eur. J. Biochem. 145: 659-676.

Mulder, M., De Wit, E., and Havekes, L.M. (1991) The binding of human lipoprotein lipase treated VLDL by the human hepatoma cell line Hep G2. Biochim. Biophys. Acta 1081: 308-314.

Nilsson-Ehle, P., Garfinkel, A.S., and Schotz, M.C. (1980) Lipolytic enzymes and plasma lipoprotein metabolism. Annu. Rev. Biochem. 49: 667-693.

O'Brien, K.D., Gordon, D., Deeb, S., Ferguson, M., and Chait, A. (1992) Lipoprotein lipase is synthesized by macrophage-derived foam cells in human coronary atherosclerotic plaques. J. Clin. Invest. 89: 1544-1550.

Olaisen, B., Teisberg, P., and Gedde-Dahl, T. (1982) The locus for apolipoprotein E (apo E) is linked to the complement component C3. C(3) locus in chromosome 19 in man. Hum. Genet. 62: 233-236.

Olivecrona, T., and Bengtsson-Olivecrona, G. (1983) In: Angel, A, Hollenberg, C.H., and Roncari D.A.K., eds. The adipocyte and obesity. New York: Raven Press, pp. 117-126.

Olivecrona, T., and Bengtsson-Olivecrona, G. (1989) Heparin. In: Lane and Lindahl, eds. Edward Arnold, pp. 335-361.

Olivecrona, T., and Bengtsson-Olivecrona, G. (1989) Lipases involved in lipoprotein metabolism. Curr. Opin. Lipidol. 1: 116-122.

Olivecrona, T., and Bengtsson-Olivecrona, G. (1990) Lipoprotein lipase and hepatic lipase. Curr. Opin. Lipidol. 1: 222-230.

Paik, Y.K., Chang, A.J., Reardon, C.A., Davies, G.E., Mahley, R.W., and Taylor, J.M. (1985) Nucleotide sequence and structure of the human apolipoprotein E gene. Proc. Natl. Acad. Sci. USA 82: 3445-3449.

Pathak, R.K., Yokode, M., Hammer, R.E., Hoffman, S.L., Brown, M.S., and Goldstein, J.L. (1990) Tissue-specific sorting of the human LDL-receptor in polarized epithelia of transgenic mice. J. Clin. Invest. 85: 1542-1547.

Plump, A.S., Smith, J.D., Hayek, T., Aalto-Setälä, K., Walsh, A., Verstuyft, J.G., Rubin, E.M., and Breslow, J.L. (1992) Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. Cell 71: 343-353.

Rall, S.C., Weisgraber, K.H., and Mahley, R.W. (1981) Human apolipoprotein E. J. Biol. Chem. 257: 4171-4178.

Rall, S.C., Weisgraber, K.H., Innerarity, T.L., and Mahley, R.W. (1982) Structural basis of receptor binding heterogeneity of apolipoprotein E from type III hyperlipoproteinemic subjects. Proc. Natl. Acad. Sci. USA 79: 4695-4700.

Rall, S.C., Weisgraber, K.H., Innerarity, T.L., et al. (1983) Identical structural and receptor binding defects in apolipoprotein E2 in hypo-, normo- and hypercholesterolemic dysbetalipoproteinemia. J. Clin. Invest. 71: 1023-1031.

Rall, S.C., Weisgraber, K.H., Innerarity, T.L., Bersot, T.P., Mahley, R.W., and Blum, C.B. (1983) Identification of a new structural variant of human apolipoprotein E, E2(Lys146 → Gln) in a type III hyperlipoproteinemic subject with the E3/E2 phenotype. J. Clin. Invest. 72: 1288-1297.

Rall, S.C., Jr., Newhouse, Y.M., Clarke, H.R.G., Weisgraber, K.H., McCarthy, B.J., Mahley, R.W., and Bersot, T.P. (1989) Type III hyperlipoproteinemia associated with apolipoprotein E phenotype E3/3. Structure and genetics of an apolipoprotein E3 variant. J. Clin. Invest. 83: 1095-1101.

Reardon, M.F., Fidge, N.H., and Nestel, P.J. (1978) Catabolism of very low density lipoprotein B apoprotein in man. J. Clin. Invest. 61: 850-860.

Rodrigues, B., Spooner, M., and Severson, D.L. (1992) Free fatty acids do not release lipoprotein lipase from isolated cardiac myocytes of perfused hearts. Am. J. Physiol. 262: E216-E223.

Rubinstein, A., Gibson, J.C., Paterniti, J.R., Kakis, G., Little, A., Ginsberg, H.N., and Brown, W.V. (1985) Effect of heparin-induced lipolysis on the distribution of apolipoprotein E among lipoprotein subclasses. Studies with patients deficient in hepatic triglyceride lipase and lipoprotein lipase. J. Clin. Invest. 75: 710-721.

Russell, D.W., Yamamoto, T., Schneider, W.J., Slaughter, C.J., Brown, M.S., and Goldstein, J.L. (1983) cDNA cloning of the bovine low density lipoprotein receptor: feedback regulation of a receptor mRNA. Proc. Natl. Acad. Sci. USA 80: 7501-7505.

Russell, D.W., Schneider, J.W., Yamamoto, T., Luskey, K.L., Brown, M.S., and Goldstein, J.L. (1984) Domain map of the LDL receptor: sequence homology with epidermal growth factor precursor. Cell 37: 577-585.

Saheki, S., Takahashi, I., Murase, M., Takeuchi, N., and Uchida, K. (1991) Composition of very low density lipoproteins and *in vitro* effect of lipoprotein lipase. Clin. Chim. Acta 204: 155-166.

Santamarina-Fojo, S. (1992) Genetic dyslipoproteinemias: role of lipoprotein lipase and apolipoprotein C-II. Curr. Opin. Lipidol. 3: 186-195.

Sarda, L., and Desnuelle, P. (1958) Action de la lipase pancréatique sur les esters en émulsion. Biochim. Biophys. Acta 30: 513-521.

Saxena, U., Klein, R.G., and Goldberg, I.J. (1991) Identification and characterization of the endothelial cell surface lipoprotein lipase receptor. J. Biol. Chem. 266: 17516-17521.

Saxena, U., Klein, M.G., and Goldberg, I.J. (1991) Transport of lipoprotein lipase across endothelial cells. Proc. Natl. Acad. Sci. USA 88: 2254-2258.

Schaefer, E.J., Gregg, R.E., Ghiselli, G., Forte, T.M., Ordovas, J.M., Zech, L.A., and Brewer, H.B. (1986) Familial apolipoprotein E deficiency. J. Clin. Invest. 78: 1206-1219.

Schayek, E., and Eisenberg, S. (1991a) Mechanisms of inhibition by apolipoprotein C of apolipoprotein E-dependent cellular metabolism of human triglyceride-rich lipoproteins through the low density lipoprotein receptor pathway. J. Biol. Chem. 266: 18259-18267.

Schayek, E., Lewin-Velvert, U., Chajek-Straul, T., and Eisenberg, s. (1991b) Lipolysis exposes unreactive endogenous apolipoprotein E-3 in human and rat plasma very low density lipoproteins. J. Clin. Invest. 88: 553-560.

Schneider, W.J., Kovanen, P.T., Brown, M.S., Goldstein, J.L., Utermann, G., Weber, W., Havel, R.J., Kane, J.P., Innerarity, T.L., and Mahley, R.W. (1981) Familial dysbetalipoproteinemia. J. Clin. Invest. 68: 1075-1085.

Scow, R.O., and Olivecrona, T. (1977) Effect of albumin on products formed from chylomicron triacylglycerol by lipoprotein lipase in vitro. Biochim. Biophys. Acta 487: 472-486.

Semb, H., and Olivecrona, T. (1989) The relation between glycosylation and activity of guinea pig lipoprotein lipase. J. Biol. Chem. 264: 4195-4200.

Semenkovich, C.F., Chen, S.H., Wims, M., Luio, C.C., Li, W.H., and Chan, L. (1989) Lipoprotein lipase and hepatic lipase mRNA tissue specific expression, developmental regulation, and evolution. J. Lipid Res. 30: 423-431.

Semenkovich, C.F., Luo, C.-C., Nakanishi, M.K., Chen, S.H., Smith, L.C., and Chan, L. (1990) *In vitro* expression and site-specific mutagenesis of the cloned human lipoprotein lipase gene. Potential N-linked glycosylation site asparagine 43 is important for both enzyme activity and secretion. J. Biol. Chem. 265: 5429-5433.

Sherill, B.C., and Dietschy, J.M. (1978) Characterization of the sinusoidal transport process responsible for the uptake of chylomicrons by the liver. J. Biol. Chem. 253: 1859-1867.

Sherill, B.C., Innerarity, T.L., and Mahley, R.W. (1980) Rapid hepatic clearance of the canine lipoproteins containing only the E apolipoprotein by a high affinity receptor. J. Biol. Chem. 255: 1804-1807.

Shore, V.G., and Shore, B. (1973) Heterogeneity of human plasma very low density lipoproteins. Separation of species differing in protein components. Biochemistry 12: 502-507.

Sigurdsson, G.A., Lewis, N., and Lewis, B. (1975) Conversion of very low density lipoprotein to low density lipoprotein. J. Clin. Invest. 56: 1481-1490.

Smit, M., De Knijff, P., Frants, R.R., Klasen, E.C., and Havekes, L.M. (1987) Familial dysbetalipoproteinemic subjects with the E3/E2 phenotype exhibit an E2 isoform with only one cysteine residue. Clin. Genet. 32: 335-341.

Smit, M., De Knijff, P., Rosseneu, M,. Bury, J., Klasen, E., Frants, R., and Havekes, L. (1988) Apolipoprotein E polymorphism in the Netherlands and its effect on plasma lipid and apolipoprotein levels. Hum. Genet. 80: 287-292.

Smit, M., De Knijff, P., Van der Kooij-Meijs, E., Groenendijk, C., Van den Maagdenberg, A.M.J.M., Gevers Leuven, J.A., Stalenhoef, A.F.H., Stuyt, P.M.J., Frants, R.R., and Havekes, L.M. (1990) Genetic heterogeneity in familial dysbetalipoproteinemia. The E2(Lys146 → Gln) variant results in a dominant mode of inheritance. J. Lipid Res. 31: 45-53.

Smith, L.C., and Pownall, H.J. (1984) Lipases. In: Borgström, B., and Brockman, H.L., eds. Amsterdam: Elsevier, pp. 263-305.

Sparkes, R.S., Zollman, S., Klisak, I., Kirchgessner, T.G., Komaromy, M.G., Mohondes, T., Schotz, M.C., and Lusis, A.J. (1987) Human genes involved in lipolysis of plasma lipoproteins: mapping of loci for lipoprotein lipase to 8p22 and hepatic lipase to 15q21. Genomics 1: 138-144.

Stalenhoef, A.F.H., Malloy, M.J., Kane, J.P., and Havel, R.J. (1986) Metabolism of apolipoproteins B-48 and B-100 of triglyceride-rich lipoproteins in patients with familial dysbetalipoproteinemia. J. Clin. Invest. 78: 722-728.

Stein, O., Stein, Y., Goodman, D.S., and Fidge, N.H. (1969) The metabolism of chylomicron cholesteryl ester in rat liver. A combined radioautographic-electron microscopic and biochemical study. J. Cell Biol. 43: 410-431.

Steinmetz, A., Assefbarkhi, N, Eltze, C., Ehlenz, K., Funke, H., Pies, A., Assmann, G., and Kaffarnik, H. (1990) Normolipemic dysbetalipoproteinemia and hyperlipoproteinemia type III in subjects homozygous for a rare genetic apolipoprotein E variant (apoE1). J. Lipid Res. 31: 1005-1013.

Stow, J.L., Kjellen, L., Unger, E., Hook, M., and Farquhar, M.G. (1985) Heparan sulfate proteoglycans are concentrated on the sinusoidal plasmalemmal domain and in intracellular organelles of hepatocytes. J. Cell Biol. 100: 975-980.

Strickland, D.K., Ashcom, J.D., Williams, S., Burgess, W.H., Migliorini, M., and Argraves, W.S. (1990) Sequence identity between the alpha-2-macroglobulin receptor and low density lipoprotein receptor-related protein suggests that this molecule is a multifunctional receptor. J. Biol. Chem. 265: 17401-17404.

Tall, A.R., and Small, D.M. (1978) Plasma high density lipoproteins. N. Engl. J. Med. 299: 1232-1236.

Tata, F., Henry, I., Markham, A.F., Wallis, S.C., Weil, D., Grzeschik, H.K., Junien, C., Williamson, R., and Humphries, S.E. (1985) Isolation and characterization of a cDNA clone for human apolipoprotein CI and assignment of the gene to chromosome 19. Hum. Genet. 69: 345-349.

Thuren, T., Wilcox, R.W., Sisson, P., and Waite, M. (1991) Hepatic lipase hydrolysis of lipid monolayers. Regulation by apolipoproteins. J. Biol. Chem. 266: 4853-4861.

Utermann, G., Pruin, N., and Steinmetz, A. (1979) Polymorphism of apolipoprotein E. III. Effect of a single polymorphic gene locus on plasma lipid levels in man. Clin. Genet. 15: 63-72.

Utermann, G., Langenbeck, U., Beisiegel, U., Weber, W. (1980) Genetics of the apolipoprotein E system in man. Am. J. Hum. Genet. 32: 339-347.

Utermann, G. (1985) Genetic polymorphism of apolipoprotein E. Impact on plasma lipoprotein metabolism. Crepaldi, G., Tiengo, A., and Baggio, G., eds. Diabetes, obesity, and hyperlipodemias-III. Amsterdam: Elsevier Science Publishers, pp. 1-28.

Vilaro, S., Llobera, M., Bengtsson-Olivecrona, G., and Olivecrona, T. (1988) Lipoprotein lipase uptake by the liver: localization, turnover, and metabolic role. Am. J. Physiol. 254: G711-G722.

Vileli, E., and Joven, J. (1991) Lipoprotein lipase binding to plasma lipoproteins. Med. Sci. Res. 19: 111-112.

Wang, C.-S., Bass, H.B., Downs, D., and Whitmer, R.K. (1981) Modified heparin-Sepharose procedure for determination of plasma lipolytic activities of normolipidemic and hyperlipidemic subjects after injection of heparin. Clin. Chem. 27: 663-668.

Wang, C.-S., McConnathy, W.J., Kloer, H.U., and Alaupovic, P. (1985) Modulation of lipoprotein lipase activity by apolipoproteins. Effect of apolipoprotein C-III. J. Clin. Invest. 75: 384-390.

Wang, C.-S., Hartsuck, J.A., Downs, D., and Bass, H.A. (1990) Acylglycerol reactivity and reaction mechanism of bovine milk lipoprotein lipase. Biochim. Biophys. Acta 1043: 143-148.

Wang, C.-S., Hartsuck, J., and McConnathy, W.J. (1992) Structure and functional properties of lipoprotein lipase. Biochim. Biophys. Acta 1123: 1-17.

Wardell, M.R., Brennan, S.O., Janus, E.D., Fraser, R., and Carrel, R.W. (1987) Apolipoprotein E2-Christchurch(136Arg → Ser). New variant of human apolipoprotein E in a patient with type III hyperlipoproteinemia. J. Clin. Invest. 80: 483-490.

Wardell, M.R., Weisgraber, K.H., Havekes, L.M., and Rall, S.C. Jr. (1989) Apolipoprotein E3-Leiden contains a seven-amino acid insertion that is a tandem repeat of residues 121-127. J. Biol. Chem. 264: 21205-21210.

Weisgraber, K.H., Innerarity, T.L., and Mahley, R.W. (1978) Role of the lysine residues in plasma lipoproteins in high affinity binding to cell surface receptors on human fibroblasts. J. Biol. Chem. 253: 9053-9062.

Weisgraber, K.H., Innerarity, T.L., and Mahley, R.W. (1982) Abnormal lipoprotein receptor binding activity of the human E apoprotein due to cysteine-arginine interchange at a single site. J. Biol. Chem. 257: 2518-2521.

Weisgraber, K.H., Rall, S.C. Jr., Innerarity, T.L., Mahley, R.W., Kuusi, T., and Ehnholm, C. (1984) A novel electrophoretic variant of human apolipoprotein E. Identification and characterization of apolipoprotein E1. J. Clin. Invest. 73: 1024-1033.

Weisgraber, K.H. (1990) Apolipoprotein E distribution among human plasma lipoproteins: Role of the cysteine-arginine interchange at residue 112. J. Lipid Res. 31: 1503-1511.

Weisgraber, K.H., and Shinto, L.H. (1991) Identification of the disulfide-linked homodimer of apolipoprotein E3 in plasma. J. Biol. Chem. 266: 12029-12034.

Wetterau, J.R., Aggerbeck, L.P., Rall, S.C., Weisgraber, K.H., Wu, X.-S.C., Lindgren, F.T. (1988) Human apo E3 in aqueous solution two domains. J. Biol. Chem. 263: 6240-6248.

Willnow, T., Goldstein, J.L., Orth, K., Brown, M.S., and Herz, J. (1992) Low density lipoprotein receptor-related protein and gp 330 bind similar ligands, including plasminogen activator-inhibitor complex and lactoferrin, an inhibitor of chylomicron remnant clearance. J. Biol. Chem. 267: 26172-26180.

Wilson, C., Wardell, M.R., Weisgraber, K.H., Mahley, R.W., and Agard, D.A. (1991) Three-dimensional structure of the LDL receptor-binding domain of human apolipoprotein E. Science 252: 1817-1822.

Windler, E., Chao, Y.-S., and Havel, R.J. (1980) Determinants of hepatic uptake of triglyceride-rich lipoproteins and their remnants in the rat. J. Biol. Chem. 255: 5475-5480.

Windler, E.E.T., Greeve, J., Daerr, W.H., and Greten, H. (1988) Binding of rat chylomicrons and their remnants to the hepatic low-density-lipoprotein receptor and its role in remnant removel. Biochem. J. 252: 553-561.

Winkler, F.K., D'Arcy, A., and Hunziker, W. (1990) Structure of human pancreatic lipase. Nature 343: 771-774.

Yang, C.-Y., Gu, Z.-W., Yang, H.-X., Rohde, M.F., Gotto, A.M., and Pownall, H.J. (1989) Structure of bovine milk lipoprotein lipase. J. Biol. Chem. 264: 16822-16827.

Yla-Herttuala, S., Lipton, B.A., Rosenfeld, M.E., Goldberg, I.J., Steinberg, D., and Witztum, J.L. (1991) Macrophages and smooth muscle cells express lipoprotein lipase in human and rabbit atherosclerotic lesions. Proc. Natl. Acad. Sci. USA 88: 10143-10147.

Zannis, V.I., and Breslow, J.L. (1981) Human very low density lipoprotein apolipoprotein E isoprotein polymorphism is explained by genetic variation and postttranslational modification. Biochemistry 20: 1033-1041.

Ziere, G.J., Van Dijk, M.C.M., Bijsterbosch, M.K., and Van Berkel, T.J.C. (1992) Lactoferrin uptake by the rat liver. J. Biol. Chem. 267: 11229-11235.

Chapter 2

THE BINDING OF HUMAN LIPOPROTEIN LIPASE TREATED VLDL BY THE HUMAN HEPATOMA CELL LINE HEP G2

Monique Mulder, Elly de Wit, and Louis M. Havekes

Gaubius Institute TNO, P.O. Box 612,
2300 AP Leiden, The Netherlands

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Summary

It has been suggested that besides the LDL-receptor, hepatocytes possess an apoE- or remnant receptor. To evaluate which hepatic lipoprotein receptor is involved in VLDL remnant catabolism, we studied the binding of VLDL remnants to Hep G2 cells.

Native VLDL was obtained from type IIb hyperlipidemic patients and treated with bovine milk lipoprotein lipase (LPL). This LPL-treated VLDL (LPL-VLDL) was used as representative for VLDL remnants. Our results show that LPL-VLDL binds with high affinity to Hep G2 cells. Competition experiments showed that the binding of ^{125}I-labelled LPL-VLDL is inhibited to about 30% of the control value by the simultaneous addition of an excess of either unlabelled LDL or LPL-VLDL.

Preincubation of Hep G2 cells with LDL resulted in a reduction of the binding of LDL and LPL-VLDL to 34 and 55% of the control value, whereas preincubation of the cells with heavy HDL (density between 1.16 and 1.21 g/ml) stimulated the binding of LDL and LPL-VLDL to about 230% of the control value. Preincubation of the cells with insulin (250 nM/l) also stimulated the binding of both LDL and LPL-VLDL (175 and 143% of the control value, respectively).

We conclude that LPL-VLDL binds to the LDL-receptor of Hep G2 cells, and that no evidence has been obtained for the presence on Hep G2 cells of an additional receptor that is involved in the binding of VLDL remnants.

Introduction

After entering the bloodstream, most of the triacylglycerols of chylomicrons and very low density lipoproteins (VLDL) are hydrolysed by the action of lipoprotein lipase lining the capillary endothelium. As compared with the native chylomicrons and VLDL, the resulting remnant particles (chylomicron- and VLDL remnants) are reduced in size concomitant with an elevated buoyant density and an altered lipid and apolipoprotein composition [for a review see reference 1]. Both remnant particles are rapidly taken up by the liver hepatocytes through recognition by high affinity lipoprotein receptors [2].

Liver hepatocytes possess two different lipoprotein receptors. One receptor recognizing both apoB and apoE, designated B,E receptor or LDL receptor, and another receptor recognizing only apoE and designated as E or chylomicron-remnant receptor [3]. Recently, an LDL receptor related protein (LRP) with molecular weight of about 500 kd was found [4,5]. This protein could be a candidate for the remnant receptor [6].

It has been reported that in many animal species chylomicron-remnants are taken up by hepatocytes exclusively through this putative remnant receptor [7]. This finding is sustained by the observations that the rate of uptake of chylomicron remnants is not influenced by interventions that affect the number of LDL receptors like cholesterol feeding [8,9], bile acid infusion [10] and administration of 17-α-ethinyl-estradiol [11]. However, in other reports it

is stated that in *in vitro* experiments chylomicron remnants bind to hepatic LDL receptors also [12].

There is no consensus concerning the hepatic receptor that mediates the uptake of VLDL and VLDL remnants. Harkes et al. [13] showed that in the rat liver almost all β-VLDL is taken up via the putative remnant receptor as the binding of β-VLDL could not be displaced by LDL. On the contrary, Windler et al. [14] showed that VLDL remnants interact with the LDL receptor in the liver with the possible exception of very large VLDL [15]. It has been reported that in LDL receptor deficient (WHHL) rabbits and in homozygous FH patients [16] the clearance of VLDL and VLDL remnants is disturbed, also indicating that the LDL receptor is involved in VLDL and VLDL remnant uptake.

Using the human hepatoma cell line Hep G2 as a model for human hepatocytes, Eisenberg et al. [17] and Friedman et al. [18] suggest that part of VLDL and VLDL remnants is taken up by a receptor different from the LDL receptor. Dashti et al. [19] reported that the binding of VLDL to Hep G2 cells is effectively inhibited by a simultaneous addition of an excess of LDL, indicating that the uptake of VLDL by Hep G2 cells is mediated by the LDL receptor.

In this paper we studied the binding of LPL-treated VLDL, taken as representative for VLDL remnants, to Hep G2 cells, in order to clarify the nature of the receptor involved in binding of VLDL remnants to Hep G2 cells. Our results show that LPL-VLDL is exclusively bound to the LDL receptor and that the affinity of these particles for the LDL receptor increases as cholesterol/triglyceride ratios increase.

Materials and Methods

Materials

Fetal calf serum (FCS) and Dulbecco's modified Eagle's medium (DMEM, cell culture medium) were obtained from Flow Laboratories (Irvine, U.K.). Penicillin, streptomycin, CHOD-PAP-mono-test kit and Test-Combination were purchased from Boehringer Mannhein (Mannheim, F.R.G.).

Human serum albumin (HSA) was obtained from Sigma Chemical Company (St. Louis, MO, USA). Sodium [^{125}I]iodide (specific activity 13.3 μCi/μg) was purchased from Amersham (Buckinghamshire, U.K.). 1M MgEGTA stock solution was prepared by mixing (1:1) solutions of 1M $MgCl_2$ and 1M EGTA.

Multiwell cell culture dishes were from Costar (Cambridge, MA, USA).

Lipoproteins

Human serum was prepared from freshly collected blood from patients with type IIb hypertriglyceridemia or from healthy donors, after an overnight fast. ApoE phenotyping was performed using Western blotting according to Havekes et al. [20]. Lipoproteins (VLDL and LDL) were isolated according to Redgrave [21] followed by tube slicing. Protein contents of the lipoprotein fractions were determined according to Lowry et al. [22]. Triglycerides

and total cholesterol were determined using enzymatic methods (Boehringer, Mannheim, FRG). The relative apolipoprotein composition of the lipoproteins was determined using electrophoresis on gradient SDS-polyacrylamide slab gels [23] (3-14%, with 0.1% sodium dodecyl sulphate) (SDS-PAGE) stained with Coomassie Brilliant blue, followed by densitometric scanning at 560 nm on a Shimadzu CS-910 densitometric scanner. Using this technique, we could not detect the presence of apoE and apoC's in LDL samples implying that less than 0.1% (w/w) of the LDL protein consists of apoE or apoC's. This was further confirmed by radial immunodiffusion ouchterlony techniques.

Preparation of Lipoprotein Lipase treated VLDL (LPL-VLDL)

LPL-VLDL were prepared by incubating total serum with lipoprotein lipase (LPL) purified from bovine milk [24]. An amount of LPL was added, equal to the amount necessary for hydrolysis of 50% of the triglycerides present in complete serum, within one hour.

The serum was incubated with lipoprotein lipase in the presence of 10% (w/v) free fatty acid free human serum albumin (HSA), and Tris-HCl buffer (final concentration 0.1 M, pH 8.5) for 90 minutes at 37°C. To stop the reaction the mixture was put on ice and solid KBr was added to adjust the solution to a density of 1.21 g/ml. The solution was then placed under a discontinuous gradient of salt solutions, of densities 1.063 and 1.019 g/ml, respectively with a volume ratio of 0.95:1:1 from bottom to top. After centrifugation in a swinging bucket rotor (SW40) for 16 hours at 200.000 x g the top fraction containing the LPL-VLDL was collected.

Labelling of the Lipoproteins

The lipoprotein preparations were immediately used for iodination by the [^{125}I]iodine monochloride method described by Bilheimer et al. [25]. After iodination, the lipoproteins were dialysed against phosphate-buffered saline for 4h (4 times 500ml). Thereafter they were stabilized by the addition of HSA (1% w/v) and further dialysed overnight against culture medium supplemented with 20 mM Hepes buffer (pH 7.4) penicillin and streptomycin. The stabilized ^{125}I-labelled lipoproteins were stored at 4°C. Their specific radioactivity ranged from 50-150 cpm/ng of lipoprotein protein. With LDL the label present in the lipid fraction was less than 1%, whereas with LPL-VLDL about 20% of the label was found in the lipid fraction. With both labelled lipoproteins less than 0.1% of the label was trichloro-acetic-acid-soluble. Although stable for periods greater than 1 month under these conditions [26], the iodinated lipoproteins were used within 10 days. To check for degradation of the labelled lipoproteins upon storage, after 10 days SDS-PAGE was performed followed by autoradiography. To check whether the binding properties of the labelled lipoproteins were affected by storage, binding studies were performed immediately after isolation and labelling of the lipoproteins and after 10 days of storage. No proteolysis or significant changes in binding properties occurred during storage for 10 days.

When not labelled with [^{125}I]iodine, lipoproteins were stabilized immediately with the addition of 1% HSA and subsequently extensively dialysed against culture medium supplemented with Hepes, penicillin and streptomycin.

Culturing of Hep G2 cells

The cells were cultured at 37°C in 25 cm^2 flasks containing 2 ml of DMEM culture medium supplemented with 10% heat-inactivated FCS, 20 mM Hepes, 25 mM $NaHCO_3$, 100 U/ml penicillin and 100 μg/ml streptomycin under 5% CO_2/95% air. The medium was renewed twice a week. About 1 week before the experiment the cells were trypsinized and transported to 2 cm^2 Multiwell dishes, with a split ratio of 1:6. Twenty hours before the assay, the medium was replaced with culture medium containing 1% HSA instead of 10% FCS and the additions as indicated.

Measurement of receptor-mediated association of labelled lipoproteins

Shortly before the experiment, the cells, were washed three times and incubated in the same culture medium supplemented with HSA, streptomycin and penicillin but without further additions, for a period of 30 min at 37°C.

The experiment was started by the addition of ^{125}I-labelled lipoprotein in the absence or in the presence of unlabelled lipoprotein as indicated. After 3 hours of incubation at 37°C the cells were cooled to 0°C and the labelled lipoprotein that became cell-associated was measured exactly as described previously [27]. The receptor-mediated (*specific*) cell-association was calculated by subtracting the amount of labelled lipoproteins that was cell associated after incubation in the presence of a 30-fold excess of unlabelled lipoprotein (*aspecific*) from the amount of labelled lipoprotein that was cell associated after incubation in the absence of unlabelled lipoproteins (*total* cell association).

Results

As presented in Table 1, LPL treatment of VLDL resulted in an increase of the cholesterol/triglyceride ratio from 0.67 ± 0.35 to 1.70 ± 1.25 (mol/mol; $n = 16$). The apoE/apoC ratio increased from 0.50 ± 0.19 to 2.90 ± 1.18 as a result of loss of apoC mainly, whereas the apoE/apoB ratio slightly decreased. In the LDL fraction apoB48, apoE and apoC could not be detected by SDS-PAGE. Only apoB100 was present. Although the cholesterol/triglyceride ratio of the VLDL increases after LPL treatment, the ratio in the LDL fraction is considerably higher (12.48 ± 3.69). Thus the composition of LPL-VLDL does not ressemble the composition of LDL as far as lipids and apolipoproteins are concerned. For the isolation of lipoproteins, plasma was taken from subjects after a 14 hour period of fasting. Nevertheless, to evaluate the presence of chylomicrons in the VLDL samples the ratio of apoB48/apoB100 was determined. The percentage of apoB48 was 3.5 ± 1.8% of the total amount of apoB.

Labelled LPL-VLDL was incubated with Hep G2 cells to measure the receptor-mediated association (Fig. 1). The high affinity binding of LPL-VLDL was saturated at about 50 μg/ml protein.

Table 1. Relative composition of VLDL, LPL-VLDL and LDL.

Ratio	VLDL	LPL-VLDL	LDL
TC/TG	0.67 ± 0.35 (n = 16)	1.70 ± 1.25 (n = 16)	12.48 ± 3.69 (n = 14)
ApoE/ApoC	0.50 ± 0.19 (n = 8)	2.90 ± 1.18 (n = 4)	n.d.*
ApoE/ApoB	2.56 (n = 2)	2.04 (n = 2)	n.d.*
ApoB48/ApoB100	0.035 ± 0.018 (n = 4)	0.035 ± 0.015 (n = 3)	n.d.*

The cholesterol/triacylglycerol ratio (TC/TG) is expressed as molar/molar ratio. Relative amounts of apolipoproteins are obtained as a result of densitometric scanning of SDS polyacrylamide gels. The percentage composition of apoB, apoE and apoC in native VLDL was 62, 13 and 25%, respectively, and changed after LPL treatment into 82, 13 and 5%, respectively. n represents the number of VLDL and LPL-VLDL samples analyzed. TC, total cholesterol; TG, triacylglycerols.

* ApoB48, apoE and apoC are not detectable in the LDL fraction by SDS gel electrophoresis.

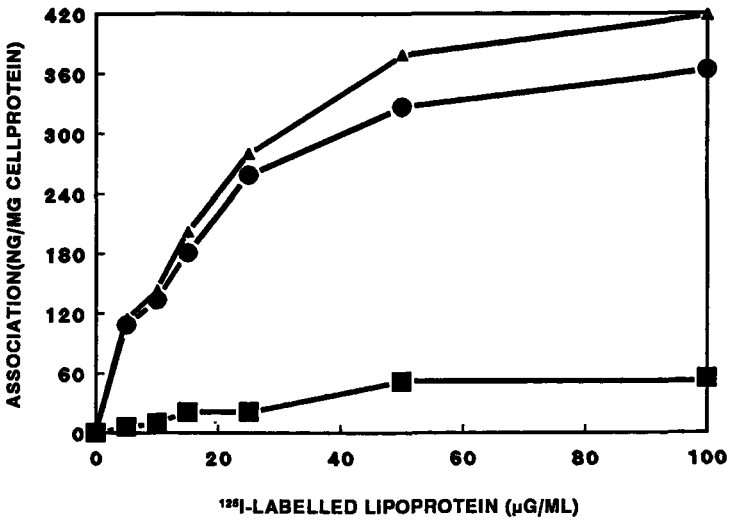

Figure 1. Representative experiment of the association of ^{125}I-labelled LPL-VLDL to Hep G2 cells. After preincubation for 20 hours in medium supplemented with 1% HSA (w/v) the Hep G2 cells were incubated for 3 hours with ^{125}I-labelled LPL-VLDL at 37°C. Total (▲), receptor-mediated (specific, ●) and receptor-independent (aspecific, ■) cell association were determined as described in Materials and Methods. In this particular experiment, the cholesterol/triglyceride ratio of the LPL-VLDL was 0.98 (mol/mol). Each value represents the mean of triplicate measurements.

To evaluate whether LPL-VLDL binds to the LDL receptor, competition experiments were performed. As shown in Fig. 2, a gradual increase of the cholesterol/triglyceride ratio in VLDL, as a result of increasing time of incubation with LPL, resulted in an increased

efficiency of competition with ^{125}I-labelled LDL for binding to the LDL receptor in Hep G2 cells. Unlabelled LDL was the most efficient competitor. In Fig. 2 the amounts of unlabelled lipoproteins added are expressed as μg/ml of lipoprotein protein. Comparable results were obtained when the amounts of unlabelled lipoproteins were expressed either as μg cholesterol/ml or as μg apolipoprotein B/ml.

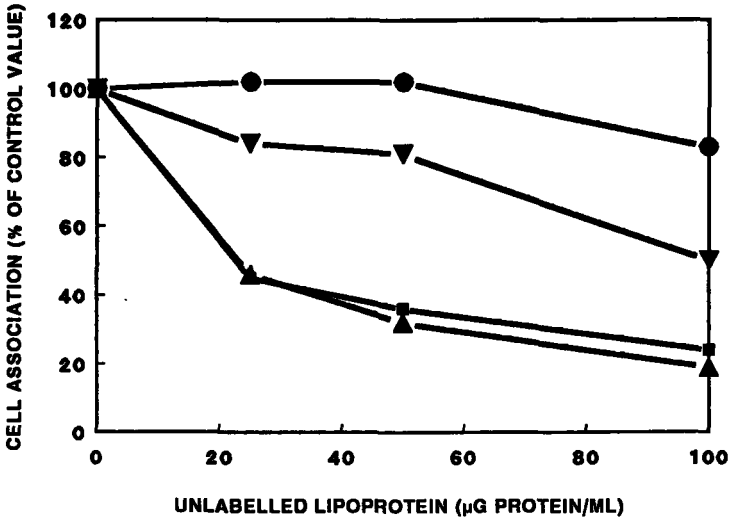

Figure 2. Competition of the association of ^{125}I-labelled LDL to Hep G2 cells by simultaneous addition of increasing amounts of unlabelled VLDL samples with different cholesterol/triglycerides ratios. After preincubation for 20 hours in medium supplemented with 1% (w/v) HSA the Hep G2 cells were incubated for 3 hours with 10 μg/ml of ^{125}I-labelled LDL protein in the presence of the indicated amount of the different unlabelled LPL-treated VLDL samples followed by measuring the association of ^{125}I-labelled LDL. The association of ^{125}I-LDL in the absence of unlabelled lipoprotein was defined as 100%. Each value is the mean of quadruplicate measurements. The various LPL-treated VLDL samples were obtained by incubation of serum with LPL for increasing periods of time followed by isolation by ultracentrifugation. The cholesterol/triglyceride ratios were: ●, 0.55; ▼, 0.81 and ■,1.29; ▲ represents competition with unlabelled LDL.

To investigate whether LPL-VLDL binds to another receptor in addition to the LDL receptor, further competition experiments were performed. The results presented in Table 2 show that 300 μg/ml of unlabelled LDL and LPL-VLDL were equally efficient in competing with either ^{125}I-labelled LDL or LPL-VLDL. The association of labelled LPL-VLDL was inhibited by the addition of 300 μg/ml of unlabelled LDL to 27% of the control value. This inhibition was enhanced by the simultaneous addition of either 300 μg/ml of unlabelled LPL-VLDL (14%) or an extra 300 μg/ml of unlabelled LDL (18%). These results suggest that the binding of LPL-VLDL is mediated by the LDL receptor, whereas no evidence has been obtained for the presence on Hep G2 cells of an additional receptor that is involved in the binding of LPL-VLDL.

To further substantiate this hypothesis, we studied the effect of preincubation of Hep G2 cells with LDL or heavy HDL on the binding of LDL and LPL-treated VLDL (Table 3).

Table 2. The ability of unlabelled LDL and LPL-VLDL to compete with both ^{125}I-labelled LDL and ^{125}I-labelled LPL-VLDL for association to Hep G2 cells.

Unlabelled lipoprotein added (μg protein/ml)	^{125}I-labelled lipoprotein (10 μg/ml, % of control value)	
	LDL	LPL-VLDL
None	100	100
LDL (300)	24 ± 10	27 ± 9
LDL (600)	14 ± 6	18 ± 1
LPL-VLDL (300)	27 ± 12	23 ± 10
LPL-VLDL + LDL (300 + 300)	n.d.	14 ± 4

After preincubation for 20 h in medium supplemented with 1% (w/v) HSA the cells were incubated for 3 h at 37°C, with 10 μg/ml of ^{125}I-labelled lipoprotein and unlabelled lipoprotein as indicated. Thereafter, the cell association was measured as described in Materials and Methods. Each value represents the percentage of the respective value obtained after incubation without unlabelled lipoprotein. Each value is the mean ± S.D. of at least four independent experiments, carried out in quadruplicate, n.d., not determined.

Table 3. Receptor-mediated association of ^{125}I-LDL or ^{125}I-labelled LPL-VLDL (10 μg/ml) to Hep G2 cells after preincubation of the cells with or without LDL, heavy HDL or insulin.

Cells preincubated with	Receptor-mediated association (% of control value)	
	^{125}I-LDL	^{125}I-LPL-VLDL
LDL (300 μg/ml)	34 ± 6	55 ± 28
Heavy HDL (100 μg apoA-I/ml)	221 ± 63	237 ± 56
Insulin (50 nM)	127 ± 5	128 ± 24
Insulin (250 nM)	175 ± 11	143 ± 25

After preincubation for 20 h in medium supplemented with 1% (w/v) HSA the cells were incubated for 3 h at 37°C, with 10 μg/ml of ^{125}I-labelled lipoprotein and unlabelled lipoprotein as indicated. Thereafter, the cell association was measured as described in Materials and Methods. Each value represents the percentage of the respective value obtained after incubation without unlabelled lipoprotein. Each value is the mean ± S.D. of at least four independent experiments, carried out in quadruplicate, n.d., not determined.

Preincubation of the cells with 250 μg/ml of LDL resulted in a reduction of the binding of ^{125}I-LDL to 34% of the control value, whereas preincubation with heavy HDL (100 μg apolipoprotein AI/ml) stimulated the binding of ^{125}I-LDL to 221% of the control value, similar to our results published previously [28,29]. Table 3 represents similar results for the binding of ^{125}I-labelled LPL-VLDL (55% and 237% of the control value, respectively).

Preincubation of the cells with insulin also resulted in a dose dependent increase of the binding of both ^{125}I-LDL and ^{125}I-labelled LPL-VLDL.

Because the LDL receptor is found to be calcium-dependent, we studied the effect of EGTA on the receptor-mediated association of LDL and LPL-VLDL. Figure 3 shows that with increasing amounts of EGTA present in the medium, the association of LDL is inhibited. At about 3 mM of EGTA the inhibition of the receptor-mediated association of LDL is nearly complete. The receptor-mediated association of LPL-VLDL was almost equally sensitive to EGTA, although the maximum inhibition of the receptor-mediated association was less pronounced.

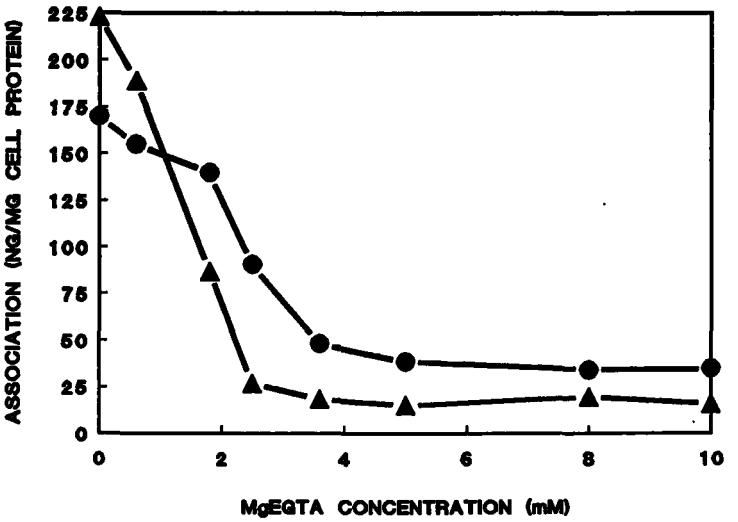

Figure 3. Effect of EGTA on the receptor-mediated association of ^{125}I-LDL and ^{125}I-LPL-VLDL to Hep G2 cells. Increasing amounts of EGTA were added to the culture medium as indicated, whereafter the receptor-mediated association of ^{125}I-LDL (▲) and ^{125}I-LPL-VLDL (●) (10 μg/ml) were measured in triplicate.

Discussion

From the literature the data concerning the nature of the hepatic lipoprotein receptor that mediates the uptake of VLDL remnants are not uniform. Several observations suggest that VLDL particles are removed from the circulation by the chylomicron-remnant receptor [13,17,30], while others find that the LDL receptor is responsible for the removal of VLDL derived lipoproteins [16,19,31]. In this paper we attempted to clarify the nature of the receptor involved in the binding of VLDL remnants. Since apoE is the ligand for binding of VLDL remnants to the receptor [17,32,33], and the isoform apoE2 is impaired in this respect [34], we used VLDL from subjects with apoE3 and/or apoE4 isoforms. These subjects, however, do not contain substantial amounts of VLDL remnants. Therefore, as an alternative for VLDL remnants, we used LPL-treated VLDL. For the treatment of VLDL with LPL, we used fasted complete serum rather than isolated VLDL in order to let the apolipoproteins

and lipids redistribute between the different lipoprotein fractions during the lipolysis of VLDL triglycerides. The composition of the LPL-VLDL samples (Table 1) resembled the composition of VLDL remnants (β-VLDL and IDL) normally isolated from subjects with familial dysbetalipoproteinemia or type III hyperlipoproteinemia [1].

Our results show that LPL-VLDL binds with high affinity to Hep G2 cells and that the increase in cholesterol/triglyceride ratio results in a gradual increase of the affinity for the LDL receptor. Factors responsible for this increase in binding affinity may be either loss of apoC which is known to inhibit the binding [13,14] or conformational changes of apoE as a result of a different lipid composition of the particle [35-37].

To avoid contamination of the LPL-VLDL samples with considerable amounts of chylomicron remnants, we used fasted serum. Our results show that only about 3.5% of the total amount of apoB in the VLDL samples consists of apoB48 (Table 1), indicating that the presence of chylomicrons is minimal. Furthermore, SDS polyacrylamide gelelectrophoresis followed by autoradiography revealed that in LPL-VLDL which became cell associated, the proportion of apoB48 relative to apoB100 did not increase compared with the relative amount of apoB48 in the stock LPL-VLDL samples (data not shown). We therefore may exclude the possibility that residual chylomicrons do contribute considerably to the binding results presented.

Besides the LDL receptor another receptor, recognizing only apoE and designed as E-or chylomicron remnant receptor, has been suggested as being present in hepatocytes [3]. Recently, an LDL receptor related protein (LRP) with molecular weight of about 500kD has been found [4,5] and could be a candidate for this apoE- or remnant receptor [6]. WHHL rabbits and patients with familial hypercholesterolemia, have a defect in the removal of VLDL remnants resulting from a lack of the LDL receptor, indicating that also *in vivo* the LDL receptor is necessary for the removal of VLDL remnants [16]. On the contrary, Harkes et al. [13] have shown that the uptake of β-VLDL *in vivo* in the rat is mediated completely by the remnant receptor. These contradictory results could be explained by species differences. It is suggested that the catabolism of VLDL in the rat resembles that of chylomicrons, while in man VLDL catabolism resembles the LDL catabolism [38].

Eisenberg and co-workers [17,18] have suggested that also in Hep G2 cells a receptor distinct from the LDL receptor is involved in VLDL and VLDL remnant (IDL) uptake.

As well as species differences, these conflicting results may be due to inter-individual variations of the VLDL fractions regarding the amount of apoE per particle and/or the conformation of apoE, which might be influenced by the (lipid) composition of the lipoprotein particle [35-37].

Friedman et al. [18] have proposed the presence of an apoE3 specific binding site on Hep G2 cells as in their competition experiments they found incomplete competition of labelled IDL with unlabelled LDL. However, this incomplete competition could be due to the relatively low amounts of unlabelled LDL used as competitor (up to five-fold excess) together with a possible lower affinity of LDL to the receptor as compared to that of IDL.

To investigate whether an additional receptor is involved in the binding of VLDL remnants, we also performed competition experiments (Table 2). For this, we used large

amounts of unlabelled LDL (upto 30-fold) to attain maximal inhibition of receptor-mediated association. From these competition experiments, we conclude that the binding of LPL-VLDL to Hep G2 cells is mediated exclusively by the LDL receptor and that no evidence has been obtained for the presence of an additional receptor on Hep G2 cells that is involved in the binding of LPL-VLDL. This similarity in binding properties is not due to a similar composition of LPL-VLDL and LDL, as indicated in Table 1. Preincubation experiments, with LDL, heavy HDL and insulin show that the binding of LPL-VLDL is regulated similarly to that of the binding of LDL. As it has been reported that the apoE receptor is not influenced by interventions that affect the number of LDL receptors [8-11], this observation also argues against the presence in Hep G2 cells of an additional receptor involved in the binding of LPL-VLDL. Previous studies have shown that Hep G2 cells offer a suitable model system to study the metabolism of lipoproteins [26,27,28]. However, whether the present data of LPL-VLDL uptake by Hep G2 cells holds true for the *in vivo* situation in the liver remains to be answered.

Acknowledgements

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References

1 Lipoprotein and lipid metabolism disorders. In: The metabolic basis of inherited diseases. Scriver CR, Beaudet AL, Sly WS, Valle D. (eds) New York McGraw-Hill Book Co 1989; pp. 1129-1302.
2 Sherill, B.C., Innerarity, T.L., and Mahley, R.W. (1980) J. Biol. Chem. 255,1804-1807.
3 Mahley, R.W., Hui, D.Y., and Innerarity, T.L. (1981) J. Clin. Invest. 68, 1197-1206.
4 Herz, J., Hatemann, U., Rogna, S., Myklebost, O., Gausepohl, H. and Stanley, K.K. (1988) EMBO J. 7, 4119-4127.
5 Beisiegel, U., Weber, W., Ihrke, G., Here J. and Stanley, K.K. (1989) Nature 314, 162-164.
6 Soutar, A.K. (1989) Nature 341, 106-107.
7 Kita, T., Goldstein, J.L., Brown, M.S., et al. (1982) Proc. Natl. Acad. Sci. USA 9, 3623-3627.
8 Melchior, G.W., Mahley, R.W. and Buckhold, D.K. (1981) J. Lipid Res. 22, 598-609.
9 Thompson, K.H. and Zilversmit, D.B. (1983) J. Nutr. 113, 2002-2010.
10 Angelin, B., Raviola, C.A., Innerarity, T.L., et al. (1983) J. Clin. Invest. 71, 816-831.
11 Arbeeny, C.M. and Rifici, V.A. (1984) J. Biol. Chem. 259, 9662-9666.
12 Florén, C.H. (1988) Scand. J. Clin. Invest. 48, 553-556.
13 Harkes, L., Van Duijne, A. and Van Berkel, T.J.C. (1989) Eur. J. Biochem. 180, 241-248.
14 Windler, E., Kovanen, P.T., Chao, Y.-S., et al. (1980) J. Biol. Chem. 255, 10464-10471.
15 Havel, R.J., Yamada, N. and Shames, D.M. (1987) Am. Heart J. 113, 470-474.
16 Kita, T., Goldstein, J.L., Brown, M.S., Watanabe, Y., Hornick, C.A. and Havel, R.J. (1982) Proc. Natl. Acad. Sci. USA 79, 5693-5697.

17 Eisenberg, S., Friedman, G. and Vogel, T. (1988) Arteriosclerosis 8, 480-487.
18 Friedman, G., Gravish, D., Vogel, T., and Eisenberg, S. (1990) Biochem. Biophys. Acta 1044, 118-126.
19 Dashti, N. and Wolfbauer, G. (1986) Biochim. Biophys. Acta 875, 473-486.
20 Havekes, L.M., de Knijff, P., Beisiegel, U., Havinga, J., Smit, M., and Klasen, E. (1987) J. Lipid Res. 28, 455-463.
21 Redgrave, T.G. (1970) J. Clin. Invest. 49, 465-471.
22 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
23 Laemmli, U.K. (1970) Nature (Lond.) 227, 680-685.
24 Pagano Mirani-Oostdijk, C. Havekes, L., Den Boer, P., De Wit, E. and Terpstra, J. (1985) Scand. J. Clin. Lab. Invest. 45, 207-215.
25 Bilheimer, D.W., Eisenberg, S. and Levy, R.I. (1972) Biochim. Biophys. Acta 260, 212-221.
26 Havekes, L. (1981) Clin. Chim. Acta 116, 131-135.
27 Havekes, L., Van Hinsbergh V., Kempen, H.J. and Emeis, J. (1983) Biochem. J. 214, 951-958.
28 Havekes, L., Verboom, H., De Wit, E., Yap, S.H. and Princen, H.M.G. (1986) Hepatology 6, 1356-1360.
29 Havekes, L.M., Schouten, D., de Wit, E., Cohen, L.C., Griffioen, M., Van Hinsbergh, V.W.M. and Princen, H.M.G. (1986) Biochim. Biophys. Acta 875, 236-246.
30 Cortner, J.A., Coates, P.M., Le, N.-A., Cryer, O.R., Ragni, M.C., Faulkner, A. and Langer, T. (1987) J. Lipid Res. 28, 195-206.
31 Huettinger, M., Corbett, J.R., Schneider, W.J., Willersar, J.T., Brown, M.S. and Goldstein, J.L. (1984) Proc. Natl. Acad. Sci. USA 81, 7594-7603.
32 Hui, D.Y., Innerarity, T.L., Milne, R.W., Marcel, Y.L. and Mahley, R.W. (1984) J. Biol. Chem. 259, 15060-15068.
33 Arbeeny, C.M., Rijici, V.A., Handley, D.A. and Eder, H.A. (1987) Metabolism 36 no. 11, 1106-1113.
34 Schneider, W.J., Kovanen, Brown, M.S., Goldstein, J.L., Utermann, G., Weber, W., Havel, R.J., Kane, J.P., Innerarity, T.L., and Mahley, R.W. (1981) J. Clin. Invest. 68, 1075-1085.
35 Bradley, W.A., Hwang, S.-L.C., Karlin, J.B., Liu, A.H.Y., Prasad, S.C., Gotto, A.M. and Gianturco, S.H. (1984) J. Biol. Chem. 259, 14728-14735.
36 Krul, E.S., Tikkanen, M.J., Cole, T.G., Davie, J.M. and Schonfeld, G. (1985) J. Clin. Invest. 75, 361-369.
37 Innerarity, T.L., Hui, D.Y., Bersot, T.P., and Mahley, R.W. (1986) in Lipoprotein Deficiency Syndromes. (Angel, A., and Frohlich, J., eds) pp. 273-288, Plenum Publishing, New York.
38 Faergeman, O., Havel, R.J. (1975) J. Clin. I. 55, 1210-1218.

Chapter 3

INEFFICIENT DEGRADATION OF TRIGLYCERIDE-RICH LIPOPROTEIN BY Hep G2 CELLS IS DUE TO A RETARDED TRANSPORT TO THE LYSOSOMAL COMPARTMENT

Paola Lombardi*, Monique Mulder*, Rune R. Frants§ and Louis M. Havekes*.

*TNO Institute of Ageing and Vascular Research, Gaubius Laboratory, Leiden;
§Department of Human Genetics, University of Leiden, The Netherlands.

Submitted.

Summary

Binding studies at 37°C showed that lipoprotein lipase treated very low density lipoproteins (LPL-VLDL) and very low density lipoproteins (VLDL), once taken up via the low density lipoprotein (LDL) receptor, are poorly degraded by Hep G2 cells as compared with LDL. Incubation of cells with ^{125}I-labeled VLDL and LPL-VLDL for 2 hours at 4°C, followed by a chase period at 37°C, showed that the membrane-bound VLDL and LPL-VLDL are internalized within about 20 min, at the same rate as LDL. Incubation of cells with labeled LDL, LPL-VLDL and VLDL at 18°C for 4.5 hours resulted in the accumulation of these particles in the early endosomes. It is known (Lombardi et al. (1993) *Biochem. J.* **290**, 509-514) that, at this temperature, the transport to the late endosomes and lysosomes, followed by degradation of the particles, does not occur. However, after washing the cells and a temperature shift to 37°C, the labeled LDL present in the early endosomes are transported to the late endosomal-lysosomal compartment almost completely within 15 min. Strikingly, for LPL-VLDL and for VLDL, only about 50% or less of the label was moved to the late endosomal-lysosomal compartment within 45 min.

To evaluate the effect of the inefficient degradation of VLDL and LPL-VLDL on cellular cholesterol homoeostasis, acyl-CoA:cholesterol acyltransferase (ACAT) activity was measured. Incubation with 30 μg/ml of LDL induced a 2.5-fold increase in ACAT activity, whereas the incubation with similar amounts of both VLDL and LPL-VLDL failed to stimulate this enzyme.

We conclude that the low degradation efficiency of VLDL and LPL-VLDL by Hep G2 cells is due to the retarded transport of these particles from the early endososmes to the late endosomal-lysosomal compartment. As a result, under the conditions applied, VLDL and LPL-VLDL degradation does not contribute to increase the cellular free cholesterol pool enough to stimulate ACAT activity.

Introduction

Very low density lipoprotein (VLDL) are triglyceride-rich, apolipoprotein (apo) E and apoB100 containing, lipoprotein particles that are synthesized and secreted by the liver. After entering the bloodstream, VLDL particles interact with lipoprotein lipase (LPL), which catalyzes the hydrolysis of triglycerides. The resulting remnant particles are smaller, more dense and have an altered lipid and apolipoprotein composition, as compared with native VLDL particles (for review, see ref. 1). The VLDL remnants are further lipolysed and converted into Intermediate Density Lipoproteins (IDL) and, finally, Low Density Lipoprotein (LDL). During VLDL lipolysis, a fraction of the remnants is directly cleared from the plasma via hepatic LDL receptors, where apoE, the major protein constituent of these particles, acts as a ligand (2-6).

Many lipoprotein particles that contain apoE have several copies of this protein and are

thought to react more avidly with the LDL receptor than LDL (7,8). A single lipoprotein particle containing several molecules of apoE could interact multivalently with a single LDL receptor; alternatively, lipoproteins containing several molecules of apoE may interact with more than one LDL receptor. In either case, particles that contain apoE in addition to apoB100 will bind to the LDL receptor with higher affinity than those that contain only one apoB100 molecule (9).

Recent studies by Tabas et al. (10) have shown that the multivalent binding of β-VLDL through apoE to the LDL receptor in mouse peritoneal macrophages leads to a divergent endocytotic pathway as compared to LDL. They found that LDL is rapidly targeted to perinuclear lysosomes near the center of the cell, whereas, after its uptake, β-VLDL is localized in more distributed vescicles. This differential distribution was found to be coupled to a slower degradation of β-VLDL concomitant with a higher capability to stimulate acyl-CoA:cholesterol acyltransferase (ACAT).

In previous studies we found that the degradation of VLDL and LPL-treated VLDL, the latter taken as representative of VLDL-remnants, by Hep G2 cells is extremely low as compared to that of LDL (unpublished observations). A low degradation efficiency of VLDL by Hep G2 cells has also been described by other investigators (11). In the present study, we addressed the question as to whether this inefficient degradation might be due to an altered intracellular processing of these particles, possibly due to their multivalent binding via apoE. The present results clearly show that after internalization, the transport of VLDL as well as of LPL-treated VLDL to the late endosomal-lysosomal compartment is indeed severely retarded. In addition, we found that these lipoproteins fail to stimulate intracellular ACAT activity.

Materials and Methods

Materials

Fetal calf serum (FCS) and Dulbecco's modified Eagle's medium (DMEM, cell culture medium) were obtained from Flow Laboratories (Irvine, U.K.). Human serum albumin (HSA) was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). $Na^{125}I$ (spec. act. 13.3 μCi/μl) was purchased from Amersham (Buckinghamshire, U.K.). Multiwell cell culture dishes were from Costar (Cambridge, MA, U.S.A.). Percoll (density 1.13 g/ml) was obtained from Pharmacia (Upsala, Sweden).

Lipoproteins

LDL and VLDL were isolated from serum of normolipidemic donors by density gradient ultracentrifugation according to Redgrave et al. (12). Lipoprotein lipase-treated VLDL (LPL-VLDL) were prepared by incubating total serum with lipoprotein lipase (LPL) purified from bovine milk (13), essentially as described before (5). Briefly, the amount of LPL added was equal to the amount necessary for hydrolysis of 50% of the triacylglycerols present in complete serum within 1 hour. The incubation was performed in the presence of 10% (w/v)

fatty acid free human serum albumin (HSA) and Tris-HCl buffer (final concentration 0.1M, pH 8.5) for 90 minutes at 37°C. To stop the reaction, the mixture was put on ice and solid KBr was added to adjust the solution to a density of 1.21 g/ml. LPL-VLDL, with density less than 1.019 g/ml, were then isolated by density gradient ultracentrifugation (12).

The lipoprotein preparations were immediately used for iodination by the ^{125}iodine monochloride method described by Bilheimer (14). After iodination, the lipoproteins were dialysed against phosphate buffered saline (PBS, pH 7.4) and stabilized with 1% (w/v) HSA. The specific activities ranged from 100 to 250 cpm/ng of protein. The stabilized ^{125}I-labeled lipoproteins were stored at 4°C and used within two weeks. With all labeled lipoproteins, less than 1% of the radioactivity was soluble in 10% (w/v) trichloroacetic acid (TCA). Whenever unlabeled lipoproteins were used, they were immediately stabilized after isolation with 1% (w/v) HSA followed by extensive dialysis against PBS and, subsequently, DMEM supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml).

Lipoprotein-depleted serum (LPDS) was obtained by ultracentrifugation of serum at a density of 1.21 g/ml followed by extensive dialysis of the infranatant against PBS and, subsequently, DMEM supplemented with penicillin and streptomycin.

β-VLDL was separated by sequential ultracentrifugation from the serum of cholesterol-fed rabbits ($d < 1.006$ g/ml) and extensively dialyzed against PBS and, subsequently, DMEM supplemented with penicillin and streptomycin.

Cell culture

Hep G2 cells were cultured in 25 cm^2 flasks in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 20 mM Hepes, 10 mm $NaHCO_3$, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in equilibration with 95% air/5% CO_2. Six to seven days prior to each experiment, cells were seeded in 2 cm^2 multiwell dishes.

Twenty-four hours before the assays, the cells were washed with DMEM-1% HSA and further incubated with DMEM containing 10% LPDS (v/v) instead of FCS.

Binding assays

Binding experiments were performed essentially a described previously (5,15).

Each experiment was started by washing the cells three times in DMEM-1% HSA. Thereafter, cells were incubated in the same medium with the addition of 20 μg/ml of labeled lipoproteins, in the presence or absence of a 30-fold excess of unlabeled LDL. Temperature and duration of the respective incubations are described in the text and figure legends. After incubation with labelled lipoproteins, cells were cooled to 0°C. Degradation was measured exactly as described (16) and, after removal of the incubation medium, the cells were washed extensively (17). To measure total cell association, the washed cells were dissolved in 0.2M NaOH and an aliquot of the cell lysate was counted for radioactivity. Another aliquot of the cell lysate was used for protein determination according to Lowry et al. (18).

Values for the specific (receptor-mediated) cell association and degradation were calculated by subtracting the amount of labeled lipoprotein that was cell associated or degraded in the presence of a 30-fold excess of unlabeled LDL (nonspecific binding) from

the amount of labelled lipoprotein that was cell-associated or degraded in the absence of an excess of unlabeled LDL (total binding).

Measurement of intracellular transport of lipoproteins

Subcellular fractionation of Hep G2 cells was performed by Percoll density gradient centrifugation as described (19). Cells were seeded in 100 mm dishes. After incubation for 4.5 hours at 18°C in the presence of 20 μg/ml of ^{125}I-labeled LDL, LPL-VLDL or VLDL, cells were washed twice with DMEM-1% HSA to remove the unbound ligand and further incubated at 37°C for the indicated periods of time in medium without the labeled lipoproteins. Cells were then washed extensively with 0.28 M sucrose, 2 mM $CaCl_2$, 0.01 M Tris-HCl pH 7,6 and scraped from the dishes with a rubber policeman in the same buffer (1 ml/dish). Then, cells were homogenised in a Dounce homogeniser by 20 complete strokes with a tight fitting pestle. The homogenates were centrifuged at 280 x g for 10 minutes in order to remove remaining intact cells. A 80% (v/v) Percoll solution and homogenization buffer were added to the supernatants to a final Percoll concentration of 20% and to a final volume of 12 ml. After thorough mixing, the samples in Percoll were placed in cellulose nitrate tubes fitting a 50Ti rotor (Beckman) and centrifuged at 10,000 x g for 45 minutes. Fractions of ~ 0.3 ml were collected by aspiration from top to bottom and the radioactivity in each sample was counted. The density of each fraction was measured in a PAAR-DMA-45 density meter equipped with a DMA-602M small sample cell (~ 170 μl). The distribution of the lysosomal marker (acid phosphatase) was measured by the method of Torriani (20).

Measurement of intracellular cholesterol esterification (ACAT activity)

Cholesterol esterification was measured by determining the incorporation of [1-^{14}C] oleic acid into labeled cholesteryl oleate, essentially as described (21), with some minor modifications. Cells cultured in 10 cm^2 wells were first preincubated for 20 hours in culture medium containing 10% lipoprotein deficient serum (LPDS) and then with the indicated amounts of lipoproteins for 4 hours. Subsequently, to 2 ml of incubation medium, 100 μl of a 1 mM solution of [^{14}C]oleate (2340 dpm/nmol) complexed to albumin was added and cells were incubated for another 2 hours at 37°C. Cells were then washed four times with ice-cold PBS and harvested in 1 ml by scraping. After addition of [^{3}H]cholesterol (60.000 dpm/sample), as internal standard, lipid extraction as described by Bligh and Dyer (22) was performed. Lipids were analyzed by thin layer chromatography on pre-coated silica plates. The developing solvent was composed of chloroform/methanol (98:2) (v/v) first, followed by chloroform/hexane (45:65) (v/v). The lipid spots were detected by autoradiography, scraped off and counted for radioactivity in a Packard 1900CA Tri-Carb liquid scintillation analyzer equipped with software validated for ^{14}C/^{3}H double labeled samples. Recovery of the internal standard was 65-85%.

Time course of receptor-mediated association and degradation of LDL, LPL treated VLDL and VLDL by Hep G2 cells

The time-course of receptor-mediated association and degradation of labeled LDL, LPL-VLDL and VLDL at 37°C are shown in Fig. 1. For all three lipoprotein samples, the cell-association increased progressively over the first three hours, before a plateau is reached. The degradation started after a lag period of 60 to 90 minutes and proceeded at a slower rate in case of both LPL-VLDL and VLDL, as compared with LDL. When the degradation efficiency is calculated as the amount of ligand degraded relative to the amount of ligand that became cell-associated (Fig. 2), it is apparent that after 5 hours of incubation the degradation efficiency of LPL-VLDL and VLDL is only 50% and 20%, respectively, of that of LDL.

We reasoned that more information about the intracellular processing of LPL-VLDL and VLDL might help explaining this difference in degradation efficiency. Therefore, the next experiments were designed to investigate whether the reduced degradation efficiency of LPL-VLDL and VLDL was due to: (*i*) a lower internalization rate, (*ii*) a less efficient transport of the apoE-binding lipoproteins from the early endosomal compartment to the late endosomal or lysosomal compartment or (*iii*) an impairment in the lysosomal degradation itself.

Rate of internalization of surface bound LDL, LPL-VLDL and VLDL by Hep G2 cells

The rate of internalization was examined by first incubating the cells at 4°C with 20 μg/ml of labeled LDL, LPL-VLDL and VLDL for two hours to allow the lipoproteins to bind to the plasma membrane, thus without subsequent internalization. Thereafter, cells were washed and further incubated at 37°C. As shown in Fig. 3, the maximal internalization is reached within about 20 minutes for all the three lipoprotein samples, indicating that the internalization rate of LPL-VLDL and VLDL does not differ from that of LDL.

We hypothesize therefore that LPL-VLDL and VLDL, once internalized, (i) cannot be further transported to the late endosomal and/or lysosomal compartment, or (ii) they cannot be degraded in the lysosomes either due to an impairment in the late endosome-lysosome fusion or to a defect in the lysosomal degradation itself.

Rate of transport of LDL, LPL-VLDL and VLDL from the early endosomes to the late endosomal or lysosomal compartment in Hep G2 cells

To evaluate whether the transport of LPL-VLDL and VLDL from the early endosomal compartment to the late endosomal or lysosomal compartment is impaired, cells were first incubated with labeled lipoproteins for 4.5 hours at 18°C. At this temperature, it has been demonstrated that degradation of LDL is inhibited owing to an impairment in the dissociation of the internalized LDL from the receptor (23) and to a block in endosome-lysosome fusion (24). As a result, the cell-associated lipoproteins will accumulate in the early endosomal compartment, without being degraded (25).

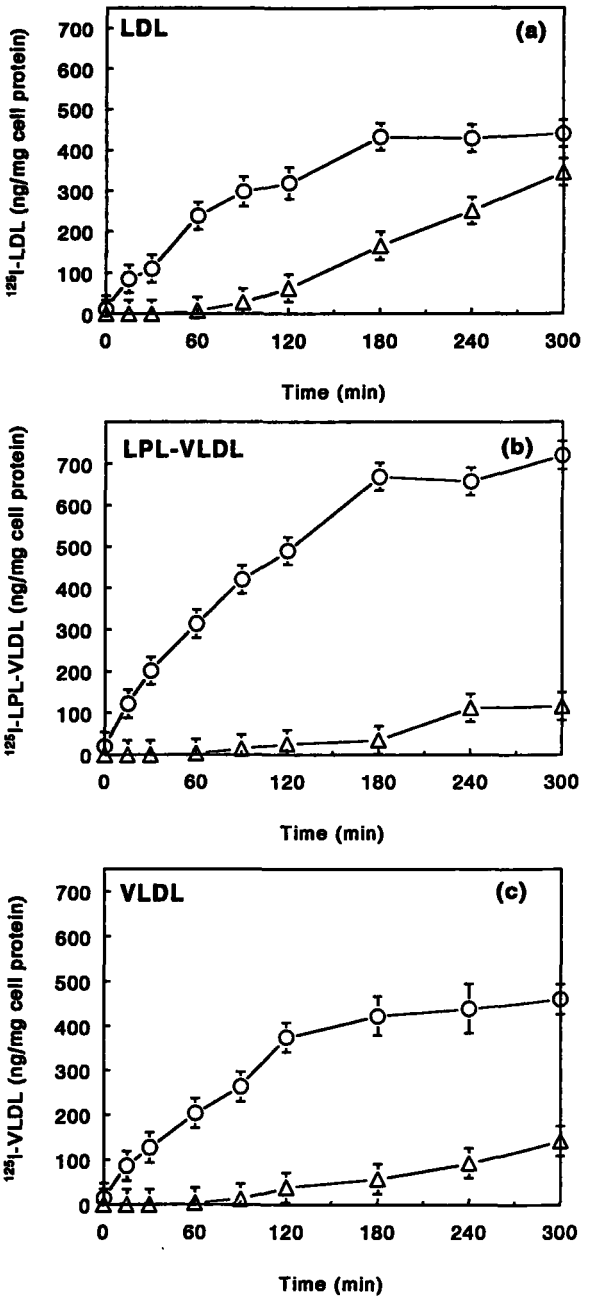

Figure 1. Time course at 37°C of the receptor-mediated association (○) and degradation (△) of LDL (A), LPL-VLDL (B) and VLDL (C) in Hep G2 cells. Cells were incubated with 20 μg/ml of ^{125}I-LDL or ^{125}I-LPL-VLDL or ^{125}I-VLDL ± 30 fold excess of unlabelled LDL at 37°C for the indicated periods of time. Thereafter, the receptor-mediated association and degradation were measured as described in Materials and Methods. Values are means ± S.D. of triplicate incubations.

After the incubation in the presence of labeled LDL, LPL-VLDL or VLDL at 18°C, cells were washed in order to remove the unbound ligand and further incubated at 37°C for the indicated periods of time (Fig. 4). With LPL-VLDL and VLDL the major portion of the initial amount of label accumulated in the endosomes is still cell associated after 5 hours at

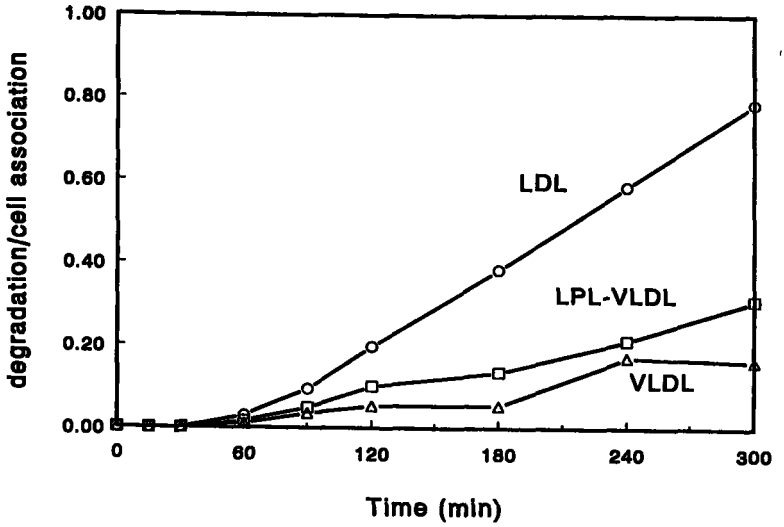

Figure 2. Degradation efficiency of LDL (○), LPL-VLDL (□) and VLDL (△). Values are calculated from the results presented in Fig. 1 as ratio degradation/association.

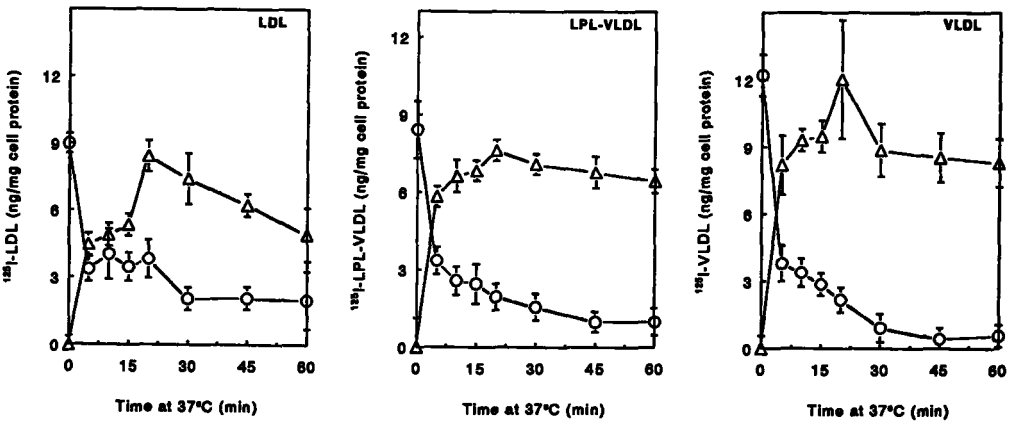

Figure 3. Internalization rate of LDL, LPL-VLDL and VLDL in Hep G2 cells. After 2 hours of incubation at 4°C in the presence of 20 μg/ml of ^{125}I-LDL, ^{125}I-LPL-VLDL or ^{125}I-VLDL ± 30 fold excess of unlabelled LDL, cells were chased for the indicated periods of time at 37°C and the amount of lipoprotein bound to the plasma membrane (○) or intracellularly-present (△) was measured as described in Materials and Methods. Values are means ± S.D. of triplicate incubations.

37°C (Figs. 4B and 4C), whereas for LDL (Fig. 4A) about 70% of the internalized LDL is degraded within 5 hours after the temperature shift from 18°C to 37°C. For LDL, the decrease in cell association is fully complementary to the amount of LDL degraded. This implies that all intracellularly present LDL is secreted, after being degraded. Strikingly, for LPL-VLDL and VLDL the sharp decline of the cell association curve in the first hour of incubation after the temperature shift from 18°C to 37°C suggests that some 20% and 40%

of the intracellularly-accumulated particles are excreted as intact particles into the medium, thus escaping the degradation route (retroendocytosis). At each time point the amount of lipoprotein that became retroendocytosed is calculated according to the formula: retroendocytosis = 100% - (% lipoprotein associated + % lipoprotein degraded) (see broken line in Fig. 4).

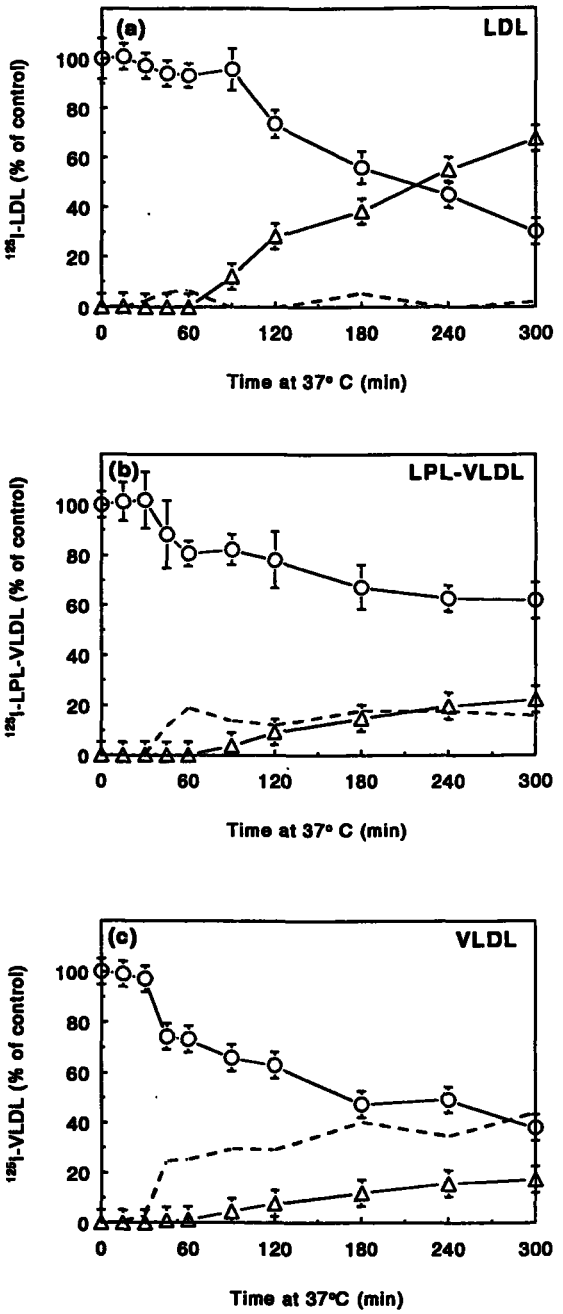

Figure 4. Intracellular processing of LDL (A), LPL-VLDL (B) and -VLDL (C). Cells were preincubated at 18°C for 4.5 hours in the presence of 20 μg/ml of ^{125}I-labeled lipoproteins ± 30-fold excess of unlabelled LDL and then chased for the indicated periods of time at 37°C. Receptor-mediated association (○) and degradation (△) were measured. The amount of lipoprotein associated at time 0 was taken as 100%. At each time point the amount of the retroendocytosed lipoprotein (broken line) was calculated according to the formula: retroendocytosis = 100% - (% lipoprotein associated + % lipoprotein degraded). Values are means ± S.D. of triplicate incubations. The 100% values of the cell-association at time 0 at 37°C are 74 ± 8, 127 ± 4, 62 ± 4 ng/mg cell protein for LDL, LPL-VLDL and VLDL, respectively.

The low degradation efficiency of LPL-VLDL and VLDL is clearly shown in Fig. 5. From these results we conclude that the impairment in LPL-VLDL and VLDL degradation occurs downstream of the early endosomal compartment and is partly due to retroendocytosis of the lipoprotein particles.

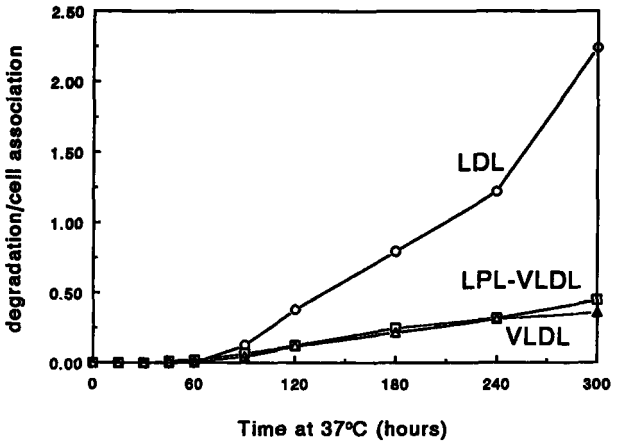

Figure 5. Degradation efficiency of LDL (○), LPL-VLDL (□) and VLDL (△). Values are calculated from the results presented in Fig. 4 as ratio degradation/association.

To investigate as to whether LPL-VLDL and VLDL are either retained in the sorting endosomes or normally delivered to the late endosomal or lysosomal compartment, but not further degraded, cells were incubated for 4.5 hours at 18°C in the presence of labeled lipoprotein, followed by a temperature shift to 37°C and homogenization at the indicated time points. Thereafter, cell homogenates were subcellularly fractionated by Percoll gradient centrifugation (Fig. 6) (19). Due to their difference in buoyant density, the early and sorting endosomes (top fractions) were separated from the late endosomal and lysosomal fractions (bottom fractions) (26). Figure 6 shows the distribution of label in the gradient fractions for each lipoprotein tested at one time point (15 minutes) after the temperature shift. With LDL (Fig. 6A), after 15 minutes at 37°C, almost all the radioactivity was found in the high density bottom fractions, which represent the late endosomal fractions and lysosomal fractions. The latter were identified by the presence of acid phosphatase activity (horizontal bar). Strikingly, LPL-VLDL, and even more dramatically VLDL, move much more slowly to the bottom fractions upon incubation at 37°C. After 15 minutes at 37°C, more than 50% of LPL-VLDL (Fig. 6B) and almost all VLDL (Fig. 6C) was still present in the light, early endosomal fractions. The rate of accumulation of the labeled lipoproteins in the high density fractions at different time points is summarized in Fig. 7. Within 15 minutes after the temperature shift to 37°C, the intracellular trafficking of LDL towards the late endosomal-lysosomal compartment was nearly complete, while for LPL-VLDL and VLDL, even after 45 minutes, the entire process towards the late endosomal-lysosomal compartment has not been completed. Apparently, LPL-VLDL and VLDL are much more slowly transported to the late endosomes or lysosomes than LDL.

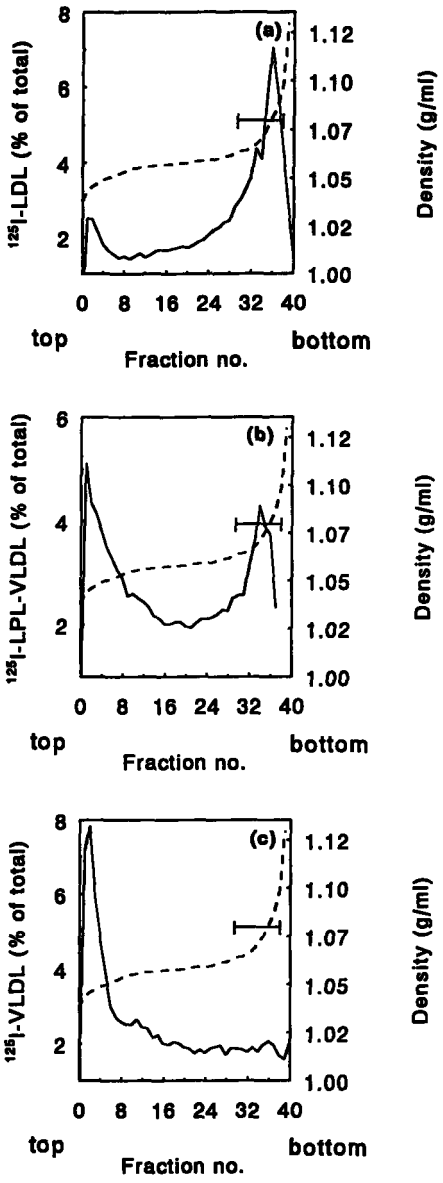

Figure 6. Subcellular distribution of LDL (A), LPL-VLDL (B) and VLDL (C) in Hep G2 cells. Cells were incubated for 4.5 hours at 18°C with 20 μg/ml ^{125}I-labeled lipoprotein in DMEM-1% HSA medium. After washing to remove unbound ligand, the cells were incubated at 37°C for 15 minutes after which the cells were homogenised and subjected to subcellular fractionation as described in Materials and Methods. Fractions were measured for radioactivity. The dotted line represents the density profile of the gradient; the horizontal bar indicates the samples representing the lysosomal fractions as evaluated by acid phosphatase activity measurement.

Cellular cholesterol esterification (ACAT activity)

In order to determine if there was a correlation between the retarded transport of LPL-VLDL and VLDL to the late-endosomal or lysosomal compartment and the potency of these particles to stimulate ACAT, ACAT activity was measured after incubation of Hep G2 cells with either LDL, LPL-VLDL, VLDL or rabbit β-VLDL. In macrophages, β-VLDL are known to be a much more potent stimulator of ACAT than LDL, although this effect is not due to a greater delivery to the cell of β-VLDL cholesterol (27). As shown in Fig. 8, after 6 hours of incubation with 30 μg/ml of LDL, a 2.5-fold increase of the enzyme activity was

obtained, as compared to the control level of ACAT activity in Hep G2 cells. Similar amounts of β-VLDL stimulated ACAT up to 5-fold. LPL-VLDL and VLDL did not influence cellular ACAT activity at all. The same results were obtained when cells were incubated for a prolonged time (20 hours instead of 6 hours) and in the presence of higher amounts of lipoprotein (up to 100 and 150 μg of lipoprotein protein/ml). Results similar to those presented in Fig. 8 were obtained when the amount of lipoprotein added was expressed as μg cholesterol/ml.

The poor ability of LPL-VLDL and VLDL to stimulate ACAT is in accordance with the retarded transport of these particles to the late-endosomal/lysosomal compartment.

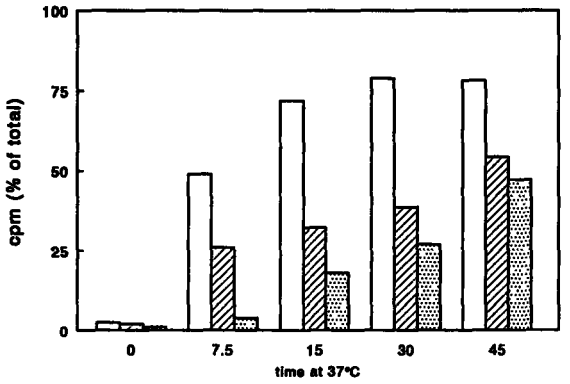

Figure 7. Accumulation of LDL, LPL-VLDL and VLDL in the late endosomal and lysosomal fractions as a function of time. Cell homogenates were fractionated on Percoll gradients as described in Fig. 6. For each time-point the fractions representing the lysosomal fractions (fractions 30-38, see Fig. 6) were pooled and expressed as the percentage of the total amount of radioactivity present in the homogenate. Open bars represent LDL; hatched bars represent LPL-VLDL and dotted bars represent VLDL.

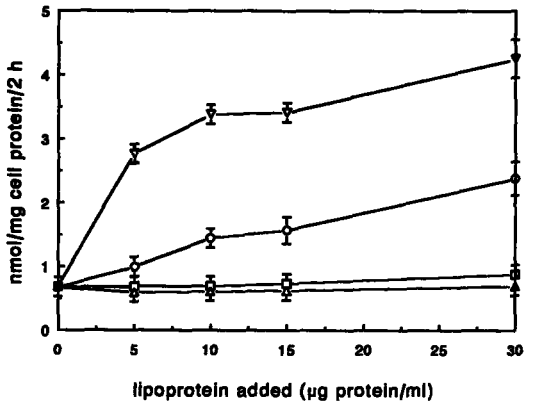

Figure 8. Cellular cholesterol esterification (ACAT activity) upon incubation with different lipoprotein fractions. Cells were incubated for 6 hours with DMEM, 10% LPDS containing the indicated amounts of rabbit β-VLDL (▽), LDL (○), LPL-VLDL (□) and VLDL (△). During the last 2 hours of the 6 hours incubation at 37°C, to the cells [^{14}C]oleate (2340 dpm/nmol) was added. At the end of the 6 hours incubation, the cells were assayed for cholesteryl [^{14}C]oleate content as described in Materials and Methods. Values are means ± S.D. of triplicate incubations.

Discussion

In the present study we have shown that normal VLDL and lipolyzed VLDL, taken as representative of VLDL remnants, once bound and taken up by the LDL receptor in Hep G2 cells, are poorly degraded as compared to LDL. A low degradation efficiency has also previously been reported for both VLDL (11) and VLDL remnants (28). In the latter study, the authors propose that either a rapid dissociation of IDL/receptor complexes at the cell surface might take place, prior to internalization, or IDL might be internalized but a major fraction recycles back to the cell surface (retro-endocytosis), possibly together with the receptor protein, thus preventing the routing to the lysosomes. Our present data rule out the first hypothesis, clearly showing that VLDL and LPL-VLDL are quantitatively taken up by Hep G2 cells (Fig. 3). Although not directly measured, but calculated from the results presented in Fig. 4, we found that for VLDL and LPL-VLDL some 40 and 20%, respectively, of the total amount of particles intracellularly-present is retro-endocytozed back into the medium, thus escaping the lysosomal pathway. However, even when the amount of lipoprotein that is retro-endocytosed is considered, this cannot fully account for the much lower degradation efficiency found for VLDL and LPL-VLDL as compared with LDL (Figs. 2 and 5).

Both at 37° and at 18°C, VLDL and LPL-VLDL are taken up by Hep G2 cells at a normal rate as compared with LDL (Figs. 1, 3 and 4). The present results provide evidence that the relatively poor degradation of VLDL and LPL-VLDL is due to a slower delivery of these particles to the lysosomal compartment (Figs. 6 and 7). The retarded intracellular routing of these particles might be the result of the polyvalent binding of apo E in VLDL and LPL-VLDL to the receptor. Recently, such a mechanism has been postulated for β-VLDL in mouse peritoneal macrophages (10). It is hypothesized that the high-affinity polyvalent ApoE binding to the LDL receptor results in a greater resistence to the acid-mediated release of the ligand from the receptor. If this is the case, the rate limiting step in the processing of VLDL and LPL-VLDL indeed would take place in the sorting endosomes, thus raising the question of the fate of the receptors bound to the ligand. Previous studies have indicated that receptor cross-linking can block ligand-receptor recycling (29,30), sometimes triggering the delivery of the multivalent-bound receptors to the lysosomes for degradation. Our results, however, cannot discriminate between the two possiblities that either the receptor is relatively slowly recycled back to the plasma membrane or, eventually, partly degraded in the lysosomes.

In order to verify the effect of the slower processing and degradation of VLDL and LPL-VLDL on cellular cholesterol homoeostasis, we measured ACAT activity, which is known to be a sensitive measure for the amount of cholesterol in the regulatory cellular cholesterol pool. Eisenberg et al. (11) and Krul et al. (31) have found that incubation of cells with VLDL did not lead to a stimulation of ACAT activity. Our results are in line with their results. Both VLDL and LPL-VLDL were not able to stimulate the intracellular cholesterol-esters synthesis (Fig. 8). In contrast with this, Krul et al. (31) and Evans et al. (32) showed that VLDL isolated from hypertriglyceridemic (or type IV) subjects (HTG-VLDL) was a

potent stimulator of ACAT. They showed that HTG-VLDL contains more apoE and more cholesterol per particle. However, a higher cholesterol content per HTG-VLDL particle, as compared with normal VLDL, cannot explain the discrepancy between their results and our results regarding the stimulation of ACAT activity. We observed that the cholesterol and apoE content (expressed as ratio cholesterol to triglycerides and apoE to apoB100, respectively) of the LPL-VLDL particles used in our study are in the same order of magnitude as that of the HTG-VLDL used by Evans et al. (results not shown) (32). Furthermore, expressing the amount of lipoprotein added in Fig. 8 as the amount of cholesterol added, instead of the amount of protein, did not considerably change the results shown.

Recently, Xu and Tabas (33,34) have found that in macrophages the cellular cholesterol level first have to reach a critical threshold of about 25% above the basal level, before ACAT activity is stimulated. If the same 25% increase in cellular cholesterol level is required in Hep G2 cells in order to stimulate ACAT activity, our results indicate that, under the conditions applied, VLDL and LPL-VLDL do not increase the ACAT substrate pool enough for exerting an effect on the ACAT activity. Since the amount of uptake of VLDL and LPL-VLDL is comparable with the uptake of LDL (Fig. 1), also when based on the amount of cholesterol uptake (not shown), we conclude from our results that the ACAT substrate pool is supplied with lipoprotein-derived cholesterol only after the lipoproteins have been degraded. Hence, the cellular degradation of VLDL and LPL-VLDL is too inefficient to increase cellular cholesterol esterification.

A low degradation efficiency of VLDL and LPL-VLDL would also imply a relatively poor down-regulation of the LDL receptor activity upon incubation of cells with these lipoproteins. Epidemiological studies suggest that the down-regulation of the LDL receptor activity in the liver by VLDL and VLDL-remnants depends, at least partly, on the polymorphism of apoE (35-37). Whether, besides affecting the binding of the lipoproteins to the receptor, apoE polymorphism also interferes with the efficiency of cellular degradation of VLDL or VLDL-remnants, as a consequence of a retarded intracelluar transport to the lysosomal compartment, is currently under investigation.

Acknowledgements

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References

1. Havel, R.J., and Kane, J.P. (1989) In *The metabolic bases of inherited diseases* (Scriver, C.R., Beaudet, A.C., Sly, W.S., and Valle D. eds) pp 1129-1138, McGrow Hill, New York.
2. Beltz, W.F., Kesaniemi, Y.A., Howard, B.V., and Grundy, S.M. (1985) *J. Clin. Invest.* **76**, 575-585.

3. Kesaniemi, Y.A., Beltz, W.F., and Grundy, S.M. (1985). *J. Clin. Invest.* **76**, 586-595.
4. Demant, T., Bedford, D., Packard, C., and Shepherd, J. (1991) *J. Clin. Invest.* **88**, 1490-1501.
5. Mulder, M., de Wit, E., and Havekes, L.M. (1991) *Biochim. Biophys. Acta* **1081**, 308-314.
6. Mahley R. (1988) *Science* **240**, 622-630.
7. Windler, E., Kovanen, P.T., Chao, Y., and Havel, R. (1980) *J. Biol. Chem.* **255**, 10464-10471.
8. Pitas, R.E., Innerarity, T.L., Arnold, K.S., and Malhey, R.W. (1979) *Proc. Acad. Sci.USA* **76**, 2311-2315.
9. Havel, R.J., and Hamilton R.L. (1988) *Hepatology* **8**, 1869-1704.
10. Tabas, I., Myers, J.N., Innerarity, T., Xiang-Xi, X., Arnold, K., Boyles, J., and Maxfield, F.R. (1991) *J. Cell Biol.* **115**, 1547-1560.
11. Eisenberg, S., Friedman, G., and Vogel T. (1988) *Arteriosclerosis* **8**, 8480-8487.
12. Redgrave, T.G., Roberts, D.C.K., and West, C.E. (1975) *Anal. Biochem.* **65**, 42-49.
13. Pagano Mirani-Oostdijk, C., Havekes, L., Den Boer, P., De Wit, E., and Terpstra, J. (1985) *Scand.J. Clin. Lab. Invest.* **45**, 207-215.
14. Bilheimer, B.W., Eisenberg, S., and Levy R.I. (1972) *Biochim. Biophys. Acta* **260**, 212-221.
15. Havekes, L.M., de Wit P.M.C., van Hinsbergh, V.W.M., and Princen H. (1983) *Biochem. J.* **214**, 951-958.
16. Goldstein, J.L., and Brown, M.S. (1974) *J. Biol. Chem.* **249**, 5153-5162.
17. Goldstein, J.L., Basu, S.K., Brunschede, G.J., and Brown, M.S. (1976) *Cell* **7**, 85-95.
18. Lowry, O.H., Rosenbrough, M.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem.* **193**, 265-275.
19. Harford, J., Bridges, K., Ashwell, G., and Klausner, R.D. (1983) *J. Biol. Chem.* **258**, 3191-3197.
20. Torriani A. (1960) *Biochim. Biophys. Acta* **38**, 460-466.
21. Havekes, L.M., de Wit, E., and Princen, H. (1987) *Biochem. J.* **247**, 739-746.
22. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol.* **37**, 911-917.
23. Wolkoff, A.W., Klausner, R.D., Ashwell, G., and Harford, J. (1984) *J. Cell Biol.* **98**, 375-381.
24. Dunn, W.A., Hubbard, A.L., and Aronson, N.N. Jr. (1980) *J. Biol. Chem.* **255**, 5971-5978.
25. Lombardi, P., Mulder, M., de Wit, E., van Berkel, T.J.C., Frants, R.R., and Havekes, L.M. (1993) *Biochem. J.* **290**, 509-514.
26. Stoorvogel, W., Strous G.J., Geuze, H.J., and Schwartz, A. (1991) *Cell* **65**, 417-427.
27. Tabas I, Lim S., Xiang Xi X., and Maxfield, F.R. (1990) *J. Cell Biol.* **111**, 929-940.
28. Friedman, G., Gavish, D., Vogel, T., and Eisenberg, S. (1990) *Biochim. Biophys. Acta* **1044**, 118-126.
29. Mellmann, I., and Plutner, H. (1984) *J. Cell Biol.* **98**, 1170-1177.
30. Anderson, R.G.W., Brown, M.S., Beisiegel, U., and Goldstein, J.L. (1982) *J. Cell Biol.* **93**, 523-551.
31. Krul, E.S., Tikkanen, M.J., Cole, T.G., Davie, J.M., and Schonfeld, G. (1985) *J. Clin. Invest.* **75**, 361-369.
32. Evans, A.J., Sawyez, C.G., Wolfe, B.M. and Huff, M.W. (1992) *J. Biol. Chem.* **267**, 10743-10751.
33. Xu, X.X., and Tabas, I. (1991) *J. Biol. Chem.* **266**, 1740-1748.
34. Liscum, L., and Dahl, N.K. (1992) *J. Lipid Res.* **33**, 1239-1254.
35. Smit, M., de Knijff, P., Rosseneu, M., Bury, J., Klasen, E., Frants, R.R., and Havekes, L.M. (1988) *Hum. Genet.* **80**, 287-292.
36. Utermann, G. (1987) *Am. Heart J.* **113**, 433-440.
37. Boerwinkle, E., and Utermann, G. (1988) *Am. J. Hum. Genet.* **42**, 104-112.

Chapter 4

TREATMENT OF E2E2 HOMOZYGOUS FAMILIAL DYSBETALIPOPROTEINEMIC SUBJECTS WITH GEMFIBROZIL DOES NOT ENHANCE THE BINDING OF THEIR D < 1.019 LIPOPROTEIN FRACTION TO THE LOW DENSITY LIPOPROTEIN RECEPTOR

Monique Mulder[1], Augustinus H.M. Smelt[2], Shui P. Zhao[2], Rune R. Frants[3], and Louis M. Havekes[1]

[1]IVVO-TNO Gaubius Laboratory, Leiden,
[2]Departments of Cardiology and Internal Medicine, University Hospital Leiden,
[3]Department of Human Genetics, Leiden University, The Netherlands

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Abstract

Six E2E2 homozygous Familial Dysbetalipoproteinemic (FD) patients were treated with gemfibrozil (2*600 mg/day) for a period of four weeks. For all subjects, normalization of serum cholesterol concentrations upon treatment, did not result in a significant change in the cholesterol/triglyceride ratio of the d < 1.019 lipoprotein fraction. In addition, the binding efficiency of this lipoprotein fraction to the LDL-receptor on Hep G2 cells, did not change consistently upon treatment. We conclude that, normalization of the serum cholesterol concentration in FD patients by treatment with gemfibrozil is the result of an effect of gemfibrozil on the synthesis of d < 1.019 lipoproteins rather than an effect on the receptor-mediated clearance of these particles.

Introduction

In normal subjects the chylomicron- and very low density lipoprotein (VLDL) remnants are rapidly removed from the circulation by receptor mediated endocytosis in the liver or by conversion into low density lipoproteins (LDL) [1]. Apolipoprotein E (apoE), a major constituent of these lipoproteins, acts as ligand for binding to the lipoprotein receptors [2,3]. ApoE is a polymorphic protein of which by use of isoelectric focusing three common genetic isoforms (E2, E3, and E4) can be detected [4,5]. ApoE2 differs from the most common apoE3 isoform by a cysteine for an arginine substitution at residue 158 [E2(arg158 → cys)], while apoE4 exhibits an arginine for a cysteine substitution at residue 112 [E4(cys112 → arg)]. By far the major part of familial dysbetalipoproteinemic (FD) patients exhibit homozygosity for E2. The accumulation of chylomicron- and VLDL-remnants (β-VLDL) in the plasma of these patients [6], is due to a defective interaction of apoE2 with the hepatic lipoprotein receptors [7,8]. Although most of the FD subjects display the E2E2 phenotype, only about 4% of all E2E2 homozygotes in the general population will develop hyperlipoproteinemia at later age [9]. It is concluded therefore, that additional genetic and/or environmental factors like age, hypothyroidism, or diabetes are needed for the expression of the disease [9,10]. In *in vitro* experiments it has been shown that, after complexation with phospholipid vesicles apoE2(arg158 → cys), displays only 1% of the binding activity of the common apoE3 isoform, irrespective of whether apoE2 is isolated from normo- or from hyperlipidemic E2E2 homozygous subjects [11,12]. This dramatic reduction in *in vitro* binding activity is due to the arginine for cysteine substitution at position 158, which is supposed to alter the conformation of the receptor binding domain, centered around residues 139-146 [13,14]. It is supposed that in intact lipoprotein particles the binding activity of E2(arg158 → cys) can be modulated by a variety of conditions. Cysteamine treatment, converting the cysteine residue at position 158 into a lysine analogue, enhances the binding activity. Similarly, removal of the carboxy-terminal part of the protein, by cleavage with thrombine, also enhances the binding of apoE2, whereas a combination of both treatments

fully restores the binding activity [15].

Innerarity et al. [16] reported that the binding of β-VLDL from a single E2E2 homozygous FD patient improved upon dietary treatment. In this particular patient, serum cholesterol and triglyceride levels as well as body weight were dramatically reduced upon treatment. Similar to these results, Chappell and Lindgren also found that the binding affinity of lipoproteins with density $d < 1.006$ isolated from E2E2 homozygous hyperlipidemic subjects increased upon treatment of the patients with a low-calorie diet for a period of seven days [17].

Although in FD patients the plasma lipid levels are commonly asssumed to be highly sensitive to caloric intake [10], in the majority of our population of FD patients, no dramatic reductions of serum cholesterol levels and body weight were achieved upon mild dietary treatment. For this reason, our FD patients are normally treated with lipid lowering drugs, like gemfibrozil. We wondered whether this treatment of E2E2 homozygous FD patients also improves the binding efficiency of lipoproteins with density $d < 1.019$ to the LDL-receptor.

We had the unique opportunity to monitor, in a group of six E2E2 homozygous hyperlipidemic FD patients, the composition and the binding affinity of β-VLDL before and after treatment with gemfibrozil (2*600 mg/day) for a period of four weeks. We found that after normalization of the serum cholesterol concentrations, the binding efficiency of lipoproteins with density $d < 1.019$ did not change consistently.

Materials and Methods

Patients

Six E2E2 homozygous hyperlipidemic FD patients, 4 females and 2 males, were recruited for the study. All patients were classified as having a lipoprotein profile characteristic for FD. All subjects displayed elevated serum cholesterol and triglyceride levels, and β-VLDL were present in the sera of all of them (Fig. 1). The presence of secondary hyperlipidemia, caused by renal, thyroid or liver disease, or diabetes or excessive alcohol intake was excluded. The clinical data of these patients are presented in Table 1. Three of the patients displayed a normal body mass index, and three of them were slightly overweight. None of the patients received any medication less than nine weeks before the start of the study. At least nine weeks prior to the study, the patients were advised to consume a low fat diet (30% of total calories, of which 10% saturated fat) with less than 300 mg cholesterol per day, to obtain reduction of the serum lipid levels as much as possible. However, no significant reduction of serum cholesterol and triglyceride levels were obtained during this 9 weeks pre-period. Gemfibrozil was given in a dose of 2*600 mg per day, for a period of four weeks. The experimental design of the study is shown in Fig. 2. None of the patients exhibited any remarkable side effects during the study period. Blood was collected after an overnight fast. Serum was separated from the cells by centrifugation at 500 g for 15 min at room temperature.

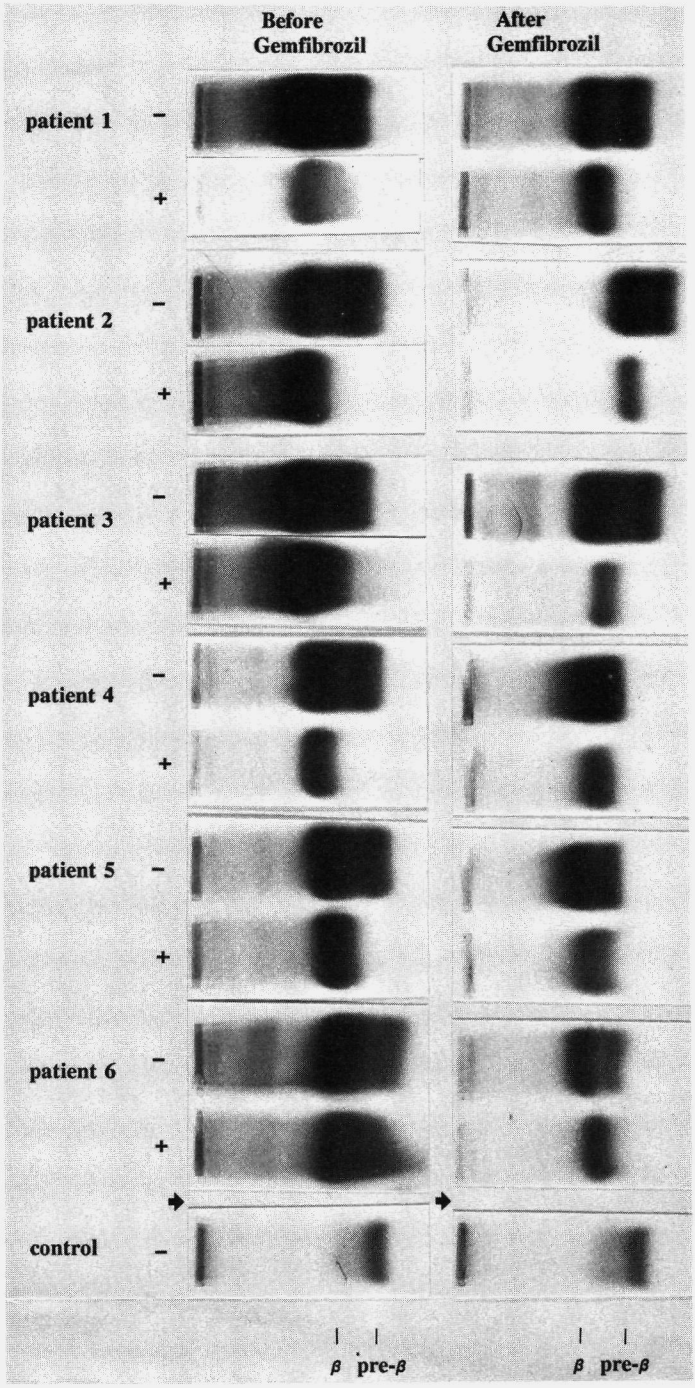

Figure 1. Agarose gel electrophoresis of the d < 1.019 lipoprotein fraction of the six patients before and after treatment with gemfibrozil. The lanes indicated by an - represent the native d < 1.019 lipoproteins, the lanes indicated by a + represent the LPL-treated d < 1.019 lipoproteins. The origin and the direction of the electrophoresis are indicated by the arrows.

Table 1. Clinical data of the six patients.

Patient No.	Sex	Age (yr)	BMI (kg/m^2)	Chol (mmol/l)	Tg (mmol/l)	[E] (mg/dl)	d<1.019 Chol (mmol/l)	LDL Chol (mmol/l)	HDL Chol (mmol/l)	d<1.019 Ratio
1	M	51	23.0	9.8	6.3	20	7.4	1.3	1.1	2.1
2	M	63	21.4	7.6	3.4	28	4.3	2.5	0.8	1.1
3	F	56	26.5	12.7	5.0	35	9.9	1.8	1.0	1.9
4	F	54	28.5	7.8	4.8	19	5.3	1.6	0.9	1.2
5	F	55	25.8	8.6	4.2	29	6.1	1.6	0.9	1.7
6	F	57	22.4	17.5	8.6	36	14.2	2.3	1.0	1.1
Mean		56	24.6	10.7	5.4	28	7.9	1.9	0.9	1.5
(SD)		(4)	(2.7)	(3.8)	(1.8)	(7)	(3.7)	(0.5)	(0.1)	(0.4)

Note: Each value represents the mean of two samples obtained 1 week apart, as depicted in Fig. 2.
Abbreviations: BMI, body mass index; Chol, total plasma cholesterol; Tg, total plsama triglycerides; [E], plasma apo E; d < 1.019 Chol, amount of cholesterol in the d < 1.019 lipoprotein fraction, calculated by subtracting the amount of cholesterol in the LDL and HDL fractions from the total amount of cholesterol; LDL chol, LDL cholesterol; HDL Chol, HDL cholesterol; d < 1.019 ratio, ratio of cholesterol to triglyceride in the d < 1.019 lipoprotein fraction (mmol/mmol).

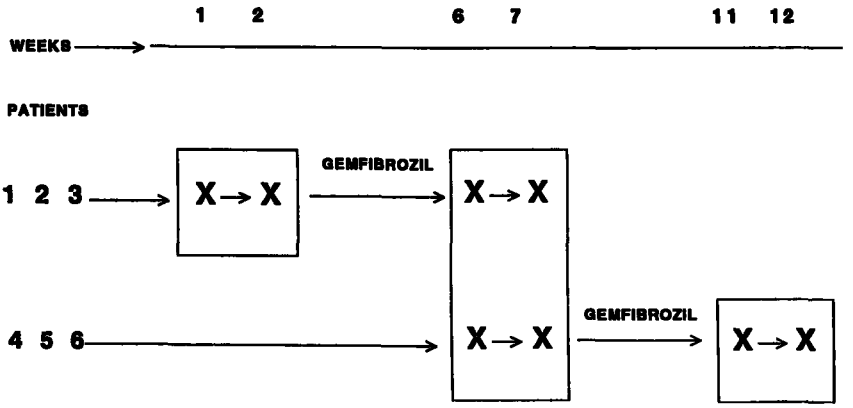

Figure 2. Experimental design. Blood samples were taken from three of the six selected subjects (1, 2 and 3), prior to treatment (week 1). Lipid analyses in serum as well as in the d < 1.019 fraction were performed. The binding to Hep G2 cells was tested of lipoproteins with density d < 1.019, as described in Materials & Methods. After one week the whole precedure was repeated (week 2). Thereafter the patients were treated for 4 weeks with gemfibrozil. Again blood samples were taken from these three patients and at this time also for the other three patients (4,5 and 6) (week 6). The experimental procedure was performed, and repeated after one week (week 7). The second group of three patients started receiving medication, and after 4 weeks for these patients, the experimental procedure was repeated (week 11 and 12).

Analytical methods

ApoE phenotyping was performed using a rapid micro-method based on isoelectric focusing of delipidated plasma followed by immunoblotting using a polyclonal anti-apoE antiserum [18].

Lipoproteins (LDL, HDL) were isolated according to Redgrave [19]. For isolation of VLDL + IDL the procedure was adapted, exactly as previously described [20]. Briefly, the density of the serum was adjusted to 1.21 g/ml, whereafter the solution was placed under a gradient of salt solutions, of densities 1.063 and 1.019 g/ml, respectively with a volume ratio of 0.95:1:1 from bottom to top. After centrifugation the 1 ml top fraction was collected. Protein contents of the lipoprotein fractions were determined according to Lowry et al. [21]. Total cholesterol, free cholesterol and triacylglycerols were determined using enzymatic methods (Boehringer Mannheim, F.R.G.). The amount of cholesterol in the $d < 1.019$ fraction was calculated by substracting the amount of cholesterol in the LDL ($1.019 < d < 1.063$) and in the HDL ($1.063 < d < 1.21$) fraction from the total amount of plasma cholesterol. Absolute amounts of apoE and apoB were determined using ELISA according to the methods described by Bury et al. [22] and Kaptein et al. [23], respectively.

LPL-treatment of lipoproteins was performed by incubating serum with lipoprotein lipase (LPL), purified from bovine milk [24]. Serum was incubated with a fixed amount of LPL per mmol of triglycerides, in the presence of 10% (w/v) free fatty acid free human serum albumin (HSA) and Tris-HCl buffer (final concentration 0.1 M, pH 8,5) for 2 hours at 37°C. To stop the reaction, the mixture was put on ice and solid KBr was added to adjust the solution to a density of 1.21 g/ml. The solution was then placed under a gradient of salt solutions and isolated as described before. Agarose electrophoresis was performed as described by Demacker et al. [25].

Labeling of LDL

Shortly before each experiment LDL was isolated from one and the same normolipidemic E3E3 homozygote control subject. LDL preparations were immediately used for iodination by the [^{125}I]iodine monochloride method described by Bilheimer et al. [26]. After iodination, the LDL was dialysed against phosphate-buffered saline for 4 h (4 times 500 ml). Thereafter it was stabilized by the addition of human serum albumin (HSA) (1% w/v) and further dialysed against culture medium supplemented with 20 mM Hepes buffer (pH 7.4) 100 U/ml penicillin and 100 μg/ml streptomycin. The stabilized lipoproteins were stored at 4°C. Their specific radioactivity ranged from 150 to 500 cpm/ng of lipoprotein protein. Less than 1% of label was present in the lipid fraction. Although stable for periods greater than 1 month under these conditions, the iodinated LDL were used within 10 days. When not labeled with [^{125}I]iodine, lipoproteins were stabilized immediately by the addition of 1% HSA (by weight) and subsequent extensive dialysis against culture medium supplemented with Hepes, penicillin and streptomycin.

Culturing of Hep G2 cells

The cells were cultured at 37°C in 25 cm^2 flasks containing 2 ml of DMEM culture medium supplemented with 10% (by volume) heat-inactivated foetal calf serum (FCS), 20 mM Hepes, 25 mM $NaHCO_3$, 100 U/ml penicillin and 100 μg/ml streptomycin under 5% CO_2/95% air. The medium was renewed twice a week. About 1 week prior to the assay, the cells were trypsinized and transferred to 2 cm^2 multiwell dishes, using a split ratio of 1:6. Twenty hours

before the start of the experiment, DMEM supplemented with 1% of HSA instead of DMEM supplemented with FCS, was added to the cells.

Measurement of receptor-mediated association of ^{125}I-LDL to Hep G2 cells

The experiment was started by the addition of ^{125}I-labeled LDL in the absence or in the presence of unlabeled d < 1.019 lipoproteins isolated from each of the six FD subjects or unlabeled control LDL. After 3 hours of incubation at 37°C, the cells were cooled to 0°C and the ^{125}I-labeled LDL that became cell associated was measured exactly as described previously [27]. The association of ^{125}I-labeled LDL was expressed as percentage of the association in the absence of unlabeled lipoproteins (100%).

Results

The clinical data of the six patients are presented in Table 1. Prior to treatment, all individuals showed lipoprotein patterns typical for FD. All patients exhibited high concentrations of serum cholesterol (mean ± SD: 10.7 ± 3.8 mmol/l) and serum triglyceride (mean ± SD; 5.4 ± 1.8 mmol/l), due to the accumulation in the plasma of VLDL + IDL cholesterol (mean ± SD; 7.9 ± 3.7 mmol/l). Furthermore, the patients displayed high plasma apoE concentrations (mean ± SD; 28 ± 7 mg/dl), and low LDL-cholesterol concentrations (mean ± SD; 1.9 ± 0.5 mmol/l). The ratio of cholesterol/triglyceride in d < 1.019 lipoproteins was elevated (1.5 ± 0.4 mmol/mmol), compared to the ratio normally found in this lipoprotein fraction (about 0.5 mmol/mmol). The presence of pre-β-VLDL as well as β-VLDL could be demonstrated in the d < 1.019 lipoprotein fraction of all patients as evaluated by agarose electrophoresis (Fig. 1). The d < 1.019 lipoprotein fraction of a normolipidemic control subject displayed only the pre-β band. After LPL treatment, the d < 1.019 lipoproteins moved almost completely to the β position, except for patient 6 who was extremely hyperlipidemic (Table 1).

The experimental design is shown in Fig. 2. From patient 1, 2 and 3, two base line blood samples were collected in week 1 and week 2. After receiving gemfibrozil, in a dose of 2 times 600 mg/day, for a period of 4 weeks, again blood samples were collected at an interval of 1 week (week 6 and 7). At these time points also base line blood samples were collected from patients 4, 5 and 6. This second group of patients was also treated with gemfibrozil for a period of four weeks, and blood samples were collected in week 11 and week 12.

Upon treatment of the patients with gemfibrozil, no significant alterations in body mass index were observed (results not shown). The serum cholesterol concentrations of all subjects, except subject number 6, were normalized upon treatment (Table 2). Most of the reduction of the serum cholesterol level was found to be due to a decrease in the amount of cholesterol present in the d < 1.019 lipoprotein fraction (7.9 ± 3.7 to 3.3 ± 1.6 mmol/l). The mean LDL-cholesterol level remained constant, whereas, in agreement with previous observations [27], HDL-cholesterol increased upon treatment with gemfibrozil (0.9 ± 0.1 to 1.2 ± 0.2 mmol/l).

Table 2. Comparison of the six FD patients before and after treatment with gemfibrozil with respect to plasma lipid and lipoprotein levels and relative composition of the d < 1.019 lipoproteins.

	Patient no.						
	1	2	3	4	5	6	Mean ± SD
Plasma Chol (mmol/l)							
Before	9.8	7.6	12.7	7.8	8.6	17.5	10.7 ± 3.8
After	5.9	4.9	6.5	5.4	5.5	10.8	6.5 ± 2.0
Plasma Tg (mmol/l)							
Before	6.3	3.4	5.0	4.8	4.2	8.6	5.4 ± 1.8
After	4.2	2.3	2.1	1.7	1.2	2.2	2.3 ± 1.0
d < 1.019 Chol (mmol/l)							
Before	7.4	4.3	9.9	5.3	6.1	14.2	7.9 ± 3.7
After	3.1	2.1	3.2	2.3	2.6	6.4	3.3 ± 1.6
LDL Chol (mmol/l)							
Before	1.3	2.5	1.8	1.6	1.6	2.3	1.9 ± 0.5
After	1.4	1.8	2.1	2.0	1.7	2.8	2.0 ± 0.5
HDL Chol (mmol/l)							
Before	1.1	0.8	1.0	0.9	0.9	1.0	0.9 ± 0.1
After	1.4	1.0	1.2	1.1	1.2	1.6	1.2 ± 0.2
Ratio Chol/Tg in d < 1.019 (mmol/mmol)							
Before	2.1	1.1	1.9	1.2	1.7	1.1	1.5 ± 0.4
After	0.8	0.9	1.8	1.0	2.1	2.4	1.5 ± 0.7
Chol/apoB in d < 1.019 (mmol/10 ng)							
Before	1.5	1.2	2.1	1.2	2.7	4.5	2.2 ± 1.3
After	1.2	0.8	1.6	1.0	1.8	1.1	1.3 ± 0.4
apoE (mg/dl)							
Before	20	28	35	19	29	36	28 ± 7
After	20	26	29	22	19	34	25 ± 6
apoB (mg/dl)							
Before	51	60	74	82	68	143	8.0 ± 3.3
After	44	36	62	92	61	125	7.0 ± 3.3
apoE/apoB ratio in plasma (mg/mg)							
Before	0.4	0.5	0.5	0.2	0.4	0.3	0.4 ± 0.1
After	0.5	0.7	0.5	0.2	0.3	0.3	0.4 ± 0.2

Note: Each value before and after treatment with gemfibrozil is the mean of two samples obtained 1 week apart, as depicted in Fig. 2.

Abbreviations: Chol, cholesterol; Tg, triglycerides; d < 1.019 Chol, lipoprotein fraction with density less than 1.019 g/ml; LDL Chol, LDL cholesterol; HDL Chol, HDL cholesterol.

From the results presented in Table 2 it is obvious that normalization of the serum cholesterol concentration upon treatment did not induce a consistent change in mean cholesterol/triglyceride ratio in the d < 1.019 lipoprotein fractions of the six subjects (1.5 ± 0.4 and 1.5 ± 0.7 before and after treatment, respectively). Similarly, we found no significant reduction in mean serum apoE level after gemfibrozil treatment (28 ± 7 versus 25 ± 6). Upon gemfibrozil treatment the amount of cholesterol per lipoprotein particle in the d < 1.019 fraction decreased (mean ratio chol/apoB: 2.2 ± 1.3 to 1.3 ± 0.4, before and after treatment, respectively).

Figure 3 shows that before and after treatment with gemfibrozil the d < 1.019 lipoproteins were equally good substrates for LPL. Before gemfibrozil treatment lipolysis of the d < 1.019 lipoproteins resulted in a change of the mean cholesterol/triglyceride ratio from 1.5 ± 0.4 to 3.0 ± 0.7 mmol/mmol, whereas after gemfibrozil administration this ratio changed from 1.5 ± 0.7 to 2.8 ± 1.1 (mmol/mmol). These results were supported by the finding that upon lipolysis all the d < 1.019 lipoprotein fractions showed mainly β-mobility, before as well as after treatment with gemfibrozil (Fig. 1).

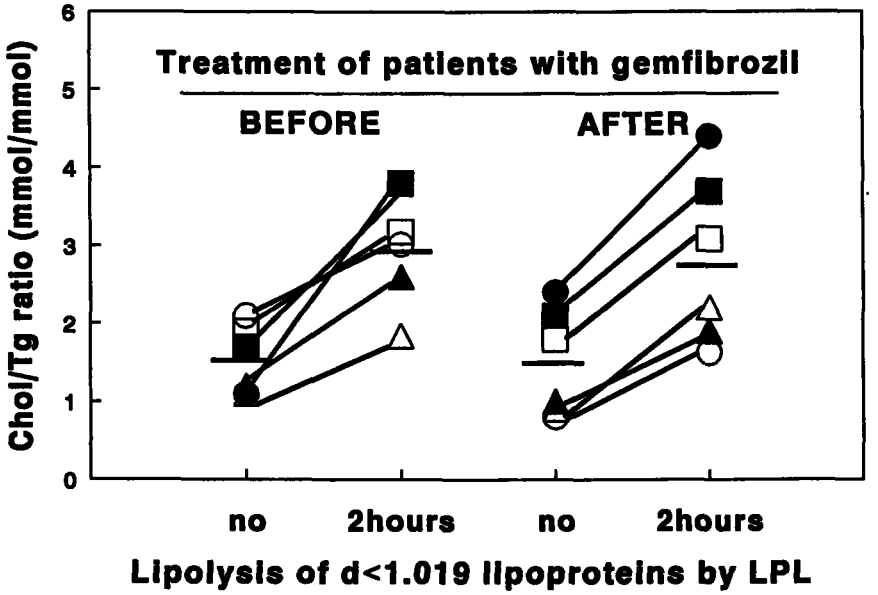

Figure 3. The change in cholesterol/triglyceride ratio of lipoproteins with density d < 1.019 upon incubation with LPL. Lipolysis was performed by incubating the sera for 2 hours at 37°C with a fixed amount of LPL per mmol of triglycerides, 10% fatty acid free HSA (w/v) and Tris-HCl buffer (0.1 M, pH 8,5). The reaction was stopped by placing the mixture on ice and by adding solid KBr to adjust the solution to a density of 1.21 g/ml, whereafter the lipoproteins with density d < 1.019 were isolated as described in Materials and Methods. Each value represents the mean of the two assays performed with an interval of one week (Fig. 2). The differences between these two independent ratios obtained were less than 8%.
Chol/Tg ratio: cholesterol/triglyceride ratio in the d < 1.019 g/ml lipoprotein fraction
Patient 1, ○ ; 2, ; 3, ; 4, ▲ ; 5, ■ ; 6, ○ .

For all six subjects we tested the ability of the d < 1.019 lipoprotein fraction (isolated before and after treatment with gemfibrozil), to compete with ^{125}I-LDL for the LDL receptor on Hep G2 cells (Fig. 4). Therefore, the association of ^{125}I-LDL to Hep G2 cells after 3 hours of incubation at 37°C, was measured in the presence of indicated amounts of d < 1.019 lipoproteins isolated from the patients. Both ^{125}I-LDL and unlabeled LDL, serving as reference competing lipoprotein, were isolated shortly before each experiment from one and the same normolipidemic subject. The efficiency of the unlabeled LDL to compete with ^{125}I-LDL remained constant. As shown in Fig. 4, the mean percentage of control binding left upon incubation with 25 μg/ml (Fig. 4A) and 100 μg/ml (Fig. 4B) of unlabeled LDL was 74 ± 8 and 48 ± 11%, respectively (six independent measurements at weeks 1, 2, 6, 7, 11 and 12, see Fig. 1). Based on the amount of lipoprotein added, the unlabeled d < 1.019 lipoprotein fractions of all six E2E2 homozygous FD patients were much less efficient than the reference LDL in competing with ^{125}I-LDL for binding to the LDL receptor on Hep G2 cells (Fig. 4A and 4B). In addition, the results presented in Fig. 4 also indicate that in these six FD subjects, normalization of the serum cholesterol concentration upon treatment with gemfibrozil, did not consistently change the binding efficiency of the d < 1.019 lipoproteins to the LDL receptor on Hep G2 cells.

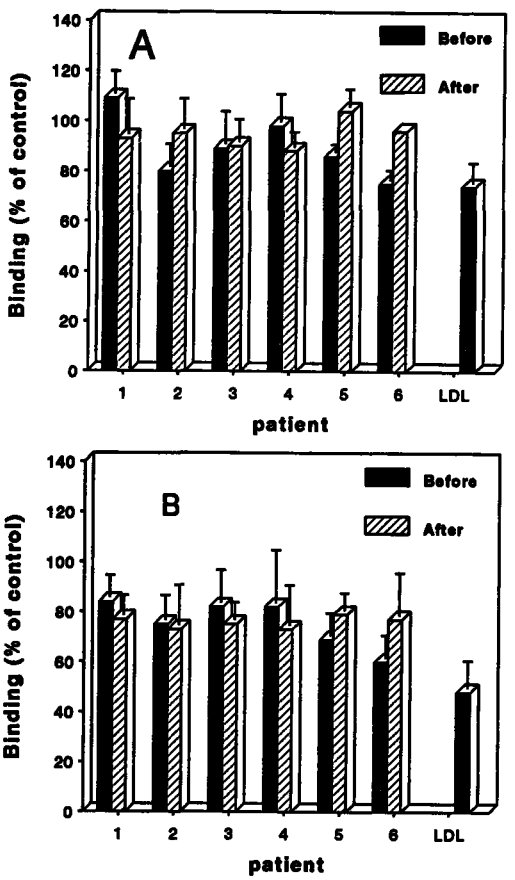

Figure 4. Competition of d < 1.019 lipoproteins isolated from the six subjects with ^{125}I-LDL for binding to the LDL receptor on Hep G2 cells. After preincubation for 20 h in medium supplemented with 1% (w/v) HSA, the cells were incubated for 3 h with 10 μg/ml of ^{125}I-labeled LDL protein in the presence of 25 μg/ml (A) or 100 μg/ml of unlabeled d < 1.019 lipoprotein or unlabeled LDL. The association in the absence of unlabeled lipoprotein was defined as 100%. Each value represents the mean of the two assays performed in triplicate with an interval of one week (Fig. 2).

Patients with familial dysbetalipoproteinemia (FD) mostly display E2E2 homozygosity and are characterized by an increased level of cholesterol in the chylomicron- and VLDL-remnant (d < 1.019 g/ml) lipoprotein fraction [10]. The major underlying metabolic defect is a disturbance in the interaction of apoE2 with hepatic lipoprotein receptors.

The basic approach to dietary treatment of FD patients is to restrict caloric intake and to reduce cholesterol and saturated fat in the diet [10]. Previously, Innerarity et al. [16] reported the treatment of a single E2E2 homozygous FD patient by severe caloric restriction. They observed considerable weight loss in this patient upon treatment concomitant with a dramatic reduction in the concentration of chylomicron- and VLDL-remnants. Strikingly, they found that this reduction is caused, at least partly, by an improved binding of the remnant lipoproteins to the LDL receptor. They hypothesized that this improved binding is the result of a conformational change of apoE2 due to a different micro-environment on the surface of the remnant particles. Similar to this, Chappell and Lindgren [17] found an increase of the binding affinity of d < 1.006 lipoproteins from three E2E2 homozygous FD patients upon significant reduction of the serum cholesterol concentration as a result of treatment with a low-calorie diet.

In our lipid clinic most E2E2 homozygous FD patients are less severe hypercholesterolemic than the patient described by Innerarity et al. [16]. In addition, a majority of our population of FD patients have a normal body mass index and dietary treatment does not result in sufficient reductions of serum cholesterol levels. Therefore, our FD patients are usually treated with gemfibrozil.

Gemfibrozil has been shown to efficiently reduce serum triglyceride and serum cholesterol levels. Triglyceride levels are reduced both by decreasing the hepatic production and by increasing the clearance from the plasma [10,28,29]. We wondered whether the hypocholesterolemic effect of gemfibrozil in FD patients is also, at least in part, the result of an improved binding efficiency of VLDL and VLDL-remnant particles to the LDL receptor.

The present paper shows however, that in this group of E2E2 homozygous FD patients treatment with gemfibrozil does not lead to a consistent improvement of the binding of d < 1.019 lipoproteins to the LDL receptor as evaluated in *in vitro* binding experiments. If taken into account individually, the change of the cholesterol/triglyceride ratio upon treatment with gemfibrozil tended to be inversly related to the change in the binding efficiency of the d < 1.019 lipoproteins. This however, was not significant and no relation with plasma cholesterol concentrations could be detected. Thus, our binding results are in contrast to the results obtained by Innerarity et al. [16] in one patient after dietary treatment. In the FD patient treated with heavy caloric restriction, Innerarity et al. [16] did find a reduction in the ratio of cholesterol to triglyceride in the VLDL fraction and in the plasma apoE level. Pauciullo et al. [30] and Fruchart et al. [31] found a reduction of plasma apoE levels upon treatment with gemfibrozil or fenofibrate (another fibric acid derivative) respectively. On the other hand, Krause and Newton found an increase of the serum apoE concentration upon treatment

with gemfibrozil, in cholesterol-fed rats [32]. In the present study, we found that the reduction of the level of cholesterol in the d < 1.019 lipoprotein fraction did not lead to a significant reduction in the mean ratio cholesterol to triglyceride in this lipoprotein fraction, nor did it affect plasma apoE levels. After treatment of the patients with gemfibrozil the d < 1.019 lipoproteins remained equally good substrates for LPL (Figs. 1 and 3). The absence of an effect of gemfibrozil treatment on these parameters sustains our observation of the absence of an effect of gemfibrozil on the ability of d < 1.019 lipoproteins to bind to the LDL receptor (Fig. 4).

Recently, it has been shown that also in normolipidemic E2E2 homozygotes the clearance of chylomicron-remnants is delayed [33,34]. This observation is in agreement with our findings, suggesting that the defective binding of the d < 1.019 lipoprotein fraction from E2E2 homozygotes is not affected by plasma cholesterol levels.

The reason for the discrepancy between our results and those of Innerarity et al. [16] could be the difference in patients. In their experiment the treatment with caloric restriction was rather extreme as the patient underwent a considerable weight loss concomitant with a dramatic fall in plasma cholesterol level from severe hypercholesterolemia to hypocholesterolemia. Our patients were treated with gemfibrozil instead of caloric restriction; they did not loose weight and the reduction in plasma cholesterol level was much less dramatic. At present no overweight and extreme hypercholesterolemic FD patients are available in our clinic to repeat the experiments performed by Innerarity et al. [16] and Chappell and Lindgren [17]. We conclude that, in contrast to the results obtained by Innerarity et al. [16], after dietary treatment and subsequent weight reduction, normalization of the serum cholesterol concentration in FD subjects by treatment with gemfibrozil does not consistently result in a change of the d < 1.019 lipoproteins in both lipid composition and the ability to bind to the LDL-receptor. Therefore, our results suggest that gemfibrozil acts on the synthesis of VLDL rather than on the receptor mediated clearance.

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References

1. Brown, MS, Kovanen PT, Goldstein JL: Regulation of plasma cholesterol by lipoprotein receptors. Science 212:628-655, 1981
2. Mahley RW, Innerarity TL: Lipoprotein receptors and cholesterol homeostasis. Biochim Biophys Acta 737:197-222, 1983

3. Sherrill BC, Innerarity TL, Mahley RW: Rapid hepatic clearance of the canine lipoproteins containing only the E apolipoprotein. J Biol Chem 255:1804-1807, 1980
4. Utermann G, Hees M, Steinmetz A: Polymorphism of apolipoprotein E and occurrence of dysbetalipoproteinemia in man. Nature 269:604-607, 1977
5. Zannis VI, Breslow JL: Human VLDL apolipoprotein E isoprotein polymorphism is explained by genetic variation and posttranslational modification. Biochemistry 20:1033-1041, 1981
6. Havel RJ, Yamada N, Shames DM: Role of apolipoprotein E in lipoprotein metabolism. Am Heart J. 113:470-474, 1987
7. Schneider WJ, Kovanen PT, Brown MS, et al: Familial dysbetalipoproteinemia. Abnormal binding of mutant apoprotein E to LDL receptors of human fibroblasts and membranes from liver and afdrenal of rats, rabbits, and cows. J Clin Invest 68:1075-1085, 1981
8. Rall SC, Weisgraber KH, Innerarity TL, et al:Structural basis for receptor binding heterogeneity of apolipoprotein E from type III hyperlipoproteinemic subjects. Proc Natl Acad Sci USA 79:4696-4700, 1982
9. Utermann G: Genetic polymorphism of apolipoprotein E. Impact on plasma lipoprotein metabolism, in G. Crepaldi, A. Tiengo, and G. Baggio (ed): Diabetes, Obesity and hyperlipidemias-III Elsevier Science Publishers. Amsterdam, 1985, pp 1-28
10. Mahley RW, Rall SC: Type III hyperlipoproteinemia (dysbetalipoproteinemia). The role of apolipoprotein E in normal and abnormal lipoprotein metabolism, in C.R. Scriver. A.L. Beaudet, W.S. Sly and D. Valle (ed): The metabolic basis of inherited disease, 6th Edition, McGraw-Hill, New York,1989, pp 1195-1213
11. Weisgraber KH, Innerarity TL, Mahley RW: Abnormal lipoprotein receptor-binding activity of the human E apoprotein due to cysteine-arginine interchange at a single site. J Biol Chem 257:2518-2521, 1982
12. Rall SC, Weisgraber KH, Innerarity TL, et al: Identical structural and receptor binding defects in apolipoprotein E2 in hypo-, normo- and hypercholesterolemic dysbetalipoproteinemia. J Clin Invest 71:1023-1031, 1983
13. Innerarity TL, Friedlander EJ, Rall SC, et al: The receptor binding domain of human apolipoprotein E: binding of apolipoprotein E fragments. J Biol Chem 258:12341-12347, 1983
14. Weisgraber KH, Innerarity TL, Harder KJ, et al: The receptor binding domain of human apolipoprotein E: monoclonal antibody inhibition of binding. J Biol Chem 258:12348-12354, 1983
15. Innerarity TL, Weisgraber KH, Arnold KS, et al: Normalization of receptor binding of apolipoprotein E2. Evidence for modulation of the binding site conformation. J Biol Chem 259:7261-7267, 1984
16. Innerarity TL, Hui DY, Bersot TP et al: Type III hyperlipoproteinemia: A focus on lipoprotein receptor-apolipoprotein E2 interactions, in Angel A, Frohlich J (ed): Lipoprotein Deficiency Syndromes, Plenum Publishing, New York, 1986, pp 273-288
17. Chappell DA, Lindgren FT: Increased receptor binding affinity of lipoproteins during *in vivo* lipolysis. Arteriosclerosis 9:728a, 1989
18. Havekes LM, de Knijff P, Beisiegel U, et al: A rapid method for apolipoprotein E phenotyping direct in serum. J Lipid Res 28:455-463, 1987
19. Redgrave TG, Roberts DCD, West CE: Separation of plasma lipoproteins by density gradient centrifugation. Anal Biochem 65:42-49, 1975
20. Mulder M, de Wit E, Havekes LM: The binding of human lipoprotein lipase treated VLDL by the human hepatoma cell line Hep G2. Biochim Biophys Acta 1081:308-314, 1990
21. Lowry OH, Rowebrough NJ, Farr AL, et al: Protein measurement with Folin phenol reagent. J Biol Chem 193:265-275, 1951
22. Bury J, Vercaemst R, Rosseneu M, et al: Apolipoprotein E quantified by enzyme-linked immunosorbent assay. Clin Chem 32:265-270, 1986
23. Kaptein A, Roodenburg L, Princen HMG: Buryrate stimulates the secretion of apolipoprotein A-I and apolipoprotein B100 by the human hepatoma cell line Hep G2. Induction of apo A-I mRNA with no change of apo B100 mRNA. Biochem J 278:557-564, 1991
24. Pagano Mirani-Oostdijk C, Havekes L, Den Boer P, et al: Diurnal course of the suitability of serum samples obtained from normal subjects and from patients with primary endogenous hypertriglyceridaemia to serve as substrate for milk lipoprotein lipase. Scand J Clin Lab Invest 45:207-215, 1985
25. Demacker PNM, Vos-Janssen HE, van't Laar A, et al: A descriptive study of the different electrophoretic patterns in agarose of human serum very low density lipoproteins. Clin. Chem. 24:1439-1444, 1978

26. Bilheimer DW, Eisenberg S, Levy RI: The metabolism of very low density lipoprotein. I. PreliminOary *in vitro* and *in vivo* observations. Biochim Biophys Acta 260:212-221, 1972
27. Havekes L, Van Hinsbergh V, Kempen HJ, et al: The metabolism *in vitro* of low density lipoproteins by the human hepatoma cell line Hep G2. Biochem J 214:951-958, 1983
28. Todd PA, Ward A: Gemfibrozil. A Review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in dyslipidaemia. Drugs 36:314-339, 1988
29. Franceschini G, Sirtori M, Vaccarino V, et al: Plasma lipoprotein changes after treatment with pravastatin and gemfibrozil in patients with familial hypercholesterolemia. J Lab Clin Med 114:250-259, 1989
30. Pauciullo P, Marotta G, Rubba P, et al: Serum lipoproteins, apolipoproteins and very low density lipoprotein subfractions during 6- month fibrate treatment in primary hypertriglyceridemia. J Intern Med 228:425-430, 1990
31. Lussier-Cacan S, Bard JM, Boulet L, et al: Lipoprotein composition changes induced by fenofibrate in dysbetalipoproteinemia type III. Atherosclerosis 78:167-182, 1989
32. Krause BR, Newton RS: Gemfibrozil increases both apoA-I and apoE concentrations. Comparison to other lipid regulators in cholesterol-fed rats. Atherosclerosis 59:95-98, 1986
33. Weintraub MS, Eisenberg S, Breslow JL: Dietary fat clearance in normal subjects is regulated by genetic variation in apolipoprotein E. J Clin Invest 80:1571-1577, 1987
34. Rubinsztein DC, Cohen JC, Berger GM, et al: CMR clearance from the plasma is normal in familial hypercholesterolemic homozygotes with defined receptor defects. J Clin Invest 86:1306-1312, 1990

Chapter 5

TRIGLYCERIDE-RICH LIPOPROTEINS OF SUBJECTS HETEROZYGOUS FOR APOLIPOPROTEIN E2(lys146 → gln) ARE POOR SUBSTRATES FOR LIPOPROTEIN LIPASE

Monique Mulder[1], Arn van den Maagdenberg[2], Hans van der Boom[1], Peter de Knijff[1], Carine Braam[1], Jan A. Gevers Leuven[1], Rune R. Frants[2], and Louis M. Havekes[1]

[1]IVVO-TNO Gaubius Laboratory, Leiden;
[2]MGC-Department of Human Genetics, Leiden University, Leiden,
The Netherlands.

Submitted.

Summary

The apoE2(lys146 → gln) variant behaves as a dominant trait in the expression of Familial Dysbetalipoproteinemia (FD). We found that lipoproteins with a density of less than 1.019 g/ml (d < 1.019 lipoproteins), of three FD patients carrying the APOE*2(lys146 → gln) allele, bound less efficiently to the LDL receptor than the corresponding lipoprotein fraction of normolipidemic APOE*3 homozygous subjects. This poor binding could not be improved by treatment of these d < 1.019 lipoproteins with lipoprotein lipase (LPL). Upon treatment with LPL, the cholesterol to triglyceride molar ratio of the d < 1.019 lipoproteins of the apoE2(lys146 → gln) FD probands increased only marginally (from 0.8 to 1.1), as compared with that of the classical apoE2(arg158 → cys) homozygous FD subjects (from 1.4 to 2.6) and the non-FD subjects (from 0.7 to 1.5). We found that the d < 1.019 lipoprotein fraction of these three apoE2(lys146 → gln) heterozygous FD probands, contained five times as much apoE per lipoprotein particle than the corresponding lipoprotein fraction of the control subjects.

We collected blood samples from family members of six FD probands carrying the APOE*2(lys146 → gln) allele. Upon treatment with LPL the ratio of cholesterol to triglyceride of the d < 1.019 lipoprotein fraction of the carriers (n = 35) and the non-carriers (n = 15) increased from 1.1 to 1.8 and from 0.7 to 1.6, respectively.

We conclude that the APOE*2(lys146 → gln) allele, under certain conditions, predisposes to an impaired LPL-mediated lipolysis of the d < 1.019 lipoprotein fraction which, consequently, results in a defective binding of these lipoproteins to the LDL receptor. In this way, the impaired lipolysis may contribute to the dominant behaviour of the apoE2(lys146 → gln) variant in the expression of FD.

Introduction

In normal subjects, chylomicron- and most of the very low density lipoprotein (VLDL) remnants are rapidly removed from the circulation by receptor-mediated endocytosis in the liver. The remaining VLDL remnants are converted into low density lipoproteins (LDL) (1). ApoE is the ligand for the binding of these remnants to hepatic lipoprotein receptors, and thus plays a crucial role in the remnant metabolism (2).

ApoE is a polymorphic protein of which, by use of isoelectric focusing, three major isoforms, E2, E3 and E4, can be separated (3,4). E2 differs from the most common apoE3 variant by exhibiting a cysteine residue at position 158 instead of an arginine. ApoE4 exhibits an arginine at residue 112 instead of a cysteine. The common apoE isoforms are encoded by three codominant alleles at a single APOE gene locus on chromosome 19 (5). Hence, six common phenotypes can be distinguished: E2E2, E3E3, E4E4, E3E2, E4E2, and E4E3.

Familial dysbetalipoproteinemia (FD) is characterized by high serum cholesterol and triglyceride concentrations, due to the accumulation in the plasma of chylomicron- and

VLDL-remnants. Patients with FD have been shown to develop premature atherosclerosis involving both coronary and peripheral arteries (6). Most FD patients (> 90%) are homozygous for apoE2(arg158 → cys) (7,8). The underlying metabolic defect in these patients is a disturbed interaction of apoE2 with hepatic lipoprotein receptors (9,10). However, of all apoE2(arg158 → cys) homozygotes, representing 1% of the total population, only 4% eventually develop hyperlipidemic FD.

Only rarely is FD associated with the E3E2 or E4E2 phenotype. Genotyping and DNA sequencing of our E3E2 heterozygous FD patients, revealed that these patients exhibited the rare APOE*2(lys146 → gln) allele (11,12). This apoE variant was first described by Rall et al. (13). Family studies have revealed that, in contrast to the most frequently-occurring APOE*2(arg158 → cys) allele, heterozygosity for the APOE*2(lys146 → gln) allele commonly cosegregates with FD, indicating that this variant is inherited as a dominant trait in the expression of the disease (12). Hence, subjects heterozygous for the APOE*2(lys146 → gln) allele frequently develop hyperlipidemic FD, despite the presence of a normal apoE3 allele.

The present paper, deals with the mechanism behind the dominant behaviour of the apoE2(lys146 → gln) variant. We found that the d < 1.019 lipoprotein fraction of the APOE*2(lys146 → gln) allele carriers was less suitable as a substrate for LPL as compared with the corresponding lipoprotein fraction of the non-carriers. A resistance to lipolysis may lead to a defective interaction of these lipoproteins with the LDL receptor, and might, therefore, contribute to the dominant behaviour of the APOE*2(lys146 → gln) allele in the expression of FD.

Materials and Methods

Subjects

The probands previously described as familial dysbetalipoproteinemic (FD) patients with heterozygosity for the APOE*2(lys146 → gln) allele (12), their relatives, the apoE2(arg158 → cys) homozygous FD patients and the other non-E2(lys146 → gln) allele carriers were admitted to the the lipid clinic in Leiden, or visited at their homes. Blood was obtained by venapuncture, after an overnight fast, and was allowed to clot for 1 hour at 37°C. Serum was then separated from blood cells by centrifugation at 500g for 10 min at room temperature.

Patients with FD were diagnosed on the basis of the presence of hyperlipidemia (cholesterol > 7.5 mmol/l; triglycerides > 2.0 mmol/l), concomitant with floating beta lipoproteins and an elevated VLDL cholesterol/plasma triglyceride ratio (> 0.69 on a molar basis). Serum cholesterol and triglyceride were determined using enzymatic methods (Boehringer Mannheim, Germany). ApoE phenotyping was performed using a rapid micro-method based on isoelectric focusing of delipidated plasma, before and after cysteamine-treatment, followed by immunoblotting using a polyclonal anti-apoE antiserum as first antibody (14). Identification of the lys146 → gln mutation as well as genotyping of the common mutation was performed by site-directed mutagenic amplification primers, as

described by van den Maagdenberg et al. (in preparation). The PCR products were digested with restriction enzyme P*vu*II according to the recommendations of the manufacturer (Pharmacia, Uppsala, Sweden). The digested fragments were then separated on a 2% agarose gel, stained with ethidium-bromide, and photographed.

Analysis of the lipoproteins

LDL were isolated according to Redgrave et al. (16). Lipoproteins with a density of less than 1.019 g/ml were isolated as previously described (17). Protein contents of the lipoprotein fractions were determined according to Lowry et al. (18). Total and free cholesterol, triglycerides and phospholipids were determined using enzymatic methods (Boehringer Mannheim, Germany). Free fatty acids were determined using a Nefa C kit from Wako Chemicals GmbH (Neuss, Germany). The relative apolipoprotein composition of the lipoproteins was estimated using SDS-polyacrylamide gradient gel electrophoresis (5-14%, with 0.1% sodiumdodecyl sulphate), followed by staining with Coomassie brilliant blue (19). Absolute concentrations of apoB were measured according to Kaptein et al. (20). ApoE concentrations were measured by ELISA. In brief, anti-human apoE antibodies were first isolated by affinity chromatography using a human apoE-Sepharose-4B column. These apoE antibodies were isolated from both goat anti-human apoE and rabbit anti-human apoE antisera. The goat anti-human apoE antibodies were used as first or catching antibodies. Rabbit antibodies were used as second antibody, whereas swine anti-rabbit IgG conjugated to peroxidase (DAKO, Denmark) was used as third antibody for detection. Phosphate-buffered saline containing either casein (0.1%, by weight, pH = 7.5) or Tween-20 (0.05%, by volume; pH = 7.5) was used as a blocking and washing buffer, respectively.

LPL-treatment of lipoproteins

LPL-treated VLDL (LPL-VLDL; with density d < 1.019) were prepared by incubating total serum with lipoprotein lipase (LPL) purified from bovine milk (21). On the day of collection, serum was subjected to lipolysis. Serum, with a set triglyceride content, was mixed with a fixed amount of bovine-LPL, 400 mg of free fatty acid-free human serum albumin (HSA) and Tris-HCl buffer. The volume was adjusted with PBS to a final volume of 4 ml containing 10% HSA (w/v) in 0.1M Tris-HCl, pH 8,5. The mixture was incubated for 2.5 hours in a waterbath at 37°C. To stop the reaction, the mixture was put on ice and solid KBr was added to adjust the density of the solution to 1.21 g/ml. The solution was then placed under a discontinuous gradient of salt solutions, of densities 1.063 and 1.019 g/ml, with a volume ratio of 0.95:1:1 from bottom to top. After centrifugation, the d < 1.019 g/ml lipoprotein fraction was obtained by taking a 1 ml fraction from the top of the tube.

Labelling of the LDL with [^{125}I]iodine

Immediately after isolation of the LDL according to Redgrave et al. (16), the lipoprotein preparations were used for iodination by the [^{125}I]iodine monochloride method according to Bilheimer et al. (22). After iodination, the LDL were dialysed against phosphate-buffered

saline for 4 hours (four times 500 ml). They were then stabilized by adding HSA (1%, w/v) and further dialysed overnight against culture medium supplemented with 20 mM Hepes buffer (pH 7.4) penicillin and streptomycin. The ^{125}I-labelled LDL were stored at 4°C. Their specific radioactivity was 200-500 cpm/ng of lipoprotein protein. The labelled LDL were used within two weeks. When not labelled with ^{125}I, lipoproteins were stabilized immediately with the addition of 1% HSA and, subsequently, extensively dialysed against culture medium as mentioned above.

Measurement of competition of lipoproteins with ^{125}I-LDL for association to Hep G2 cells
Hep G2 cells were cultured as previously described (17). Competition experiments were performed by incubating Hep G2 cells for a period of 3 h at 37°C with ^{125}I-LDL (10 μg/ml of protein) in the presence or in the absence of increasing amounts of unlabelled lipoproteins, as indicated. Cell association was measured as previously described (17).

Results

The results presented in Table 1, show that the d < 1.019 lipoproteins from apoE2(lys146 → gln) carrying FD probands competed less efficiently with ^{125}I-LDL for binding to the LDL-receptors, than did lipoproteins with the same density from apoE3 homozygotes. However, the binding of the d < 1.019 lipoproteins of apoE2(lys146 → gln) heterozygotes was not as defective as the binding of corresponding lipoproteins isolated from apoE2(arg158 → cys) homozygotes. The binding of the d < 1.019 lipoproteins of the apoE3 and apoE2(arg158 → cys) homozygotes improved after *in vitro* lipolysis, as had been observed in our previous studies (17). In contrast to these observations, the binding of the corresponding lipoproteins of apoE2(lys146 → gln) heterozygotes did not improve upon treatment with LPL (Table 1).

We wondered whether this could be due to an inefficient lipolysis of these lipoproteins. To test this hypothesis, the LPL-mediated lipolysis of d < 1.019 lipoproteins of 3 apoE2(lys146 → gln) heterozygous FD probands was compared with that of 12 apoE2 homozygous FD subjects and 14 non-FD control subjects. The results presented in Fig. 1 indicate that the ratio of cholesterol to triglyceride in the d < 1.019 fraction of three apoE2(lys146 → gln) heterozygous FD probands did not change substantially after treatment with LPL for a period of 2 hours (from 0.8 to 1.1), while that of the corresponding lipoproteins of the apoE2 homozygotes and the non-FD subjects (controls) increased significantly (from 1.4 to 2.6 and from 0.7 to 1.5, respectively). This suggests that the d < 1.019 lipoproteins of E2(lys146 → gln) carriers are relatively resistant to LPL-mediated lipolysis.

In line with this, the results presented in Fig. 2 indicate that upon incubation with LPL, the release of free fatty acids from serum of the apoE2(lys146 → gln) heterozygous FD probands is impaired, when compared with that of the control group.

Table 1. **Competition of unlabelled lipoproteins with a density of d < 1.019 (g/ml) from individuals with different apoE phenotypes, and LDL with ^{125}I-LDL for association to Hep G2 cells.**

unlabelled lipoprotein	apoE phenotype	LPL treatment	μg unlabelled lipoprotein added per ml			
			N	0	10	50
LDL			(n = 4)	100	54 ± 11	32 ± 4
d < 1.019						
lipoproteins:	E3E3	-	(n = 3)	100	66 ± 7	55 ± 4
	E3E3	+	(n = 2)	100	61	37
	E2(lys146 → gln)E3	-	(n = 5)	100	79 ± 7	60 ± 7
	E2(lys146 → gln)E3	+	(n = 1)	100	79	60
	E2E2	-	(n = 6)	100	90 ± 10	74 ± 20
	E2E2	+	(n = 6)	100	78 ± 13	59 ± 15

After preincubation for 20 h in medium supplemented with 1% (w/v) HSA, the cells were incubated for 3 h at 37°C, with 10 μg/ml of ^{125}I-LDL in the presence of unlabelled lipoproteins with a density of less than 1.019 g/ml, as indicated. Thereafter, receptor-mediated cell association was measured as previously described (17). Values represent cell association expressed as a percentage of the control cell association. The control association is the association in the absence of unlabelled lipoprotein (100%). Experiments are carried out in triplicate. Each value, except two, represents the mean ± S.D.

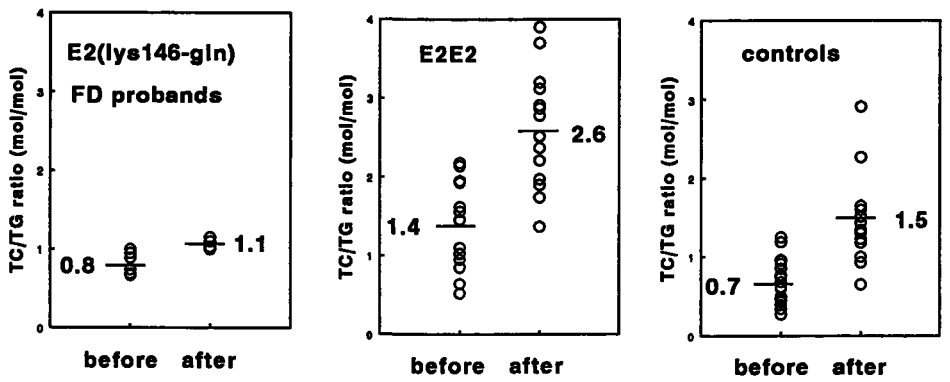

Figure 1. Ratio cholesterol/triglyceride in the d < 1.019 lipoprotein fraction, before and after incubation of serum with bovine-LPL. Sera from apoE2(lys146 → gln) heterozygotes, apoE2(arg158 → cys) homozygotes and controls, were incubated for 2 hours in the presence of LPL, as described in Materials and Methods. Lipoproteins with a density of d < 1.019 were then isolated, as described in Materials and Methods. At the time points 0 h and 2 h, samples were taken for isolation. The cholesterol/triglyceride ratios (TC/TG; mmol/mmol) were then determined. A: three FD probands heterozygous for E3E2(lys146 → gln), the sera of two of them were subjected to lipolysis twice within a three-year interval; B: twelve apoE2(arg158 → cys) homozygotes; C: fourteen controls.

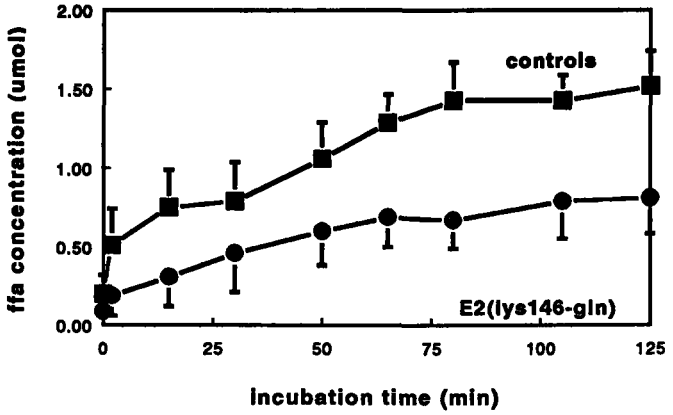

Figure 2. Free fatty acid (FFA) release upon incubation of serum from E2(lys146 → gln) heterozygous FD probands and from control subjects with LPL. An amount of serum containing equal amounts of triglyceride from each subject were adjusted by the addition of PBS to a fixed volume of 4 ml. LPL-treatment was further performed as described in Materials and Methods. Every 15 minutes, samples of 20 μl were taken in quadruplicate and immediately stored at -20°C until measurement. Free fatty acids (ffa) concentrations were measured using a NEFA C kit from Wako Chemicals GmbH, Germany. The values represent the mean ± S.D. of the three E2(lys146 → gln) heterozygous FD probands ●, and of the four control subjects (two E2E2, one E3E3 and one E3E2 subject) ■.

Comparison of the composition of the d < 1.019 lipoproteins of the E2(lys146 → gln) probands and the non-carriers revealed that the cholesterol to triglyceride ratio of the d < 1.019 lipoproteins of the E2(lys146 → gln)-carriers was not as elevated as that of E2E2 homozygous FD subjects (0.8 ± 0.2 vs. 1.4 ± 0.5, mmol/mmol). Most remarkably, the ratio of apoE over total protein in the d < 1.019 fraction of the apoE2(lys146 → gln) probands was much higher, when compared with that of the non-carriers (36 ± 5 vs. 7 ± 2% of total protein (by weight)). The amounts of apoE2(lys146 → gln) present in the serum and in the various lipoprotein fractions were comparable to the amounts of normal apoE (11).

To further investigate the possible underlying metabolic defect in APOE*2(lys146 → gln) allele carriers, we had the opportunity of studying the family members of six apoE2(lys146 → gln) heterozygous FD patients (35 carriers vs. 15 non-carriers). Figure 3 shows the distribution of plasma cholesterol and triglyceride over the various lipoprotein fractions of the APOE*2(lys146 → gln) allele carriers and their relatives not carrying the mutant allele. The APOE*2(lys146 → gln) allele carriers display a relatively large amount of their plasma cholesterol in the VLDL (d < 1.006 g/ml) fraction when compared with the controls. Remarkably, the IDL fraction (1.006 < d < 1.019) of the APOE*2(lys146 → gln) allele carriers contains only a small percentage of cholesterol and triglyceride as compared with what is observed normally in apoE2(arg158 → cys) homozygotes (23), and for subjects heterozygous for the apoE3-Leiden variant (24). The amount of cholesterol in the LDL fraction is slightly lower than that of the controls, although not as low as in apoE2(arg158 → cys) homozygotes (23). This family analysis also confirms the above mentioned observations that the ratio of apoE to apoB, in the d < 1.019 fraction of the APOE*2(lys146

→ gln) allele carriers, is highly elevated as compared with that of the non-carriers (0.28 ± 0.02 vs 0.08 ± 0.006 by weight, respectively). Similarly, in the APOE*2(lys146 → gln) allele carrying family members the ratio of cholesterol to triglycerides in the d < 1.019 lipoprotein fraction is lower than the ratio observed in apoE2(arg158 → cys) homozygous FD subjects (1.1 ± 0.4 vs. 1.4 ± 0.5, respectively), and only slightly increased in comparison with that of the non-carrying family members (1.1 ± 0.4 vs. 0.8 ± 0.4, in the carriers and the non-carriers, respectively).

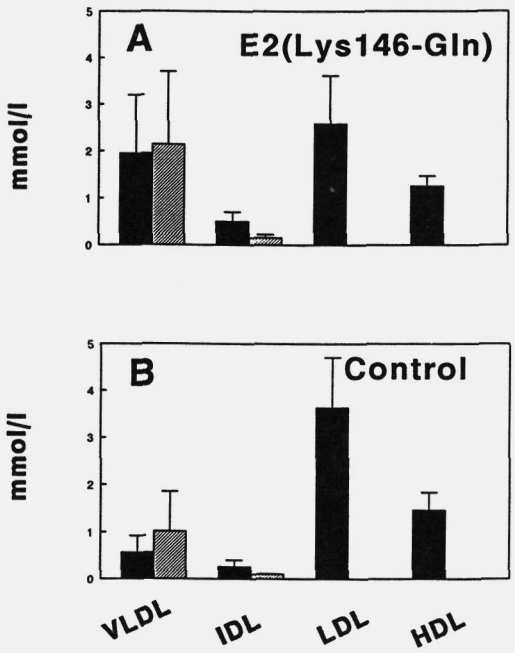

Figure 3. The distribution of plasma cholesterol over the various lipoprotein fractions. The amount of cholesterol and triglyceride in the VLDL (d < 1.006 g/ml), IDL (1.006 < d < 1.019 g/ml), LDL (1.019 < d < 1.063 g/ml), and HDL fraction is shown for apoE2(lys146 → gln) heterozygotes (A), and the controls representing their relatives not carrying the mutant allele (B). Closed bars represent cholesterol, dashed bars represent triglycerides. The values are means ± S.D.

We determined the suitability of the d < 1.019 lipoproteins, of family members carrying the mutant allele and that of the family members not carrying this allele, as a substrate for LPL. Table 2 shows the cholesterol to triglyceride ratios of the d < 1.019 lipoproteins of apoE2(lys146 → gln) carriers and non-carriers, before and after lipolysis. In both groups the ratio of cholesterol/triglyceride significantly ($p < 0.001$) increases as a result of LPL-treatment. However, for the apoE2(lys146 → gln) carriers, the relative increase of the cholesterol/triglyceride ratio upon lipolysis, is significantly ($p < 0.01$) smaller than for the non-carriers. This indicates a less efficient lipolysis of the d < 1.019 lipoproteins of subjects carrying the APOE*2(lys146 → gln) allele. However, a large variation in lipolysis efficiency was found.

In line with this, as a result of lipolysis the triglyceride to apoB ratio of the d < 1.019 fraction of the allele carriers is less dramatically decreased than is that of the d < 1.019 fraction of the non-carriers (relative decrease of 45% and 64%, respectively). These results confirm that the d < 1.019 lipoproteins of the APOE*2(lys146 → gln) allele carriers are poor substrates for LPL.

Table 2. **Ratio cholesterol/triglyceride and triglyceride/apoB in the d < 1.019 lipoprotein fraction, before and after incubation of serum with bovine-LPL.**

	TC/Tg (mean ± S.D.)		relative increase	p*
	before	after		
non-carriers	0.7 ± 0.3	1.6 ± 0.9	249%	0.01
carriers	1.1 ± 0.3	1.8 ± 0.6	164%	
	Tg/B (mean ± S.D.)		relative decrease	p*
	before	after		
non-carriers	9.0 ± 5.1	3.5 ± 1.5	61%	0.005
carriers	8.7 ± 5.0	5.5 ± 3.3	37%	

Sera from family members carrying the E2(lys146 → gln) variant (n = 35) and from family members not carrying this allele (n = 15) were incubated for 2 hours in the presence of LPL, as described in Materials and Methods. At time points 0 h and 2 h, samples were taken for isolation. Lipoproteins with density d < 1.019 were then isolated as described in Materials and Methods. Values for the cholesterol/triglyceride ratios (TC/Tg, mmol/mmol) and triglyceride/apoB (Tg/B, mmol/μg) were measured before and after lipolysis of the d < 1.019 fraction. Values are presented as the mean ± S.D. p*: differences in relative change between the carriers and the non-carriers, as calculated with the Wilcoxon signed Ranks test.

Discussion

FD is commonly associated with homozygosity for apoE2(arg158 → cys). The underlying metabolic defect is a disturbed interaction of apoE2 with lipoprotein receptors. Since homozygosity for the allele is required for the development of FD, this variant is called to be associated with a recessive mode of inheritance of FD. Heterozygosity for apoE2(lys146 → gln) is also associated with FD. In this case, FD exhibits a dominant mode of inheritance as only one defective APOE*2(lys146 → gln) allele is required. Thus, FD is expressed despite the presence of normal apoE (12), suggesting that the presence of the abnormal apoE2(lys146 → gln) variant itself is involved in the expression of FD.

It has been shown that the basic residues in the region 131 to 150 of apoE are necessary for binding to the LDL receptor (25). In line with this, the apoE2(lys146 → gln) variant displays only about 40% of the binding activity of normal apoE3, when associated with artificial phospholipid vesicles (13). Our results show that the native d < 1.019 lipoproteins of three different apoE2(lys146 → gln) heterozygous FD probands, bound less efficiently to the LDL receptor than did the corresponding lipoproteins of normal apoE3 homozygotes (Table 1). However, the d < 1.019 lipoproteins from these patients were not as deficient in

their binding to the LDL receptor as the lipoproteins from apoE2(arg158 → cys) homozygotes.

Previously, we have reported that the increase of the cholesterol to triglyceride ratio of the d < 1.019 lipoproteins of normolipidemic subjects upon treatment with LPL, leads to an enhanced binding efficiency of these lipoproteins to the LDL receptor (17). Our present results show that the d < 1.019 lipoproteins from the apoE2(lys146 → gln) heterozygous FD probands, and also of their relatives carrying the APOE*2(lys146 → gln) allele, are less suitable as a substrate for LPL than the corresponding lipoproteins from their relatives, not carrying this apoE variant. This is in contrast to that in apoE2(arg158 → cys) homozygotes, where it is the conversion IDL into LDL that is disturbed, rather than the conversion of VLDL into VLDL-remnants or IDL (26). Our results also show that the d < 1.019 lipoproteins of apoE2(arg158 → cys) homozygotes are relatively good substrates for LPL, although some studies have shown that these lipoproteins are relatively resistant to lipolysis (27-29). The relative defect in the lipolysis of the d < 1.019 lipoproteins carrying the APOE*2(lys146 → gln) allele, might be the direct cause of an impaired binding of these lipoproteins to the LDL receptor and this might, therefore, result in increased plasma levels of cholesterol and triglyceride in this density fraction. We observed a large variation in the binding efficiency (not shown) of the d < 1.019 lipoproteins, and in their susceptibility to lipolysis. Also earlier Chappell et al. (15) found that VLDL isolated from a subject heterozygous for apoE2(lys146 → gln) was not disturbed in its ability to compete with LDL for binding to the LDL receptor. We, therefore, suggest that, besides the presence of an APOE*2(lys146 → gln) allele, another environmental or genetic factor is required to render the E2(lys146 → gln) containing, d < 1.019 lipoproteins resistant to lipolysis.

ApoC2 is known to be an activator of LPL (for review see reference 30). Isoelectric focusing followed by protein-staining showed that the d < 1.019 lipoprotein fractions of the apoE2(lys146 → gln) heterozygous FD probands, do contain apoC2 (see also Fig. 2 in reference 11). Thus the lack of susceptibility of these lipoproteins to LPL-mediated lipolysis is not due to a deficiency of apoC2.

In the present study, we found that the d < 1.019 lipoproteins of the APOE*2(lys146 → gln) allele carriers contain a relatively high amount of apoE per particle, which is distributed about equally between apoE2(lys146 → gln) and normal apoE (11). The presence of high amounts of total apoE or apoE2(lys146 → gln) per lipoprotein particle can be excluded as the direct cause of poor lipolysis, since data obtained from the APOE*2(lys146 → gln) allele-carrying family members, did not show any correlation between the apoE to apoB ratio of the d < 1.019 lipoprotein fraction and its susceptibility to lipolysis (not shown). Similarly, we could not detect any correlation between the amount of free cholesterol per lipoprotein particle and its susceptibility to lipolysis, hereby excluding the possibility that the high amount of free cholesterol competes with the triglyceride for a place at the surface of the lipoprotein (31).

In summary, our results suggest that apoE2(lys146 → gln) predisposes to a retarded lipolysis of the d < 1.019 lipoprotein fraction, and, consequently, to a less efficient binding of these lipoproteins to the LDL receptor in the liver. Although the molecular mechanism

behind this poor suitability as substrate for LPL is not yet known, it may certainly help to explain the dominant mode of inheritance of FD in subjects with heterozygosity for the APOE*2(lys146 → gln) allele.

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References

1. Brown, M.S., P.T. Kovanen, J.L. Goldstein. 1981. Regulation of plasma cholesterol by lipoprotein receptors. *Science* **212**: 628-655.
2. Sherrill, B.C., T.L. Innerarity, and R.W. Mahley. 1980. Rapid hepatic clearance of the canine lipoproteins containing only the E apolipoprotein by a high affinity receptor. *J. Biol. Chem.* **255**: 1804-1807.
3. Zannis, V.I., and J.L. Breslow. 1981. Human VLDL apolipoprotein E isoprotein polymorphism is explained by genetic variation and posttranslational modification. *Biochemistry* **20**: 1033-1041.
4. Utermann, G., M. Hees, and A. Steinmetz. 1977. Polymorphism of apolipoprotein E and occurrence of dysbetalipoproteinemia in man. *Nature (London)* **269**: 604-607.
5. Shaw, D.J., J.D. Brook, A.L. Meredith, H.G. Harley, M. Sarfarazi, and P.S. Harper. 1986. Gene mapping and chromosome 19. *J. Med. Genet.* **23**: 2-10.
6. Fredrickson, D.S., J.L. Goldstein, and M.S. Brown. 1980. The familial hyperlipoproteinemias. In the metabolic basis of inherited disease. J.B. Stanbury, J.B. Wyngaarden, and D.S. Fredrickson (eds.) New York McGraw-Hill Book Co. pp. 604-655.
7. Breslow, J.L., V.I. Zannis, T.R. SanGiacomo, J.L.H.C. Third, T. Tracy, and C.J. Glueck. 1982. Studies of familial type III hyperlipoproteinemia using as a genetic marker the apoE phenotype E2E2. *J. Lipid Res.* **23**: 1224-1235.
8. Utermann, G., M. Jaeschke, and J. Menzel. 1975. Familial hyperlipoproteinemia type III. Deficiency of a specific apolipoprotein (apoEIII) in the VLDL. *FEBS Lett.* **56**: 352-355.
9. Rall, S.C., K.H. Weisgraber, T.L. Innerarity, and R.W. Mahley. 1982. Structural basis of receptor binding heterogeneity of apolipoprotein E from type III hyperlipoproteinmeic subjects. *Proc. Natl. Acad. Sci. USA* **79**: 4695-4700.
10. Schneider, W.J., P.T. Kovanen, M.S. Brown, J.L. Goldstein, G. Utermann, W. Weber, R.J. Havel, L. Kotite, J.P. Kane, T.L. Innerarity, and R.W. Mahley. 1981. Familial dysbetalipoproteinemia. Abnormal binding of mutant apolipoprotein E to low density receptors of human fibroblasts and membranes from liver and adrenals of rats, rabbits and cows. *J. Clin. Invest.* **68**: 1075-1085.
11. Smit, M., P. De Knijff, R.R. Frants, E.C. Klasen, and L.M. Havekes. 1987. Familial dysbetalipoproteinemic subjects with the E3/E2 phenotype exhibit an E2 isoform with only one cysteine residue. *Clin. Genet.* **32**: 335-341.
12. Smit, M., P. De Knijff, E. Van der Kooy-Meys, C. Groenendijk, A.M.J.M. Van den Maagdenberg, J.A. Gevers Leuven, A.F.H. Stalenhoef, P.M.J. Stuyt, R.R. Frants, and L.M. Havekes. 1990. Genetic heterogeneity in familial dysbetalipoproteinemia. The E2(lys146 → gln) variant results in a dominant mode of inheritance. *J. Lipid Res.* **31**: 45-53.

13. Rall, S.C., K.H. Weisgraber, T.L. Innerarity, T.P. Bersot, and R.W. Mahley. 1983. Identification of a new structural variant of human apolipoprotein E, E2(lys146 → gln) in a type III hyperlipoproteinemic subject with the E3/E2 phenotype. *J. Clin. Invest.* **72:** 1288-1297.
14. Havekes, L.M., P. De Knijff, Beisiegel, U., Havinga, J., Smit, M., and Klasen, E. 1987. A rapid micromethod for apolipoprotein E phenotyping directly in serum. *J. Lipid Res.* **28:** 455-463.
15. Chappell, D.A. 1989. High receptor binding affinity of lipoproteins in atypical dysbetalipoproteinemia (type III hyperlipoproteinemia). *J. Clin. Invest.* **84:** 1906-1915.
16. Redgrave, T.G., D.C.K. Roberts, and C.E. West. 1975. Separation of plasma lipoproteins by density gradient centrifugation. *Anal. Biochem.* **65:** 42-49.
17. Mulder, M., E. De Wit, and L.M. Havekes. 1991. The binding of human lipoprotein lipase treated VLDL by the human hepatoma cell line Hep G2. *Biochim. Biophys. Acta* **1081:** 308-314.
18. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193:** 265-275.
19. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227:** 680-685.
20. Kaptein, A., L. Roodenburg, and H.M.G. Princen. 1991. Butyrate stimulates the secretion of apolipoprotein A-I and B100 by the human hepatoma cell line Hep G2. *Biochem. J.* **278:** 557-564.
21. Pagano Mirani-Oostdijk, C., L. Havekes, P. Den Boer, E. De Wit, and J. Terpstra. 1985. Diurnal course of the suitabilityof serum samples obtained from normal subjects and from patients with type IV hyperlipidaemia to serve as substrate for lipoprotein lipase. *Scand. J. Clin. Lab. Invest.* **45:** 207-215.
22. Bilheimer, D.W., S. Eisenberg, and R.I. Levy. 1972. The metabolism of very low density lipoprotein proteins. I. Preliminary *in vitro* and *in vivo* observations. *Biochim. Biophys. Acta* **260:** 212-221.
23. Zhao, S-P., A.H.M. Smelt, J.A. Gevers Leuven, A.M.J.M. Van den Maagdenberg, A. Van der Laarse, F.M. Van 't Hooft. 1993. Lipoproteins in familial dysbetalipoproteinemia. Variations of serum cholesterol level associated with VLDL concentration. *Arterioscler. Thrombos.* **13:** 316-232.
24. De Knijff, P., A.M.J.M. Van den Maagdenberg, A.F.H. Stalenhoef, J.A. Gevers Leuven, P.N.M. Demacker, L.P. Kuyt, R.R. Frants, and L.M. Havekes. 1991. Familial dysbetalipoproteinemia associated with apolipoprotien E3-Leiden in an extended multigeneration pedigree. *J. Clin. Invest.* **88:** 643-655.
25. Wilson, C., M.R. Wardell, K.H. Weisgraber, R.W. Mahley, and D.A. Agard. 1991. Three-dimensional structure of the LDL receptor binding domain of human apolipoprotein E. *Science* **252:** 1817-1822.
26. Demant, T., D. Bedford, C.J. Packard, and J. Shepherd. 1991. Influence of apolipoprotein E polymorphism on apolipoprotein B-100 metabolism in normolipidemic subjects. *J. Clin. Invest* **88:**1490-1501.
27. Byung H.C., and J.P. Segrest. 1983. Resistance of a very low density lipoprotein subpopulation from familial dysbetalipoproteinemia to *in vitro* lipolytic conversion to the low density lipoprotein density fraction. *J. Lipid Res.* **24:** 1148-1159.
28. Ehnholm, C., R.W. Mahley, D.A. Chappell, K.H. Weisgraber, E. Ludwig, and J.L. Witztum. 1984. Role of apolipoprotein E in the lipolytic conversion of β-very low density lipoproteins to low density lipoproteins in type III hyperlipoproteinemia. *Proc. Natl. Acad. Sci. USA* **81:** 5566-5570.
29. Saheki, S., I. Takahashi, M. Murase, N. Takeuchi, and K. Uchida. 1991. Composition of very low density lipoproteins and *in vitro* effect of lipoprotein lipase. *Clin. Chim. Acta* **204:** 155-166.
30. Wang, C.-S, J. Hartsuck, and W. McConathy. 1992. Structure and functional properties of lipoprotein lipase. *Biochim. Biophys. Acta* **1123:** 1-17.
31. Miller, K.W., and D.M. Small. 1987. in: Plasma Lipoproteins (A.M. Gotto, Jr. (Ed.)) Elsevier Science Publishers B.V. (Biomedical Division).

Chapter 6

HEPARAN SULPHATE PROTEOGLYCANS ARE INVOLVED IN THE LIPOPROTEIN LIPASE-MEDIATED ENHANCEMENT OF THE CELLULAR BINDING OF VERY LOW DENSITY AND LOW DENSITY LIPOPROTEINS

Monique Mulder[1], Paola Lombardi[1], Hans Jansen[2],
Theo J.C. van Berkel[3], Rune R. Frants[4] and Louis M. Havekes[1]

[1]IVVO-TNO Gaubius Laboratory, AK Leiden,
[2]Dept. of Biochemistry, and Internal Medicine, Rotterdam,
[3]Center for Bio-Pharmaceutical Sciences, Leiden,
[4]MGC-Dept. Human Genetics, Leiden,
The Netherlands

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Summary

We found that LPL enhances the binding to Hep G2 cells and fibroblasts of both VLDL and apoE free LDL. In the presence of 1.7 μg/ml of purified bovine LPL, the binding of LDL and VLDL was up to 60 fold increased as compared to the control binding. In addition, LPL enhances the binding in LDL-receptor negative fibroblasts to the same extent as it does in normal fibroblasts. The presence of 10 mM of EGTA could not prevent the LPL-mediated enhancement of the binding of both LDL and VLDL to fibroblasts, indicating that the binding is calcium independent. Furthermore, up- and down regulation of the LDL receptor, did not influence the binding of these lipoproteins in the presence of LPL.

Strikingly, we found that the enhancing effect of LPL on the binding of LDL and VLDL to Hep G2 cells could be abolished by preincubation of the cells with heparinase, suggesting that heparan sulphate proteoglycans are involved in the LPL-mediated stimulation. We hypothesize that the enhancement of the cellular binding of LDL and VLDL in the presence of LPL, is caused by an LPL-bridging between proteoglycans present on the plasma membrane and the lipoproteins, and that the LDL receptor and LRP are not involved.

Introduction

In the circulation triglyceride-rich lipoproteins like chylomicrons and VLDL are partly lipolysed through the action of endothelium-bound LPL. The resulting chylomicron- and VLDL remnants are rapidly taken up by hepatic receptors, mainly through their major protein constituent apolipoprotein E (apoE), which functions as a high affinity ligand. Liver cells possess two different types of lipoprotein receptors. One recognizing both apoB and apoE, designated as LDL receptor and another recognizing only apoE and designated as apoE or remnant receptor (1). Recently, the LDL receptor related protein (LRP) described by Herz et al. (2), turned out to be a potential candidate for this putative remnant receptor (3,4) and appeared to be structurally identical to the α2-macroglobulin receptor (5). The LRP has been found in several different cell types, including Hep G2 cells and LDL receptor negative fibroblasts (3,4).

It has been reported that chylomicron remnants are taken up exclusively through the remnant receptor (6), although the involvement of the LDL receptor in chylomicron-remnant clearance has been suggested as well (7). Uptake of VLDL and VLDL remnants by the liver is reported to be mediated through the action of LDL receptor, exclusively (8,9), although others found that also the remnant receptor is involved in the processing of these lipoproteins (10). Harkes et al. (11) showed that in the rat liver almost all β-VLDL is taken up via a putative remnant receptor on parenchymal cells that is most probably different from the LRP (12).

It is commonly assumed that lipolysis of chylomicrons and VLDL by LPL renders these particles to better ligands for both hepatic lipoprotein receptor. Recently, Beisiegel et al. (13) found that LPL strongly enhances the binding of apoE containing lipoproteins to Hep G2

cells and LDL receptor negative fibroblasts not only because of its lipolytic activity but mainly because of a stimulating effect of LPL on the interaction of apoE with LRP. Since *in vivo* the major part of circulating LPL is associated with LDL (14), we wondered whether the stimulatory effect of LPL on lipoprotein binding holds true also for the interaction of LDL with the LDL receptor. Indeed, the present study shows that LPL strongly stimulates the cellular binding of LDL but, strikingly, we found that the enhancement of the binding of both VLDL and LDL by LPL is caused by a LPL-mediated bridging between proteoglycans on the plasma membrane and the lipoprotein particles rather than by a stimulation of the binding of these lipoproteins to the LRP and/or LDL receptor.

Materials and Methods

Lipoproteins

Blood was obtained from healthy volunteers, after an overnight fast. Serum was separated from the cells by centrifugation at 500g for 15 min at room temperature. LDL, lipoproteins with density d < 1.019 g/ml (called VLDL in this paper) and heavy HDL (density 1.16-1.20 g/ml) were isolated by ultracentrifugation, using the procedure as previously described (8). Protein contents of the lipoprotein fractions were determined according to Lowry et al. (15). Total cholesterol, free cholesterol, triacylglycerols, and phospholipids were determined with enzymatic colorimetric assays (Boehringer Mannheim FRG, and Wako Chemicals GmbH, Neuss, FRG).

Labelling of lipoproteins

After isolation, the lipoproteins were iodinated immediately using the [^{125}I]iodine monochloride method described by Bilheimer et al. (16). After iodination the lipoproteins were dialyzed and stored exactly as described previously (8). The specific radio-activity ranged from 150 to 500 cpm/ng protein.

Lipoprotein lipase

Bovine LPL was isolated from skimmed milk as described by Tajima et al. (17).

Binding studies

Hep G2 cells, normal fibroblasts, and LDL-receptor negative fibroblasts were cultured as previously described (8). The latter were obtained from a patient with homozygous Familial Hypercholesterolemia (18). Twentyfour hours before the start of the experiment, DMEM supplemented with 1% HSA instead of FCS was added to the cells. The binding of [^{125}I]-LDL and [^{125}I]-VLDL to the cells after a 2.5 hour incubation at 0°C, was measured exactly as previously described (8).

Heparinase was obtained from Sigma Chemical Company, St. Louis, USA. Heparinase treatment was performed by incubating the cells 40 min at 37°C in the presence of 2.4 U/ml heparinase.

Recently, it has been reported that LPL dramatically increases the binding of apoE-containing lipoproteins to the LRP, not because of its lipolytic activity but most probably because of its structural properties (13). Normally, only a low amount of LPL circulates in plasma, mainly associated with lipoproteins. As *in vivo* the major part of circulating LPL was found to be associated with the LDL fraction (14), we questioned as to whether the stimulating effect of LPL on the cellular lipoprotein binding holds true for LDL as well.

In Fig. 1A it is shown that the binding of [^{125}I]-LDL to Hep G2 cells increases with increasing concentrations of LPL protein in the medium. This stimulation of the binding is irrespective of whether LPL is heat-inactivated or not. These results indicate therefore that the cellular binding of LDL is stimulated by the addition of LPL.

In addition, the LPL-mediated enhancement of the binding of LDL is of the same order of magnitude as found for VLDL (Fig. 1B). The stimulation of the binding of these lipoproteins is observed in Hep G2 cells, normal fibroblasts and in LDL receptor negative fibroblasts, the latter cell line providing evidence against the LDL receptor being involved in the LPL-mediated stimulation.

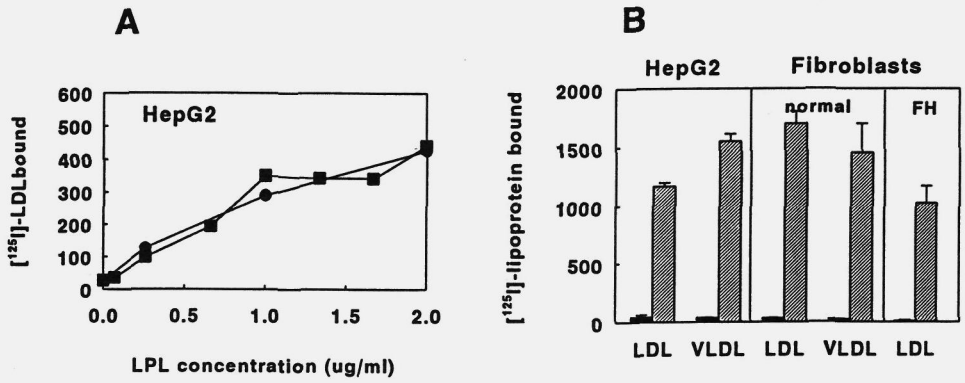

Figure 1. The effect of LPL on the binding of [^{125}I]-LDL or [^{125}I]-VLDL to Hep G2 cells, normal fibroblasts and LDL receptor negative fibroblasts. The binding (expressed as ng lipoprotein per mg of cell protein) was measured after incubation of cells for 2.5 hours at 0°C with 10 μg/ml of ^{125}I-labelled lipoproteins.

A. In the presence of different amounts of native LPL (squares) or LPL that had been inactivated by a 4 hour incubation at 50°C (dots).

B. In the presence (shaded bars) or in the absence (solid bars) of 1.7 μg/ml of LPL. The LDL receptor negative fibroblasts (FH) were obtained from a homozygous familial hypercholesterolemic (FH) patient. The specific binding was calculated by substracting from the total binding the binding in the presence of a 30 fold excess of unlabelled LDL. Each value represents the mean of three measurements.

Preincubation of Hep G2 cells and fibroblasts with LDL or heavy HDL resulted in the well known down- and upregulation of the LDL receptor activity, respectively (Table 1, (19)). On the contrary, preincubation of both cell types with either LDL or heavy HDL

(density 1.16-1.20 g/ml) hardly affected the binding of LDL and VLDL, if 1.7 μg/ml of LPL was added. This provides further evidence that the LDL receptor is not involved in the LPL-mediated stimulation of the binding of these lipoproteins.

By means of isoelectric focusing of 100 μg protein of LDL followed by immunoblotting, the presence of apoE could not be detected in any of the LDL samples used in these studies (results not shown). As it is postulated that LRP, within the apolipoproteins, only recognizes apoE (4), our results strongly suggest that the LRP is also not involved in this stimulating effect of LPL on the binding. Although a role for apoE in the uptake of the lipoproteins following the LPL-mediated binding can not definitely be excluded, as yet effects of secreted apoE as phospholipid discs (20) by Hep G2 cells on the LPL-mediated binding of lipoproteins are not very likely as: 1) LPL enhances the binding of LDL and VLDL not only to Hep G2 cells but also to normal fibroblasts and even to LDL receptor negative fibroblasts, to the same extent, and 2) in contrast to VLDL, LDL is supposed not to interact with apoE, whereas LPL enhances the binding of both LDL and VLDL.

Table 1. The binding of [^{125}I]-LDL and [^{125}I]-VLDL in the presence or in the absence of 1.7 μg/ml of LPL, under varying conditions.

			(pre)incubation with		(% of control binding)	
cell type	^{125}I-labelled *lipoproteins*	control	LDL	heavy *HDL*	EGTA	Heparinase
Hep G2 cells	LDL	100	55 ± 7	240 ± 80	34	94
	LDL + LPL	100	115 ± 6	89 ± 8	73	15
	VLDL	100	49 ± 7	137 ± 27	59	125
	VLDL + LPL	100	108 ± 4	93 ± 9	103	41
Fibroblasts	LDL	100	6 ± 4	-	22	90
	LDL + LPL	100	75 ± 10	-	85	35
	VLDL	100	24 ± 10	-	37	104
	VLDL + LPL	100	69 ± 11	-	89	50

The binding (expressed as % of the control binding) was measured after 2.5 hours of incubation of the cells at 0°C with 10 μg/ml of [^{125}]-labelled lipoproteins, in the presence of in the absence of 1.7 μg/ml of LPL, as indicated; Before the binding the cells were preincubated for 24 hours with medium containing 1% HSA (by weight) instead of FCS (control incubation, 100%); During the preincubation period either LDL (300 μg/ml) or heavy HDL (100 μg apoAI/ml); In case of the EGTA incubation, the binding experiment was performed in the presence of 10 mM EGTA; In case of heparinase treatment the cells were preincubated for 40 min at 37°C in the presence of 2.4 U/ml of heparinase. Just before the start of the experiment the cells were washed with DMEM containing 1% HSA. The binding of LDL or VLDL to the cells without any addition, and the binding of LDL or VLDL in the presence of 1.7 μg/ml of LPL, but without any further addition were taken as respective control values (100%).

As the binding of lipoproteins to the LDL receptor as well as to LRP is known to be calcium-dependent (21), we also tested the influence of EGTA on the binding in the absence

and in the presence of LPL (Table 1). In the absence of LPL, the binding of LDL and VLDL to both Hep G2 cells and fibroblasts is strongly inhibited by the presence of 10 mM EGTA, as expected (8). On the contrary, a significant influence of EGTA on the binding of these lipoproteins could not be found if the binding experiment was performed in the presence of LPL, indicating that the LPL-mediated binding of LDL and VLDL is almost completely calcium-independent. Again, these results imply that neither the LDL receptor nor the LRP are involved in the LPL-mediated enhancement of cellular binding of both LDL and VLDL.

The LPL-stimulated binding of LDL and VLDL is inhibited by the addition of an excess of unlabelled LDL to the medium, indicating that this binding represents high affinity binding. As our results clearly exclude the involvement of both lipoprotein receptors, we hypothesize that the LPL mediated stimulation of the lipoprotein binding occurs through the binding of LPL to its binding sites. It has been shown that the high affinity binding of LPL to heparan sulphate proteoglycans of plasma membranes, is inhibited by treatment of the cells with heparinase (22). We wondered whether the same holds true for the LPL-mediated lipoprotein binding. Table 1 shows that the stimulation of the binding by LPL of both LDL and VLDL, was strongly diminished after heparinase treatment of the cells. We conclude that the major part of the binding of LDL and VLDL in the presence of LPL is caused by an LPL-mediated bridging between heparan sulphate proteoglycans on the plasma membrane and the lipoproteins, rather than by a stimulation of the binding to LRP and/or LDL receptor. We hypothesize that the LPL-mediated binding of these lipoproteins might be an alternative pathway for lipoprotein uptake, that is of particular importance for patients with homozygous familial hypercholesterolemia. From the physiological point of view Hep G2 cells, as model for human hepatocytes, are the most interesting model for studying the effect of LPL on the uptake of lipoproteins, as *in vivo* LPL is very rapidly removed from the circulation by the liver. This possible physiological meaning of the suggested alternative pathway is currently under investigation.

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References

1. Brown, M.S., and Goldstein, J.L. (1983) J. Clin. Invest. 72, 743-747.
2. Herz, J., Hammann, U., Rogne, S., Myklebost, O., Gausepohl, H., and Stanley, K. (1988) EMBO J. 7, 4119-4127.
3. Beisiegel, U., Weber, W., Ihrke, G., Herz, J., and Stanley, K. (1989) Nature (London) 341, 162-164.
4. Kowal, R.C., Herz, J., Goldstein, J.L., Esser, V., and Brown, M.S. (1989) Proc. Natl. Acad. Sci. USA 86, 5810-5814.
5. Strickland, D.K., Ashcom, J.D., Williams, S., Burgess, W.H., Mirgliorini, M., and Argraves, W.S. (1990) J. Biol. Chem. 265, 17401-17404.

6. Kita, T., Goldstein, J.L., Brown, M.S., Watanabe, Y., Hornick, C.A., and Havel, R.J. (1982) Proc. Natl. Acad. Sci. USA 79, 3623-3627.
7. Windler, E., Greve, J., Daerr, W., and Greten, H. (1988) Biochem. J. 252, 553-561.
8. Mulder, M., De Wit, E., and Havekes, L.M. (1990). Biochim. Biophys. Acta 1081, 308-314.
9. Windler, E., Kovanen, P.T., Chao, Y.-S., Brown, M.S., Havel, J., and Goldstein, J.L. (1980) J. Biol. Chem. 255, 10464-10471.
10. Eisenberg, S., Friedman, G., and Vogel, T. (1988) Arteriosclerosis 8, 480-487.
11. Harkes, L., Van Duijne, A., and Van Berkel, T.J.C. (1989) Eur. J. Biochem. 180, 241-248.
12. Van Dijk, M.C.M., Ziere, G.J., Boers, W., Linthorst, C., Bijsterbosch, M.K., and Van Berkel, T.J.C. (1991) Biochem. J. 279, 863-870.
13. Beisiegel, U., Weber, W., and Bengtsson-Olivecrona, G. (1991) Proc. Natl. Acad. Sci. USA 88, 8342-8346.
14. Villeli, E., and Joven, J. (1991) Med. Sci. Res. 19, 111-112.
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
16. Bilheimer, D.W., Eisenberg, S., and Levy, R.I. (1972) Biochim. Biophys. Acta 260, 212-221.
17. Tajima, S., Yokoyama, S., and Yamamoto, A. (1984) J. Biol. Chem. 96, 1753-1767.
18. Lombardi, P., De Wit, E., Frants, R.R., and Havekes, L.M. (1990) Biochim. Biophys. Acta 1044, 127-132.
19. Havekes, L.M., Schouten, D., De Wit, E., Cohen, L.C., Griffioen, M., Van Hinsbergh, V.W.M., and Princen, H.M.G. (1986) Biochim. Biophys. Acta 875, 236-246.
20. Hamilton, R.L., Wong, J.S., Guo, L.S.S., Krisans, S., and Havel, R.J. (1990) J. Lipid Res. 31, 1589-1603.
21. Brown, M.S., Herz, J., Kowal, R.C., and Goldstein, J.L. (1991) Current Opinion in Lipidology 2, 65-72.
22. Cisar, L.A., Hoogewerf, A.J., Cupp, M., Rapport, C.A., and Bensadoun, A. (1989) J. Biol. Chem. 264, 1767-1774.

Chapter 7

LOW DENSITY LIPOPROTEIN RECEPTOR INTERNALISES LOW DENSITY AND VERY LOW DENSITY LIPOPROTEINS WHICH ARE BOUND TO HEPARAN SULPHATE PROTEOGLYCANS VIA LIPOPROTEIN LIPASE

Monique Mulder[1], Paola Lombardi[1], Hans Jansen[2], Theo J.C. van Berkel[3], Rune R. Frants[4] and Louis M. Havekes[1]

[1]IVVO-TNO Gaubius Laboratory, Leiden,
[2]Dept. of Biochemistry and Internal Medicine III, Rotterdam,
[3]Center for Bio-Pharmaceutical Sciences, Leiden,
[4]MGC-Dept. Human Genetics, Leiden,
The Netherlands

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Summary

It has previously been shown that lipoprotein lipase (LPL) enhances the binding of low density lipoproteins (LDL) and very low density lipoproteins (VLDL) to Hep G2 cells and fibroblasts, up to 80-fold. This increase in binding is LDL receptor-independent and is due to a bridging of LPL between extra-cellular heparan sulphate proteoglycans (HSPG) and the lipoproteins. In the present paper, we show that preincubation of the cells with LPL, followed by washing prior to the binding experiment, increased binding to the same extent as occurs when the binding is performed in the presence of LPL. This indicates that the formation of a complex of LPL with the lipoproteins is not a prerequisite of binding. Binding curves and Scatchard analyses reveal that both the number of binding sites and the affinity of the binding is increased 20- to 30-fold by the addition of 3.4 μg/ml of LPL. The addition of LPL also resulted in an enhanced uptake and subsequent lysosomal degradation of both LDL and VLDL when compared with binding, although to a lesser extent (up to 25-fold, when measured after 5 hours at 37°C). Strikingly, enhanced uptake did not occur in LDL receptor-negative fibroblasts. In addition, down-regulation of the LDL receptor activity by preincubation of the cells for 48 hours with either LDL or β-VLDL, resulted in a parallel decrease in the uptake of LPL-mediated HSPG-bound LDL, whereas the LPL-mediated binding itself was not diminished. These observations indicate that the uptake of LPL-mediated HSPG-bound LDL and VLDL mainly proceeds via the LDL receptor. Binding of labeled LDL to the cells at 4°C for two hours followed by a chase period at 37°C, revealed that, in absolute terms, the initial rate of internalisation of HSPG-bound LDL is comparable to that of LDL receptor-bound LDL (0.58 and 0.44 ng/minute/mg cell protein, respectively). We conclude that in LDL receptor-positive cells the LPL-mediated binding of LDL and VLDL to HSPG is followed by internalisation of the lipoproteins mainly through the rapid process of the classical LDL receptor recycling system, whereas only a minor portion is internalised via the much slower process of HSPG uptake.

Introduction

In the circulation, chylomicrons and very low density lipoproteins (VLDL) are partly lipolysed through the action of endothelium-bound lipoprotein lipase (LPL). The resulting chylomicron- and VLDL-remnants are rapidly taken up after binding to hepatic receptors, mainly through one of their major protein constituents apolipoprotein E (apoE). Liver cells possess two different types of lipoprotein receptors. One receptor recognizes both apoB and apoE and is designated B,E receptor or LDL receptor. The other receptor recognizes only apoE and is designated as apoE or remnant receptor (1). The LDL receptor related protein (LRP) described by Herz et al. (2) appeared to be a potential candidate for the remnant receptor (3,4) and was observed to be structurally identical to the α2-macroglobulin receptor (5). The LRP proved to be a multifunctional receptor. It is not yet certain whether the LRP actually is the remnant receptor.

Chylomicrons have been reported to be taken up exclusively through the remnant receptor (6), although the involvement of the LDL receptor in chylomicron remnant clearance has also been suggested (7). Uptake of VLDL and VLDL remnants by the liver is reported to be mediated exclusively through the LDL receptor (8,9), although others have found that the remnant receptor is also involved in the processing of these lipoproteins (10). Harkes et al. (11) and De Water et al. (12) have shown that in the rat liver almost all β-VLDL is taken up via a putative remnant receptor on parenchymal liver cells which is different from the liver α2-macroglobulin recognition site (13).

Recently, it has been found that the binding of chylomicrons and β-VLDL to either Hep G2 cells or LDL receptor-negative fibroblasts was strongly increased when bovine or human LPL was added to the medium (14). It has been suggested that the LPL protein stimulates the interaction of apoE with LRP. Recently, we found that the stimulating effect of LPL on lipoprotein binding also holds for apoE-free LDL (15).

In addition, we provided evidence that neither the LDL receptor nor the LRP is responsible for the LPL-mediated stimulation of the binding of LDL and VLDL. We found that the enhancing effect of LPL on the binding of these lipoproteins could be prevented by pre-incubating the cells with heparinase, which is known to prevent high affinity binding of LPL to heparan sulphate proteoglycans (HSPG) (16,17). This led us to conclude that the stimulation of the binding is caused by the bridging of LPL between proteoglycans present on the plasma membrane and the lipoproteins.

Recently, Williams et al. (18) have also reported that LPL enhances the binding of apoB100-rich lipoproteins, such as LDL and Lp(a), via binding to HSPG. They found that the LPL-mediated cell association of Lp(a) is completely LDL receptor-independent, whereas the subsequent degradation of this lipoprotein is partly LDL receptor-dependent. In the case of LDL and nascent apoB-containing lipoproteins, the LPL-mediated cell association and degradation appeared both to be independent of LDL receptor activity. Rumsey et al. (19) also reported that the LDL receptor is not involved in the LPL-mediated binding and uptake of LDL by both fibroblasts and THP-1 macrophages. With the results presented in this paper we obtained strong evidence that the LDL receptor is responsible for the major part of the uptake of (LPL-mediated) HSPG-bound LDL and VLDL, whereas only a minor part of HSPG-bound LDL and VLDL is directly internalized, thus without the LDL receptor. We also show that the rate of internalisation of HSPG-bound LDL via the LDL receptor is comparable to that of LDL which is directly bound to the LDL receptor.

Materials and methods

Lipoproteins

Blood was obtained from healthy volunteers, after an overnight fast. Serum was separated from the cells by centrifugation at 500 g for 15 min at room temperature. LDL (density 1.035-1.06 g/ml), VLDL (density d < 1.019 g/ml) and heavy HDL (density 1.16-1.20 g/ml) were isolated by ultracentrifugation, using the procedure as previously described (8).

β-VLDL was obtained from fasted serum of male Wistar rats that were maintained on a cholesterol-rich diet (Hope Farms, Woerden, the Netherlands) containing 2% cholesterol, 5% olive oil and 0.5% cholic acid. β-VLDL were isolated according to Redgrave (20) followed by a second identical centrifugation step.

Protein contents of the lipoprotein fractions were determined according to Lowry et al. (21). Total cholesterol, free cholesterol, triacylglycerols, and phospholipids were determined with enzymatic colorimetric assays (Boehringer Mannheim, Mannheim FRG, and Wako Chemicals GmbH, Neuss).

Labelling of lipoproteins

After isolation, the lipoproteins were immediately iodinated using the [^{125}I]iodine monochloride method described by Bilheimer et al. (22). After iodination the lipoproteins were dialysed and stored as described previously (8). The specific radio-activity ranged from 150 to 500 cpm/ng of protein.

Lipoprotein lipase

Bovine LPL was isolated from skimmed milk as described by Tajima et al. (23). Inactive LPL was obtained by incubation of the lipase for 4 hours at 50°C. Complete loss of activity of the enzyme was then checked using as substrate serum-activated [9,10-^{3}H] oleic acid-labelled trioleoyl-glycerol emulsified with phosphatidylcholine (24).

Binding studies

Hep G2 cells, normal fibroblasts, and LDL receptor-negative fibroblasts were cultured in 2 cm^2 multiwell dishes (Costar) using Dulbbeco's Modified Eagle's Medium (DMEM) containing 10% (v/v) foetal calf serum (FCS) as previously described (8). LDL receptor-negative fibroblasts were obtained from a patient with homozygous Familial Hypercholesterolemia (25). Twenty-four hours before the start of the experiment, DMEM supplemented with 1% (w/v) human serum albumin (HSA) instead of FCS was added to the cells. The binding of [^{125}I]-LDL and [^{125}I]-VLDL to the cells in the presence or in the absence of LPL was determined after a 2.5 hour incubation with 10 μg/ml of [^{125}I]-labelled lipoprotein at 0-4°C. After removing the medium the cells were washed five times with ice cold phosphate buffered saline (PBS) containing 1% (w/v) of bovine serum albumin (BSA), followed by one wash with PBS without BSA. Cells were then dissolved in 0.5 ml 0.2 N NaOH. Protein content was measured according to Lowry et al. (21). The radioactivity in an aliquot of the sample represents the binding.

To measure binding, intracellular-presence and degradation of lipoproteins separately, cells were incubated for 4 hours at 37°C with 10 μg [^{125}I]-LDL or [^{125}I]-VLDL per ml either in the presence or in the absence of LPL. At the end of the incubation the medium was removed for determination of lipoprotein degradation as described previously (8). The cells were then washed five times with ice cold PBS/BSA (0.1%, w/v), followed by one wash with PBS without BSA. The cells were then released from the culture dishes by incubation with trypsin (0.05%, w/v) in a 137 mM NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 5 mM D-Glucose,

0.02% EDTA buffer (pH 7.4) for 10 min at 37°C. The viability of the cells was checked, using trypan blue. Trypsin removes both cell-bound lipoproteins and cell-bound lipoprotein lipase (26). The cells were placed on ice to prevent further proteolysis, and then immediately centrifuged for 1 min at 13.000 g at 4°C. Radioactivity was determined in an aliquot of the supernatant, reflecting the binding of the labelled lipoproteins to the exterior of the cells. The cell pellet was resuspended in PBS and centrifuged for 5 min at 10.000 g. The pellet was dissolved in 0.5 ml 0.2 N NaOH. The radioactivity found in the pellet represents the amount of lipoprotein that is intracellularly present (trypsin-resistant). Protein was measured in an aliquot of the sample.

Treatment with heparinase (Sigma Chemical Company, St. Louis, MO, USA) was performed by incubating the cells at 37°C in the presence of 2.4 U/ml of heparinase.

Results

We have previously found that the LPL-mediated enhancement of the binding of LDL and VLDL occurs via bridging of LPL between heparan sulphate proteoglycans (HSPG) and lipoproteins, as it could be inhibited by pre-treatment of the Hep G2 cells with heparinase (15). In Fig. 1 it is shown that preincubation of Hep G2 cells with LPL for 1 hour at 4°C followed by washing, also results in an increase of the binding of LDL. This enhancement of the LDL-binding is similar to that found if the binding experiment is performed in the presence of LPL. Therefore, these results indicate that the complex formation between the lipoproteins and the lipase prior to the binding is not a prerequisite, and thus sustain the hypothesis that LPL forms a bridge between HSPG and lipoproteins. In Fig. 1A LPL concentrations in the μg/ml range are used. In Fig. 1B it is shown that the LPL-mediated binding of LDL is already evident at more physiological concentrations of LPL (ng/ml range).

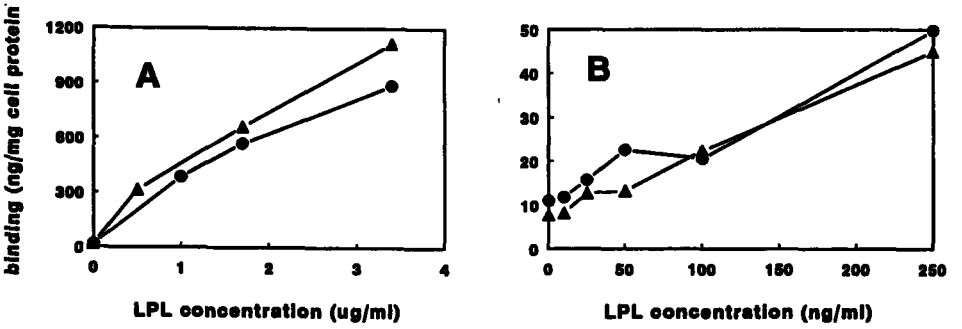

Figure 1. Effect of LPL on the binding of LDL. The binding experiment was performed either with Hep G2 cells in medium containing increasing concentrations of LPL (▲) or with Hep G2 cells that had been preincubated for a period of one hour with increasing concentrations of LPL at 0°C followed by washing in medium without LPL (●). Binding of ^{125}I-LDL was measured after 2.5 hours of incubation with 10 μg/ml of ^{125}I-LDL at 4°C, as described in Materials and Methods. Values are presented as the mean of three measurements. A: LPL concentrations in μg/ml range. B. LPL concentrations in ng/ml range.

Figure 2 shows the binding of increasing concentrations of LDL to Hep G2 cells, which had been preincubated with either medium alone or with medium supplemented with 1.7 μg/ml of LPL for a period of 1 hour at 0°C. The results show an about 20-fold higher maximum binding of LDL to the cells that had been preincubated with LPL. The scatchard plots, shown in the insert of the graphs, suggest a comparable increase of the binding affinity.

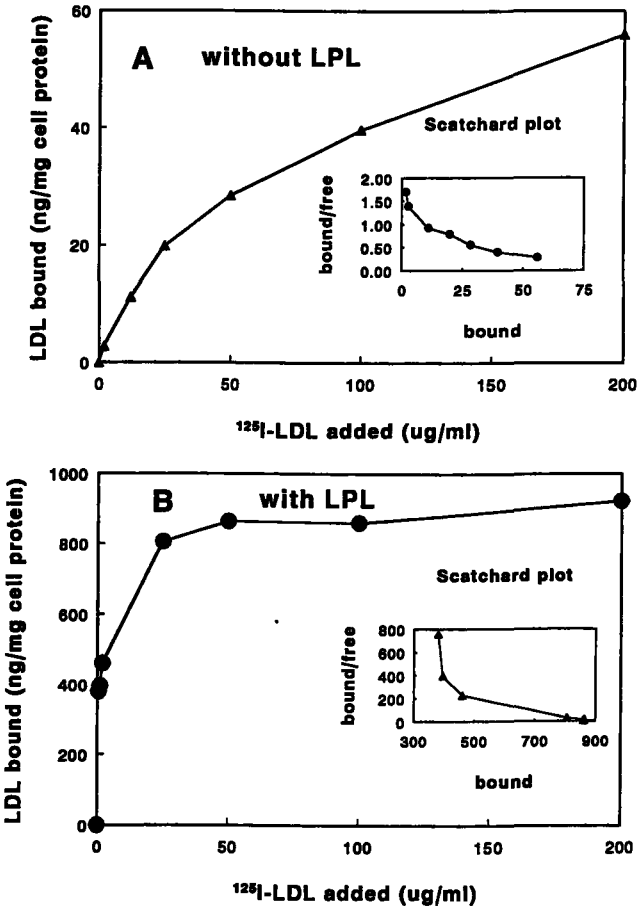

Figure 2. Binding curves of ^{125}I-LDL to Hep G2 cells preincubated with DMEM/HSA alone (A) or with DMEM/HSA supplemented with 1.7 μg/ml of LPL (B). The cells were preincubated for a period of 1 hour at 4°C in the presence of DMEM/HSA alone or DMEM/HSA supplemented with LPL. After three washes with DMEM/HSA the cells were incubated with increasing concentrations of ^{125}I-LDL at 4°C for a period of 2.5 hours. Binding was then measured as the amount of ^{125}I-LDL that became cell associated as described in Materials and Methods. Values are the mean of two measurements. Inserts represent the respective Scatchard analysis.

To investigate whether LPL also enhances the uptake of LDL and VLDL, we incubated Hep G2 cells with either ^{125}I-LDL or with ^{125}I-VLDL at 37°C either in the presence or in the absence of heat-inactivated LPL for a period of 4 hours. In Fig. 3 it is shown that, in the presence of heat-inactivated LPL, not only the binding of LDL and VLDL is enhanced (about 14-fold and 31-fold for LDL and VLDL, respectively) but also the internalisation (expressed as the amount of intracellular plus degraded lipoprotein) is increased, although to a lesser extent (6-fold and 23-fold, for LDL and VLDL, respectively). In our previous paper (15) we have shown that the major part of LPL-mediated binding is prevented by pre-treating the

cells with heparinase, indicating that the binding is mediated via HSPG. Figure 3 shows that, besides the inhibition of the LPL-mediated binding of LDL and VLDL, treatment of the cells with heparinase also resulted in inhibition of the LPL-mediated internalisation of both lipoproteins. These results indicate, therefore, that at least part of the lipoproteins which are bound via LPL to HSPG are subsequently internalised and degraded as well.

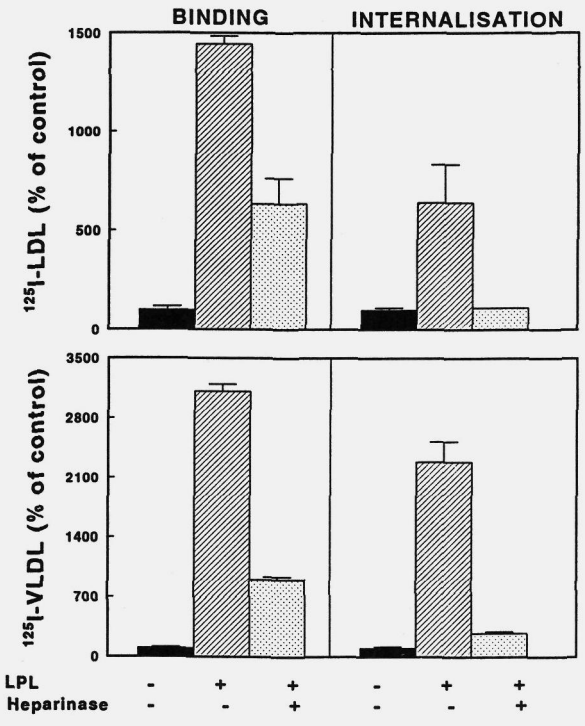

Figure 3. The effect of LPL and heparinase on the binding and internalisation of ^{125}I-LDL and ^{125}I-VLDL by Hep G2 cells. Lipoprotein binding and internalisation (expressed as intracellular plus degraded lipoprotein) was measured upon incubation of the cells with 10 μg/ml of the labelled lipoproteins at 37°C for a period of 4 hours, in the absence (solid bars, control values 100%) or in the presence (tightly dotted bars) of 3.4 μg/ml of heat-inactivated LPL. For the heparinase treatment, 2.4 U/ml of heparinase were present during the 4 hours of incubation of the cells with labelled lipoprotein in order to prevent regeneration of HSPG on the cell membrane during this incubation period (dotted bars). Binding and internalisation are expressed as a percentage of the control values (incubations in the absence of LPL), and were determined as described in Materials and Methods. Incubation with heparinase did not affect the control binding and internalisation. The values represent the mean ± standard deviation of four measurements.

As shown in Table 1, degradation of both LDL and VLDL is inhibited in the presence of 50 μM chloroquine to 24% and 36%, of the control value, respectively, when the experiment is performed in the absence of LPL, and to 32% and 38%, respectively, when performed in the presence of LPL. In the presence of 100 μM chloroquine the degradation of LDL and VLDL is further reduced to 10% and 15% in the absence of LPL, and to 14% and 18% in the presence of LPL. 10 mM NH_4Cl reduces the degradation of LDL and VLDL to less than 10%, irrespective of the presence or the absence of LPL. From these results we conclude that the (LPL-mediated) HSPG-bound LDL and VLDL are also taken up and directed to the lysosomes for degradation.

We wondered whether or not lipoprotein receptors such as the LDL receptor and/or the putative remnant-receptor are involved in the internalisation of LPL-mediated HSPG-bound LDL and VLDL. To answer this question, we first measured the binding and the internalisation of ^{125}I-LDL and ^{125}I-VLDL in the presence and in the absence of LPL in normal fibroblasts and in LDL receptor-negative fibroblasts. Figure 4 shows that, in normal

receptor-positive (Fig. 4A) and receptor-negative cells (Fig. 4B), the total amount of LDL and VLDL that is bound in the presence of LPL (hatched bars) is of the same order of magnitude. However, in contrast to the binding, the internalisation of LDL and VLDL in receptor-negative fibroblasts did not reach the same order of magnitude as that measured for receptor-positive fibroblasts. Thus, although the LPL-mediated binding of LDL and VLDL occurs via HSPG, the major part of the subsequent internalisation of these lipoproteins is mediated via the LDL receptor.

Table 1. The effect of chloroquine and ammonium chloride on the degradation of ^{125}I-LDL and ^{125}I-VLDL, in the presence and in the absence of 3.4 μg/ml of heat-inactivated LPL.

	incubation with			
^{125}I-labelled lipoproteins	no addition	chloroquine 50 μM	chloroquine 100 μM	NH_4Cl 10 mM
	% of control degradation			
LDL	100	24 ± 5	10 ± 3	2 ± 0.3
LDL + LPL	100	32 ± 1	14 ± 1	2 ± 0.1
VLDL	100	36 ± 9	15 ± 1	10 ± 0
VLDL + LPL	100	38 ± 5	18 ± 2	6 ± 0.2

Twenty four hours before the start of the experiment, cells were incubated with DMEM/HSA (1% w/v). Degradation was determined after 4 hours of incubation of the cells with 10 μg/ml of ^{125}I-labelled lipoproteins in the presence or in the absence of LPL at 37°C with DMEM/HSA alone, or DMEM/HSA supplemented with chloroquine or ammoniumchloride as indicated. The degradation of the lipoproteins by the cells without any addition, and the degradation of the lipoproteins in the presence of LPL but without any further addition were taken as respective control values (100%). Values given represent the mean ± standard deviation of four measurements. The absolute control values were in ng lipoprotein degraded/mg cell protein; LDL: 140; LDL + LPL: 302; VLDL: 65; VLDL + LPL: 300.

Further evidence for this statement is provided by the results presented in Fig. 5. Pre-incubation of Hep G2 cells with either 200 μg/ml of rat-β-VLDL or 300 μg/ml of human LDL resulted in down-regulation of the binding of ^{125}I-LDL to about 65% (Fig. 5A), whereas these conditions exerted an increase in the LPL-mediated binding to HSPG of about 1.5-fold (Fig. 5B). Pre-incubation of the cells with β-VLDL or LDL also resulted in a decreased internalisation of LDL receptor-bound ^{125}I-LDL (40 and 25% of the control value, respectively) (Fig. 5C). Strikingly, parallel results were obtained for the uptake of LPL-mediated HSPG-bound LDL (50 and 45% of the control value, respectively) (Fig. 5D). From these results we conclude that the LDL receptor is involved in the uptake of lipoproteins following the binding of these lipoproteins via a LPL-mediated bridging between lipoproteins and HSPG.

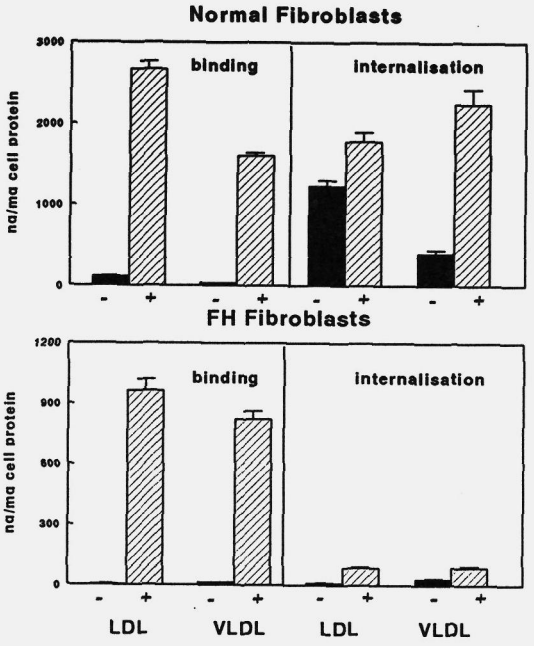

Figure 4. Effect of LPL on the binding and the internalisation of LDL and VLDL by normal fibroblasts and LDL receptor-negative fibroblasts. The cells were incubated for 4 hours at 37°C, in the presence of 10 μg/ml of ^{125}I-LDL and ^{125}I-VLDL without (solid bars) or with (hatched bars) the addition of 3.4 μg/ml of LPL. The presence of LPL is also indicated by "+" and "-" signs in the figure. Binding and internalisation are measured as described in Materials and Methods. The values represent the mean ± standard deviation of four measurements.

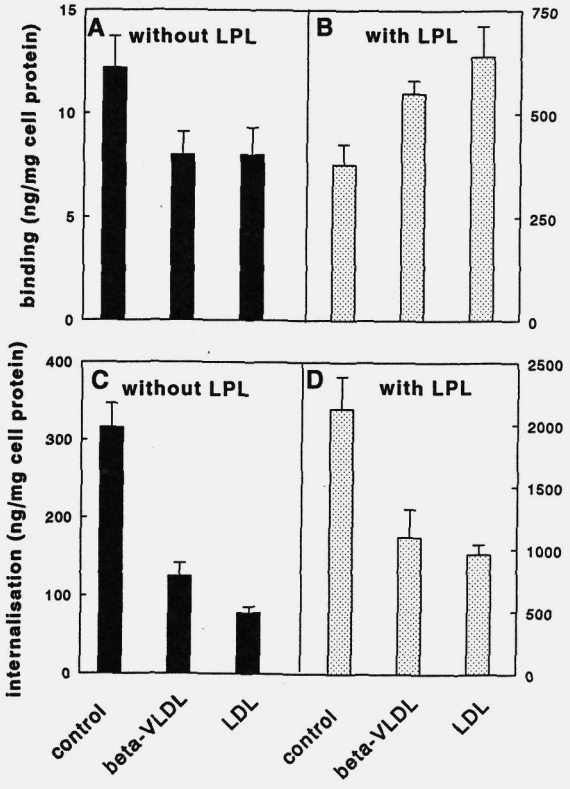

Figure 5. Effect of down-regulation of the LDL receptor on the binding and internalisation of ^{125}I-LDLin the absence and in the presence of LPL. Hep G2 cells were incubated in DMEM/HSA alone or DMEM/HSA supplemented with 200 μg/ml of rat β-VLDL or DMEM/HSA supplemented with 300 μg/ml of human LDL as indicated, at 37°C for a period of 24 hours. At the end of these incubations, the cells were washed three times with DMEM/HSA and incubated for 4 hours at 37°C with 10 μg/ml ^{125}I-LDL in the absence or in the presence of 3.4 μg/ml inactive LPL. Results are expressed as ng lipoprotein bound or internalised per mg cell protein. Values of binding and internalisation in the absence of LPL (5A and 5C) are indicated on the left Y-axis, and values obtained after incubation in the presence of LPL (5B and 5D) are indicated on the right Y-axis.

We wondered whether the rate of internalisation of HSPG-bound LDL is comparable to that of LDL receptor-bound LDL. To study this, the cells were first incubated with ^{125}I-LDL, either in the presence or in the absence of LPL, at 4°C for a period of 2 hours, followed by three washes at 4°C. To allow the lipoproteins to be internalised, the cells were then incubated at 37°C in medium, without any addition, for increasing periods of time. If expressed in absolute amounts of LDL internalised, it is obvious that the initial rate of internalisation of LPL-mediated HSPG-bound LDL is comparable to the internalisation rate of LDL receptor-bound LDL (0.58 versus 0.44 ng of LDL/minute/mg cell protein) (Fig. 6).

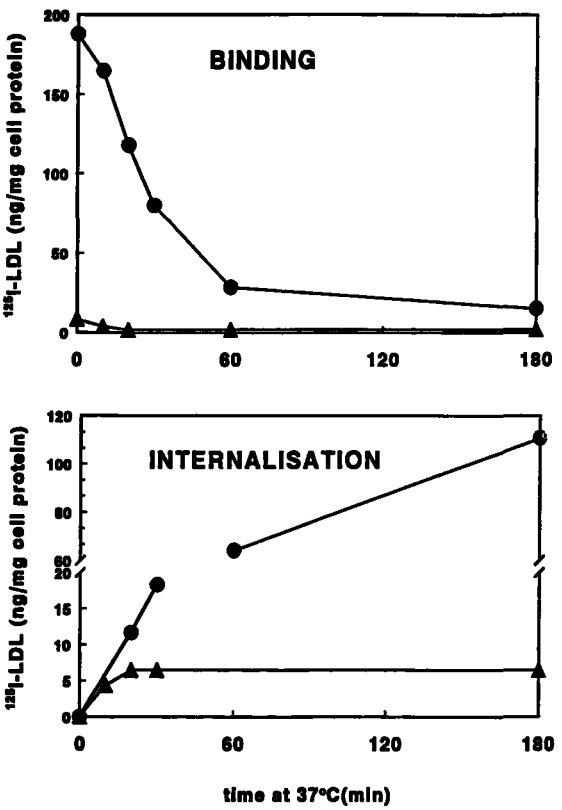

Figure 6. Rate of internalisation of ^{125}I-LDL bound either in the absence or in the presence of 3.4 μg/ml of LPL. The Hep G2 cells were incubated with 10 μg/ml of ^{125}I-LDL for 2 hours at 4°C either in the presence (●) or in the absence (▲) of 3.4 μg/ml of heat-inactivated LPL. At the end of this incubation period, the cells were washed three times with DMEM containing 1% (w/v) HSA. Cells were then further incubated at 37°C for increasing periods of time, as indicated, and the binding and internalisation were measured separately as described in Materials and Methods. Values are expressed as ng ^{125}I-LDL per mg cell protein.

Discussion

Previously, it has been reported that LPL, independently of its lipolytic activity, enhances the cellular binding of a number of lipoproteins, including chylomicrons, VLDL, chylomicron- and VLDL-remnants, β-VLDL, apoE-free LDL and HDL (14,15,27). We found that neither the LDL receptor nor the LRP is involved in the LPL-mediated binding of LDL and VLDL, but that binding occurs mainly through bridging of LPL between HSPG on the plasma membrane and lipoproteins (15). This result was confirmed recently by Williams et al. (18). Further evidence for this is provided by the observation that

preincubation of the cells with LPL followed by three washes resulted in the same increase in the binding of LDL as when the experiment was performed in the presence of the same amount of LPL (Fig. 1). The saturation curves shown in Fig. 2 indicate that the LPL-stimulated binding is due to an increase in the maximum binding and an increase in the binding affinity, of about 20-fold.

At 37°C most of the LPL-mediated binding and LPL-mediated internalisation of LDL and VLDL could be inhibited by heparinase, indicating that the LPL-mediated increase in both binding and internalisation are dependent on the presence of HSPG on the plasma membrane. Furthermore, our results show that the degradation of LDL and VLDL is lysosomal both in the absence and in the presence of LPL, which is similar to the results obtained by Williams et al. (18), with respect to LDL and Lp(a).

It has been suggested that the receptor involved in the LPL-mediated internalisation of lipoproteins may be the LRP (14). We believe however that an important role for the LRP in this respect can be excluded, as the binding of apoE-free LDL (15) and Lp(a) (18) is also enhanced by the presence of LPL, while the LRP is assumed to bind only apoE-containing lipoproteins. In addition, as already mentioned by Williams et al. (18), most of the LPL-mediated binding is abolished by heparinase or heparitinase, whereas the LRP is assumed to contain no heparan sulphate side chains.

Bihain et al. (28) have found that long chain free fatty acids rapidly increase, up to 50-fold, the uptake of LDL. It seems, however, unlikely that a significant part of the LPL-mediated binding of LDL and VLDL is dependent on this "lipolysis stimulated receptor" as at 4°C LPL displays little or no activity at all and because heat-inactivated LPL has also been found to increase the binding to a similar extent as native LPL (15).

In their experiments, Rumsey et al. (19) found that also with receptor-negative fibroblasts the incubation with LPL resulted in a dramatic stimulation of the uptake of LDL, thereby stating that LPL increases lipoprotein uptake via a pathway not involving the LDL receptor. Although less pronounced due to a shorter incubation time (4 instead of 8 hours) and lower LPL concentration used (3.4 instead of 10 μg/ml), we also show that in LDL receptor-negative fibroblasts the internalisation of LDL and VLDL is increased considerably upon incubation of the cells with LPL (Fig. 4). However, if expressed in absolute amounts of LDL taken up per mg of cell protein, our results show that the receptor-negative fibroblasts are much less efficient than control fibroblasts in LPL-mediated uptake of lipoproteins. This led us to conclude that the major portion of the (LPL-mediated) HSPG-bound lipoproteins is taken up via the LDL receptor, whereas, simultaneously, only a minor part of the LPL-mediated binding of LDL is internalised without the action of the LDL receptor. Rumsey et al. (19) were not able to draw this conclusion as from their results a comparison of LPL-mediated uptake by receptor-negative fibroblasts with that of control fibroblasts could not be made.

We found that the LPL-mediated binding of lipoproteins is not suppressed by preincubation of the cells with lipoproteins (Fig. 5). This is in fully agreement with the results reported by Williams et al. (18) and Rumsey et al. (19). However, we also found that, in contrast to the binding, the uptake of HSPG-bound LDL is suppressed parallelly to

the downregulation of the LDL receptor activity (Figs. 5C and 5D). This sustains our conclusion that the LDL receptor is indeed involved in the internalisation of LPL-mediated HSPG-bound lipoproteins. Similar results were obtained for the degradation of HSPG-bound Lp(a) by Williams et al. (18). However, they found that down-regulation of the LDL receptor in normal fibroblasts did not affect the degradation of LDL in the presence of LPL, suggesting that the subsequent uptake of HSPG-bound LDL is LDL-receptor independent. An explanation for the discrepancy between results obtained by Williams et al. (18) and our results might be that they measured total cell association, thus without discriminating between lipoproteins bound to the outer cell membrane and those that had been internalized.

As shown in Fig. 6, most of the LDL receptor-bound LDL has been internalised within 10 to 15 minutes, which is in accordance with the recycling time reported earlier for the classical LDL receptor (28). These results also indicate that the internalisation of HSPG-bound LDL, if expressed as ng of LDL/min/mg of cell protein, is as fast as the internalisation of LDL bound to the classical LDL receptor (in the absence of LPL). Similar experiments have been performed by Rumsey et al. (19). However, in contrast to our conclusion, Rumsey et al. (19) concluded that the uptake of HSPG-bound LDL is much slower than the uptake of LDL that is bound directly to the LDL receptor. Two facts may explain the discripancy between their conclusion and our conclusion regarding the rate of uptake of HSPG-bound LDL: (i) they used one hour as first sampling time point, whereas we used minutes for initial time intervals, which is in our opinion reasonable as the LDL receptor recycling time is also in the order of minutes (28); (ii) in their time course experiment, Rumsey et al. (19) expressed the rate of internalisation as "% of total radioactivity". We also found much slower internalisation when expressed as "% of total radioactivity". However our results clearly show that the internalisation of LPL-mediated HSPG-bound LDL is equally fast as that of LDL receptor-bound LDL, when expressed in absolute terms ("ng of LDL/min/mg of cell protein").

From the results presented in this paper, we propose the mechanisme for LPL-mediated uptake of LDL and VLDL as illustrated in Fig. 7: LPL enhances the binding of LDL and VLDL to cells by means of a bridging between the lipoproteins in the medium and HSPG on the plasma membrane. Thereafter, the HSPG-bound LDL and VLDL are internalised mainly via the rapid process of classical LDL receptor recycling system, if the LDL receptor is present. Simultaneously, the remaining portion of HSPG-bound lipoproteins is internalised together with HSPG, which is a much slower process with a half-life of about 7 hours (30). In LDL receptor-negative fibroblast the total amount of HSPG-bound lipoprotein is internalised via this slow process of HSPG uptake. The fact that in normal cells the uptake of LPL-mediated HSPG-bound LDL continues upto 60 minutes indicates that the LDL-receptor recycling system is saturated during 4 to 6 LDL receptor cycli and, consequently, the rate limiting step in this process.

The role of LPL in lipoprotein uptake *in vivo* is presently the subject of speculation. Williams at al. (18) suggest that LPL may serve as an atherogenic molecule in the arterial wall, by stimulating the uptake of apoB-rich lipoproteins by macrophages and smooth muscle cells, leading to foam cell formation. On the other hand, in the liver it would be anti-

atherogenic by enhancing uptake of apoB-rich atherogenic lipoproteins, such as VLDL-remnants, LDL and Lp(a). This possible dual function of LPL *in vivo* may thus relate to its location.

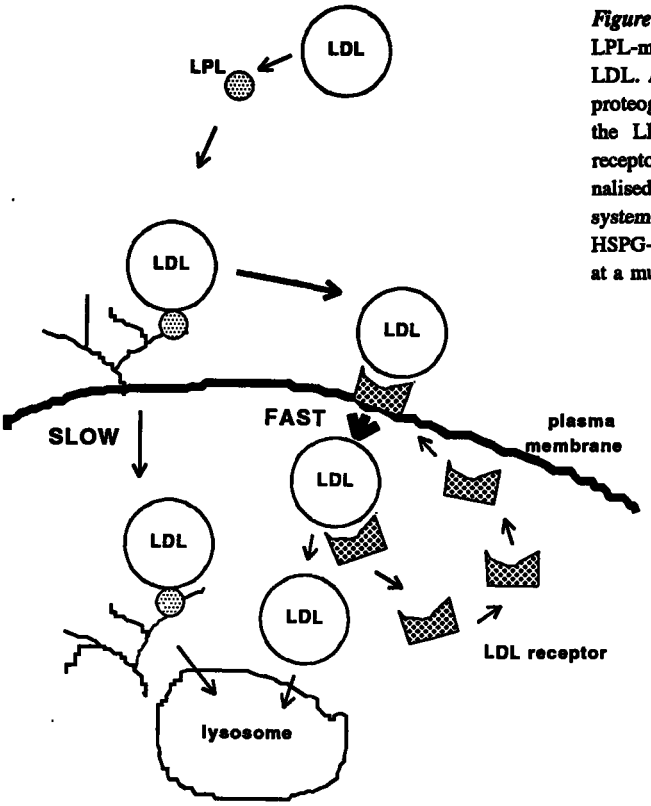

Figure 7. Proposed mechanism for LPL-mediated binding and uptake of LDL. After binding to heparan sulphate proteoglycans (HSPG), the major part of the LDL is transferred to the LDL receptor, whereafter it is rapidly internalised via the LDL receptor recycling system. Only a minor portion of the HSPG-bound LDL is taken up directly, at a much slower rate.

It has been reported that macrophages in atherosclerotic plaques synthesize LPL, which can be found anchored to their cell surface (31,32). This fact, together with the observation that *in vivo* macrophages synthesize HSPG depending on the amount of intracellular cholesterol-ester accumulation (30), strongly sustains the hypothesis that in the arterial wall LPL may, indeed, serve as an atherogenic factor.

The concentration of LPL in the circulation is normally kept low because of avid uptake in the liver (33). Although the lipase concentrations used in most of the present experiments are more than 100 times the physiological concentration that ranges between 8 and 25 ng/ml (34), we found that in the presence of 25 ng/ml of LPL the binding of LDL to Hep G2 cells also increased (about 1.5-fold, Fig. 1B). This sustains the hypothesis that *in vivo* LPL may affect lipoprotein binding. Whether this may also affect the lipoprotein catabolism *in vivo* remains subject to further investigation.

In vivo, most of the circulating LPL is associated with lipoproteins (34), mainly with LDL and HDL and, strikingly, not with VLDL or chylomicrons (35), suggesting a specific role for LPL in the directing LDL and HDL to the liver. Vilaro et al. (36) have shown that

exogenous LPL bound in the liver caused a dramatic increase in the utilisation of a perfused triacylglycerol emulsion. Possibly, LPL fulfils a metabolic role at its binding-site in the liver before it is degraded. Hepatic lipase which is present in the liver could also act in this way (37).

The relevance of our data, obtained with cells in culture, for the *in vivo* fate of lipoproteins is still unclear. We propose that LPL may accelerate the removal of the atherogenic LDL particles from the blood circulation and, simultaneously, stimulate the reverse cholesterol transport mediated by HDL. Studies are in progress to test these potential important implications for atherosclerosis.

Acknowledgements

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References

1. Brown, M.S., and Goldstein, J.L. (1983) *J. Clin. Invest.* **72**, 743-747
2. Herz, J., Hammann, U., Rogne, S., Myklebost, O., Gausepohl, H., and Stanley, K. (1988) *EMBO J.* **7**, 4119-4127
3. Beisiegel, U., Weber, W., Ihrke, G., Herz, J., and Stanley, K. (1989) *Nature (London)* **341**, 162-164
4. Kowal, R.C., Herz, J., Goldstein, J.L., Esser, V., and Brown, M.S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 5810-5814
5. Strickland, D.K., Ashcom, J.D., Williams, S., Burgess, W.H., Mirgliorini, M., and Argraves, W.S. (1990) *J. Biol. Chem.* **265**, 17401-17404
6. Kita, T., Goldstein, J.L., Brown, M.S., Watanabe, Y., Hornick, C.A., and Havel, R.J. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 3623-3627
7. Windler, E., Greve, J., Daerr, W., and Greten, H. (1988) *Biochem. J.* **252**, 553-561
8. Mulder, M., De Wit, E., and Havekes, L.M. (1990). *Biochim. Biophys. Acta* **1081**, 308-314
9. Windler, E., Kovanen, P.T., Chao, Y.-S., Brown, M.S., Havel, J., and Goldstein, J.L. (1980) *J. Biol. Chem.* **255**, 10464-10471
10. Eisenberg, S., Friedman, G., and Vogel, T. (1988) *Arteriosclerosis* **8**, 480-487
11. Harkes, L., Van Duijne, A., and Van Berkel, T.J.C. (1989) *Eur. J. Biochem.* **180**, 241-248
12 De Water, R., Kamps, J.A.A.M., Van Dijk, M.C.M., Hessels, E.M.A.J., Kuiper, J., Kruijt, J.K., and Van Berkel, T.J.C. (1992) *Biochem. J.* **281**, 62-88
13. Van Dijk, M.C.M., Ziere, G.J., Boers, W., Linthorst, C., Bijsterbosch, M.K., and Van Berkel, T.J.C. (1991) *Biochem. J.* **279**, 863-870
14. Beisiegel, U., Weber, W., and Bengtsson-Olivecrona, G. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8342-8346
15. Mulder, M., Lombardi, P., Jansen, H., van Berkel, T., Frants, R.R., and Havekes, L.M. (1992) *Biochem. Biophys. Res. Comm.* **185**, 582-587
16. Cisar, L.A., Hoogewerf, A.J., Cupp, M., Rapport, C.A., and Bensadoun, A. (1989) *J. Biol. Chem.* **264**, 1767-1774
17. Saxena, U., Klein, M.G., and Goldberg, I.J. (1991) *J. Biol. Chem.* **266**, 17516-17521
18. Williams, K.J., Fless, G.M., Snyder, M.L., Brocia, R.W., and Swenson, T.L. (1992) *J. Biol. Chem.* **267**, 13284-13292

19. Rumsey, S.C., Obunike, J.C., Arad, Y., Deckelbaum, R.J., and Goldberg, I.J. (1992) *J. Clin. Invest.* **90**, 1504-1512
20. Redgrave, T.G., Roberts, D.C.K., and West, C.E. (1975) *Anal. Biochem.* **65**, 42-49
21. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem.* **193**, 265-275
22. Bilheimer, D.W., Eisenberg, S., and Levy, R.I. (1972) *Biochim. Biophys. Acta* **260**, 212-221
23. Tajima, S., Yokoyama, S., and Yamamoto, A. (1984) *J. Biol. Chem.* **96**, 1753-1767
24 Nilsson-Ehle, P., and Schotz, M.C. (1976) *J. Lipid Res.* **17**, 536-541
25. Lombardi, P., De Wit, E., Frants, R.R., and Havekes, L.M. (1990) *Biochim. Biophys. Acta* **1044**, 127-132
26. Friedman, G., Chajek-Shaul, T., Olivecrona, T., Stein, O., and Stein, Y. (1988) *Biochim. Biophys. Acta* **711**, 114-122
27. Felts, J.M., Itakura, H., and Crane, R.T., (1975) *Biochem. Biophys. Res. Commun.* **66**, 1467-1475
28. Bihain, B., Deckelbaum, R.J., Yen, F. T., Gleeson, A.M., Carpentier, Y.A., and Witte, L.D. (1989) *J. Biol. Chem.* **264**, 17316-17321
29. Goldstein, J.L., Basu, S.K., Brunschede, G.Y., and Brown, M.S. (1976) *Cell* **7**, 85-90
30. Owens, R.T., and Wagner, W.D. (1991) *Arterioscler. Thrombos.* **11**, 1752-1758
31. O'Brien, K.D., Gordon, D., Deeb, S., Ferguson, M., and Chait, A. (1992) *J. Clin. Invest.* **89**, 1544-1550
32. Vijayagopal, P., Srinivasan, S.R., Radhakrishnamurthy, B., and Berenson, G.S. (1992) *Arterioscler. Thrombos.* **12**, 237-249
33. Olivecrona, T., and Bengtsson-Olivecrona, G. (1989) *Curr. Opin. Lipidol.* **1**, 116-122
34. Goldberg, I.J., Kandel, J.J., Blum, C.B., and Ginsberg, H.N. (1986) *J. Clin. Invest.* **78**, 1523-1528
35. Villeli, E., and Joven, J. (1991) *Med. Sci. Res.* **19**, 111-112
36. Vilaro, S., Lobera, M., Bengtsson-Olivecrona, G., and Olivecrona, T. (1988) *Am. J. Physiol.* **254**, G711-G722
37. Olivecrona, T., and Bengtsson-Olivecrona, G. (1990) *Curr. Opin. Lipidol.* **1**, 222-230

Chapter 8

THE *IN VIVO* EFFECT OF LIPOPROTEIN LIPASE ON THE CATABOLISM OF LOW DENSITY LIPOPROTEINS IN THE RAT

Monique Mulder[1], J. Kar Kruijt[2], Hans Jansen[3],
Rune R. Frants[4], Theo J.C. van Berkel[2], and Louis M. Havekes[1]

[1]IVVO-TNO Gaubius Laboratory, Leiden,
[2]Division of Biopharmaceutics, Center for Bio-Pharmaceutical Sciences, Leiden University, [3]Dept. of Biochemistry and Internal Medicine III, Erasmus University Rotterdam, [4]MGC-Dept. Human Genetics, Leiden,
The Netherlands

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Summary

In the present study we investigated the effect of lipoprotein lipase (LPL) on the *in vivo* fate of LDL in the rat. Simultaneous administration of increasing amounts of LPL together with ^{125}I-LDL to rats led to an enhanced association of ^{125}I-LDL with the liver, and was followed by subsequent release of LDL into the circulation again. Upregulation of the LDL receptor activity by treating the rats with oestradiol resulted in a markedly increased interaction of LDL with the liver, which could be further increased by simultaneous administration of LPL. The effect of LPL on the LDL-binding persisted for a longer period of time in the oestradiol-treated rats than in the control rats.

In vitro studies with freshly isolated rat liver endothelial-, parenchymal- and Kupffer cells showed that LPL enhances the interaction of LDL with all three cell types, although the magnitude of the effect of LPL on the binding of LDL to endothelial and Kupffer cells was five times higher than the effect on the binding to parenchymal cells. Only the LPL-mediated binding of ^{125}I-LDL to parenchymal cells could be inhibited by pretreating the cells with heparinase, indicating that in these cells heparan sulphate proteoglycans are involved. The LPL-mediated binding of ^{125}I-LDL to endothelial cells, Kupffer cells as well as the binding to parenchymal cells was calcium-independent. The LPL-mediated binding of ^{125}I-LDL to endothelial and Kupffer cells was inhibited completely by heparin, while that to parenchymal cells was lowered to about 10% of the maximal value.
These results indicate that LPL mediates the binding of ^{125}I-LDL to parenchymal cells mainly by attaching to heparan sulphate proteoglycans, while other proteoglycans structures or non-proteoglycan structures are involved in the LPL-mediated binding of LDL to endothelial- and Kupffer cells.

In vivo it appears that, in rats, LPL specifically enhances the association of ^{125}I-LDL to the liver. The lack of coupling to internalisation might allow further sinusoidal processing of LDL components.

Introduction

After being secreted into the circulation, chylomicrons and very low density lipoproteins (VLDL) are rapidly lipolyzed through the action of endothelium-bound lipoprotein lipase (LPL). Before being completely lipolysed the resulting chylomicron- and VLDL remnants are released into the blood. Subsequently, the remnants are rapidly removed from the circulation by the liver through the interaction of apolipoprotein E (apoE) with hepatic lipoprotein receptors (1).

Recently, it has been demonstrated that LPL, independently of its enzymatic activity, enhances the binding of several lipoproteins, such as chylomicrons, VLDL, β-VLDL, LDL, Lp(a) and to a lesser extent HDL, to cultured Hep G2 cells and fibroblasts (2-5). We have reported earlier that the LPL-mediated binding of LDL and VLDL to Hep G2 cells occurs

through bridging of LPL between the lipoproteins and heparan sulphate proteoglycans (HSPG) present on the cell membrane (6). This binding itself is independent of the LDL receptor and the LDL receptor related protein (LRP), while the increased internalisation of the lipoproteins occurs through the LDL receptor (5,7). Although a second, slow, internalisation route may utilize a LDL receptor-independent pathway (3,4).

Under physiological conditions, LPL is bound to heparan sulphate proteoglycans present on the surface of the endothelial cells lining the capillaries, where it is involved in the lipolysis of triglyceride-rich lipoproteins (8). Felts et al. (9) have suggested that after hydrolysis of the triglycerides, when the lipoprotein remnants detache from the endothelium, some LPL proteins might remain associated with the lipoprotein and function as a signal in directing the remnants to the liver. More recently, such a role for LPL in the catabolism of chylomicron remnants was suggested by Beisiegel et al. (2). It has been demonstrated that the LPL attached to the endothelial cells can be released by the injection of a triacylglycerol emulsion (10). Also, low concentrations of LPL have been detected in the circulation, and in the liver of mammals (11,12). According to data of Olivecrona et al. (8) a constant flow of LPL from the endothelium to the liver exists in order to regulate the amount of LPL, as endothelial cells themselves are not able to degrade LPL (13,14). Villeli et al. (15) reported that major part of the LPL that is present in the blood circulation is associated with LDL, and also with HDL. From the *in vitro* experiments with Hep G2 cells the question arose whether LPL could enhance the liver interaction of LDL *in vivo*. To study this, we administrated, to rats, ^{125}I-labelled LDL simultaneously with LPL. We found that LPL indeed enhances the association of ^{125}I-LDL to the liver. However, this LPL-mediated liver association of LDL was not followed by an enhanced internalisation.

Materials and Methods

Animals

Male Wistar rats of mass 225-300 g, fed *ad libitum* with regular chow, were used in this study. For determination of liver association and serum decay, rats were starved for 16 h. When indicated, 17α-ethinyloestradiol in propylene glycol at a dose of 5 mg/kg body weight (16) was injected subcutaneously every 24 h for 3 days. The experiment was performed 72 h after the first treatment.

Lipoproteins

Blood was collected from normal inidividuals after an overnight fast. Serum was separated from the red blood cells by centrifugation at 500 g for 10 min at room temperature. LDL were isolated according to Redgrave et al. (17). Protein contents of the lipoprotein fractions were determined according to Lowry et al. (18).

Labelling of lipoproteins

LDL was radioiodinated at pH 10 with carrier-free ^{125}I according to the ICl method of

Bilheimer et al. (19). Free ^{125}I was removed by Sephadex G-25 gel filtration, followed by dialysis against PBS/EDTA, pH 7.4, for 20 h at 4°C, with repeated changes of buffer.

Lipoprotein lipase

Bovine LPL was isolated from skimmed milk as described by Tajima et al. (20). Inactive LPL was obtained by incubation of the lipase for 4 hours at 50°C. Complete loss of activity of the enzyme was then confirmed, using as substrate serum-activated [9,10-^{3}H]oleic acid-labelled trioleoyl-glycerol emulsified with phosphatidylcholine (21).

Serum decay and liver association

Male Wistar rats were anaesthetized by intraperiotoneal injection of 15-20 mg of sodium pentobarbita, and the abdomen was opened. Radiolabelled ligands were injected via the inferior vena cava. At indicated times blood samples were taken from the inferior vena cava and allowed to clot for 30 min. The samples were centrifuged for 2 min at 16,000 x g, and radioactivity was determined in 100 μl serum. The total amount of radioactivity in the serum was calculated using the equation: serum volume (ml) = (0.0219 x body weight (g)) + 2.66 (22). At the indicated times, liver lobules were excised and weighted. The total amount of liver tissue tied off did not exceed 15% of the total liver mass. The radioactivity in the liver was corrected for the radioactivity in serum present in the tissue at the time of sampling (85 μl of serum/g wet weight (23)).

In vitro studies with freshly isolated parenchymal, endothelial and Kupffer cells

After the rats were anaesthetized, the liver parenchymal (PC), endothelial (EC) and Kupffer cells (KC) were isolated by differential centrifugation and counterflow elutriation as described in detail earlier (24). The liver cells were isolated by perfusion of the liver with 0.1% collagenase (type D) by the method of Seglen (25) modified as previously described (26). The PC, EC and KC were isolated by differential centrifugation and counterflow elutriation as described earlier (24). The PC, EC and KC obtained were resuspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 2% BSA (w/v), pH 7.4.

For studies *in vitro*, 2-3 mg of rat liver PC, EC or KC protein (> 95% viable, as judged by 0.2% Trypan Blue staining) was incubated for 2 h at 4°C, with 10 μg of ^{125}I-labelled lipoproteins/ml. During the incubation the air was saturated with carbogen (95% O_2/5% CO_2). At the end of the incubation the cells were washed three times with washing buffer (0.9% NaCl, 1 mM EDTA, 0.05 M Tris-HCl, 5 mM $CaCl_2$, 0.2% BSA, pH 7.4), and three times with washing buffer without BSA. Cells were lysed in 1 ml of 0.1 M- NaOH, and radio activity and protein content was determined. Competitor was dissolved in DMEM supplemented with 2% BSA, pH 7.4. Amounts of competitor were added as indicated.

Results

In Fig. 1A the effect of LPL on the association of ^{125}I-LDL to the rat liver *in vivo* is shown,

after intravenous administration to anaesthetized rats. When ^{125}I-LDL alone was given, about 3% of the injected dose was found to become associated with the liver at 3 minutes after injection. The simultaneous administration of LPL and ^{125}I-LDL increased the liver association of LDL in a concentration dependent manner. Administration of 7.5 μg of LPL resulted in an approximately 10-fold increase in the amount of LDL associated with the liver at 3 minutes after injection. The effect of LPL on the liver association of LDL was quantitatively reflected in the serum decay (Fig. 1B), indicating that LPL selectively acts upon the association of LDL with the liver.

After having reached the maximal liver association (within 3 minutes), the liver associated radioactivity decreased again, and at 15 minutes the effect of LPL had almost completely disappeared (Fig. 1A). For the reason that the radioactivity could be recovered in serum (1B), it can be concluded that the effect of LPL is not coupled to an increased internalisation of ^{125}I-LDL.

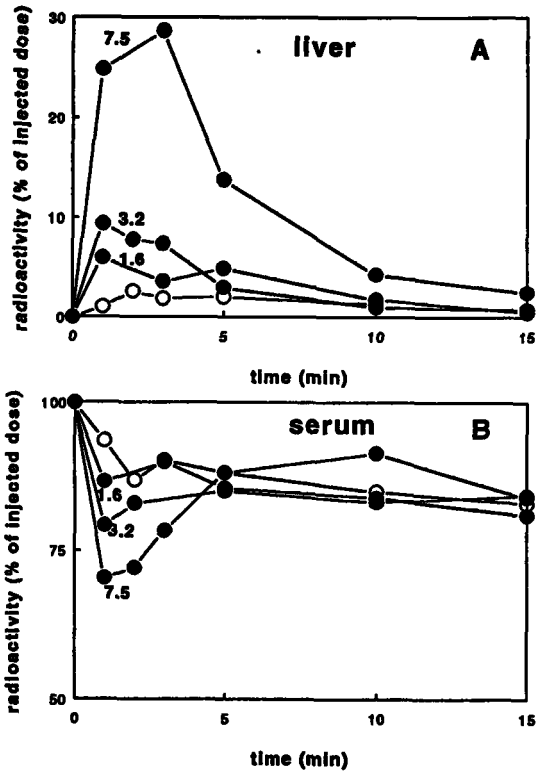

Figure 1. The effect of LPL on the liver association (A) and serum decay (B) of ^{125}I-labelled LDL. The liver association and serum decay of ^{125}I-labelled lipoprotein was determined after administration of 10 μg/ml of ^{125}I-LDL (○), or ^{125}I-LDL mixed with different amounts of LPL (1.6, 3.2, and 7.5 μg/ml; ●). Values for liver association are corrected for serum contribution.

Previously, we have reported that the majority of LDL that is bound via LPL to heparan sulphate proteoglycans, present on the cell surface of cultured Hep G2 cells and fibroblasts, are subsequently internalised through the LDL receptor (7). Under normal physiological conditions, the rat liver expresses only small numbers of LDL receptors (27). Thus, the absence of an enhancement *in vivo* of the internalisation of LDL by LPL, could be due to the absence of LDL receptors in the liver. It has been shown that treatment of rats with

oestradiol selectively induces LDL receptors on liver parenchymal cells (27). Figure 2 shows that the liver association of ^{125}I-LDL indeed does increase after oestradiol treatment. Under these conditions, the increase in the LPL-mediated liver association of ^{125}I-LDL in absolute terms is comparable with that observed in the untreated rats (Fig. 1A). The percentage of liver associated radiolabel at 60 minutes after injection, however, is not significantly different in the absence and in the presence of LPL, and also the serum radioactivity is identical (49 and 56% of the injected dose, in the absence and in the presence of LPL, respectively; data not shown).

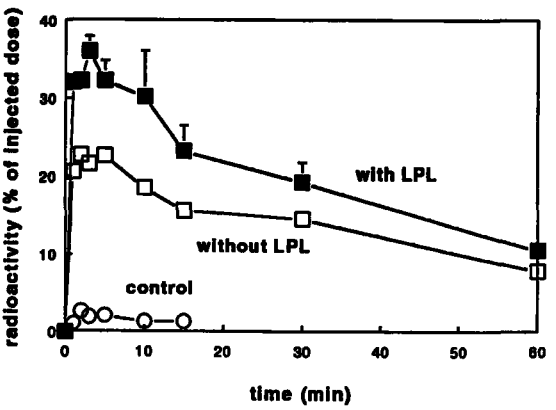

Figure 2. The effect of LPL on the liver association of ^{125}I-labelled LDL in 17α-ethinyl oestradiol-treated rats (□,■) and in control rats (○). The liver association of ^{125}I-LDL was determined after administration of either 10 μg of ^{125}I-LDL alone (□,○), or after administration of 10 μg of ^{125}I-LDL that had been mixed with 7.5 μg of LPL protein (■). Prior to the experiment, the rats were treated with oestradiol every 24 hours for a period of 3 days. The rats were injected subcutaneously with 17α-ethinyloestradiol in propylene glycol at a dose of 5 mg/kg body weight every 24 h (15).

In order to identify the particular liver cell types which could be responsible for the LPL-mediated liver association of LDL, we studied the effect of LPL on the binding of LDL to isolated liver parenchymal (PC), endothelial (EC) and Kupffer cells (KC). As shown in Fig. 3, *in vitro* LPL enhances the binding of ^{125}I-LDL to all three cell types. The procentual effect of LPL on the binding of LDL with endothelial cells and Kupffer cells was at least five times as high as with parenchymal cells (Fig. 3). As observed earlier in Hep G2 cells (7), the effect was dose dependent in all three cell types (not shown).

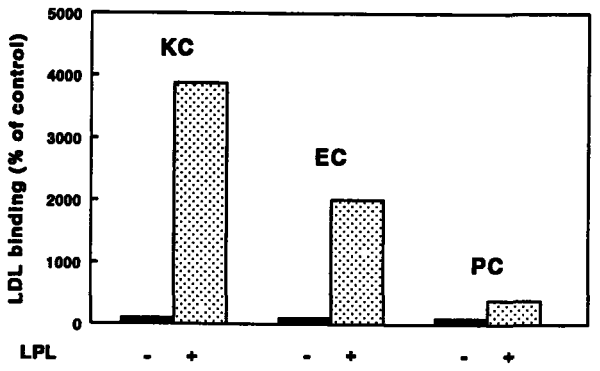

Figure 3. The effect of LPL on the binding of ^{125}I-LDL to isolated rat liver Kupffer cells (KC), endothelial cells (EC) and parenchymal cells (PC). Rat liver parenchymal, endothelial and Kupffer cells were incubated, for a period of 2 h at 4°C, with 10 μg/ml of ^{125}I-LDL in the presence or in the absence of 1.6 μg of LPL as indicated. The results represent the mean values of two experiments. Values obtained in the absence of LPL were taken as controls (100%).

Previously, we have shown that LPL enhances the binding of LDL to heparan sulphate proteoglycans and that this can be prevented by treating the cells with heparinase. Preincubation of the cells for 1 hour at 37°C with 2.4 U/ml of heparinase, partly inhibited the LPL-mediated binding of ^{125}I-LDL to the parenchymal cells, whereas the binding to the endothelial- or Kupffer cells was not affected at all by heparinase pretreatment (Fig. 4). These results indicate that heparan sulphate proteoglycans are involved in the LPL-mediated binding of ^{125}I-LDL to parenchymal cells but not to endothelial- and Kupffer cells. The properties of the LPL-mediated binding of ^{125}I-LDL to parenchymal-, endothelial-, and Kupffer cells were also compared with respect to calcium dependency, and the effect of heparin.

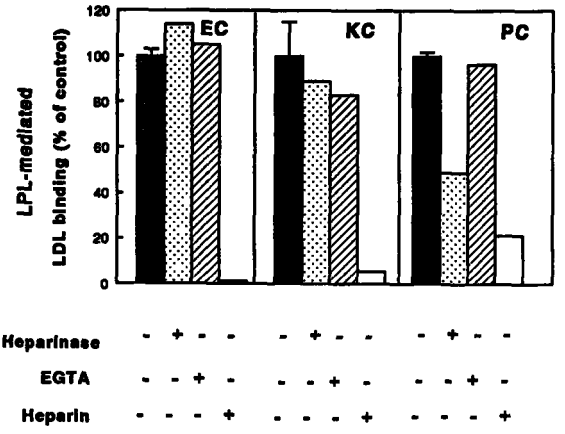

Figure 4. The effect of heparinase, EGTA, and heparin on the LPL-mediated binding of ^{125}I-LDL to isolated rat liver endothelial cells (EC), Kupffer cells (KC), and parenchymal cells (PC). The binding of ^{125}I-LDL to freshly isolated EC, KC and PC was determined after incubation of the cells for a period of 2 h at 4°C with 10 μg/ml of ^{125}I-LDL in the presence of 1.6 μg of LPL and in the presence or in the absence of either 10 mM of EGTA, 100 U of heparin, as indicated in the figure. For heparinase treatment, prior to the experiment the cells were incubated for a period of 45 min at 37°C in the presence of either medium alone or medium supplemented with 2.4 U heparinase/ml. Values represent means ± SD of three measurements.

The LPL-mediated binding of ^{125}I-LDL is, for all three cell types, calcium-independent, as it is not affected by the addition of EGTA. These results are in agreement with our earlier observations in Hep G2 cells. The LPL-mediated binding of LDL to endothelial and Kupffer cells and also most of the binding to parenchymal cells is largely inhibited in the presence of heparin.

Discussion

The effect of LPL on the binding and uptake of lipoproteins by cells in culture has been studied by various groups (2-7). However, its relevance for the metabolism *of lipoproteins in vivo* is uncertain. In the present paper we addressed the question whether LPL could influence the interaction of LDL with cells or tissues *in vivo*. Indeed, we found that the simultaneous administration of LPL with ^{125}I-LDL resulted in an apparent increase in the decay of LDL (Fig. 1B). By simultaneously analysing the liver-association, it could be

verified that this effect is caused by an increased liver association of LDL in the presence of LPL (Fig. 1A).

Previously, we have reported that after binding of LDL to HSPG present on the cell membrane of Hep G2 cells or fibroblasts, the lipoproteins are subsequently internalised mainly through the LDL receptor. Under normal physiological conditions, the rat liver displays only little LDL receptor-activity. Therefore, we analysed the possible increased liver uptake of ^{125}I-LDL that was bound through LPL to the liver, in control rats and in rats pretreated with oestradiol. Oestradiol treatment results in a selective upregulation of the number of LDL receptors in liver parenchymal cells (27). The enhancement of the liver association of ^{125}I-LDL by simultaneous injection of LPL in oestradiol-treated rats was comparable with that in the untreated animals if expressed in absolute terms. Both in control rats and after upregulation of the LDL receptor activity by oestradiol-treatment, LPL did not affect the serum concentration of ^{125}I-LDL at the later time points after injection, indicating that the catabolism of LDL is not affected by LPL. A possible explanation for the lack of an effect of LPL in the *in vivo* catabolism of LDL, might be caused by the intrahepatic cellular specificity of the effect of LPL. Oestradiol-treatment predominantly results in upregulation of the LDL receptor in the parenchymal cells (27). If *in vivo* LPL primarily affects the binding of LDL to endothelial- or Kupffer cells, the site for LDL uptake will reside at a different cell type. In agreement with this, we found that *in vitro* the increase in the binding of LDL as a result of the presence of LPL is about 5 times higher in endothelial and Kupffer cells than in parenchymal cells (Fig. 3).

When ^{125}I-labelled LPL is injected intravenously to rats, 40-60% is removed by binding to the liver cell surface during a single passage (14). Vilaro et al. (28) found that at least half of the LPL was localized at the surface of liver endothelial cells. The binding site present on these cells differed from the binding site present on the endothelium in other organs, as it was able to bind both active and inactive LPL.

We attempted to analyze the cellular localization of the binding sites for the ^{125}I-LDL/LPL complex. However, for the reason that LPL did not increase the internalisation of ^{125}I-LDL by liver cells, it was not possible to analyse the sites of uptake by separation of the liver cells after injection of the ^{125}I-LDL. Studies with freshly isolated rat liver parenchymal, endothelial and Kupffer cells revealed that LPL enhances the binding of ^{125}I-LDL to all three cell types (Fig. 3). The effect on parenchymal cells was much less pronounced than the effect on endothelial- and Kupffer cells. Only the LPL-mediated binding of LDL to parenchymal cells could be partly prevented by pretreatment of the cells with heparinase, indicating that HSPG play a role in the LPL-mediated binding of LDL to these cells. These results are in agreement with data obtained by Stow et al. (29), who have localized membrane HSPG, by immunocytochemistry, predominantly to the sinusoidal plasmalemmal domain of rat liver hepatocytes.

This left us with the question, if not HSPG, what binding sites could be involved in the LPL-mediated binding of LDL to the endothelial and Kupffer cells. As shown in Fig. 4, the LPL-mediated binding of LDL to parenchymal, endothelial and Kupffer cells was not affected by the presence of EGTA, indicating that this binding is calcium-independent. The

bindingsites involved, were found to be heparin sensitive, as the LPL-mediated binding of LDL was completely inhibited in the presence of heparin.

In conclusion, *in vivo* LPL enhances the association of LDL with the liver, in a concentration dependent fashion. However, no evidence for an increased internalisation could be obtained. As LDL remains extracellularly associated with the liver, we considered the possibility that the LPL-mediated binding of LDL to the liver might allow lipid exchange with the liver. However, so far we were not able to detect any such function. Vilaro et al. (28) have reported that exogenous LPL bound in the liver caused a dramatic increase in the utilization of a perfused triacylglycerol emulsion, with a rapid formation of free fatty acids and water-soluble metabolites. LDL is thought to be the end product of lipolysis, and it might be that LDL transfers the lipase to the liver, where it remains active untill it is finally taken up and degraded. This delivery to the liver, may prevent LPL for serving as an atherogenic molecule in the arterial wall, where it could stimulate the uptake of apoB-containing lipoproteins by macrophages and smooth muscle cells, leading to foamcell formation.

Acknowledgements

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References

1. Brown, M.S., and Goldstein, J.L. (1983) J. Clin. Invest. 72, 743-747
2. Beisiegel, U., Weber, W., and Bengtsson-Olivecrona, G. (1991) Proc. Natl. Acad. Sci. USA 88, 8342-8346
3. Williams, K.J., Fless, G.M., Snyder, M.L., Brocia, R.W., and Swenson, T.L. (1992) J. Biol. Chem. 267, 13284-13292
4. Rumsey, S.C., Obunike, J.C., Arad, Y., Deckelbaum, R.J., and Goldberg, I.J. (1992) J. Clin. Invest. 90, 1504-1512
5. Eisenberg, S., Sehayek, E., Olivecrona, T., and Vlodavsky, I. (1992) J. Clin. Invest. 90:2013-2021.
6. Mulder, M., Lombardi, P., Jansen, H., van Berkel, T., Frants, R.R., and Havekes, L.M. (1992) Biochem. Biophys. Res. Commun. 185, 582-587
7. Mulder, M., Lombardi, P., Jansen, H., van Berkel, Th.J.C., Frants, R.R., and Havekes, L.M. (1993) J. Biol. Chem. (in press)
8. Olivecrona, T., and Bengtsson-Olivecrona, G. (1990) Curr. Opin. Lipidol. 1, 222-230.
9. Felts, J.M., Itakura, H., and Crane, R.T. (1975) Biochem. Biophys. Res. Commun. 66, 1467-1475
10. Saxena, U., Witte, L.D., and Goldberg, I.J. (1989) J. Biol. Chem. 264, 4349-4355.
11. Knobler, H., Chajek-Shaul, T., Stein, O., Etienne, J., and Stein, Y. (1984) Biochim. Biophys. Acta 795, 363-371.
12. Peterson, J., Olivecrona, T., and Bengtsson-Olivecrona, G. (1985) Biochim. Biophys. Acta 837, 262-270.
13. Chajek-Shaul, T., Bengtsson-Olivecrona, G., Peterson, J. and Olivecrona, T. (1988) Am. J. Physiol. 255, E247-E254.
14. Wallinder, L., Peterson, J., Olivecrona, T., and Bengtsson-Olivecrona (1984) Biochim. Biophys. Acta 769, 513-524.

15. Villeli, E., and Joven, J. (1991) Med. Sci. Res. 19, 111-112.
16. Chao, Y.-S., Windler, E.E., Chen, G.C., and Havel, R.J. (1979) J. Biol. Chem. 254, 11360-11366.
17. Redgrave, T.G., Roberts, D.C.K., and West, C.E. (1975) Anal. Biochem. 65, 42-49.
18. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
19. Bilheimer, D.W., Eisenberg, S. and Levy, R.I. (1972) Biochim. Biophys. Acta 260, 212-221.
20. Tajima, S., Yokoyame, S., and Yamamoto, A. (1984) J. Biol. Chem. 96, 1753-1767.
21. Nilsson-Ehle, P., and Schotz, M.C. (1976) J. Lipid Res. 17, 536-541.
22. Bijsterbosch, M.K., Ziere, G.J., and van Berkel, Th.J.C. (1989) Mol. Pharmacol. 36, 484-489.
23. Caster, W.O., Simon, A.B., and Armstrong, W.D. (1955) Am. J. Physiol. 183, 317-321.
24. Van Berkel, Th.J.C., Dekker, C.J., Kruijt, J.K., and van Eijk, H.G. (1987) Biochem. J. 243, 715-722.
25. Seglen, P.O. (1976) Methods Cell Biol. 13, 29-83.
26. Casteleijn, E., van Rooij, H.C.J., van Berkel, Th.J.C., and Koster, J.F. (1986) FEBS Lett. 201, 193-197.
27. Harkes, L., and van Berkel, Th.J.C. (1983) FEBS Lett 154, 75-80.
28. Vilaro, S., Llobera, M., Bengtsson-Olivecrona, G., and Olivecrona, T. (1988) Am. J. Physiol. 254, G711-G722.
29. Stow, J.L., Kjellen, L., Unger, E., Hook, M., and Farquhar, M.G. (1985) J. Cell Biol. 100, 975-980.

Chapter 9

GENERAL DISCUSSION

The binding of lipoprotein lipase treated-VLDL and the intracellular routing of native and lipoprotein lipase treated-VLDL by Hep G2 cells

The nature of the hepatic lipoprotein receptor which mediates the uptake of VLDL remnants is disputed. Several observations suggest that VLDL and VLDL remnants are removed from the circulation by the chylomicron-remnant receptor [Harkes, 1989; Eisenberg, 1988; Cortner, 1987], while others find that the LDL receptor is responsible for the removal of these lipoproteins [Kita, 1982; Dashti, 1986; Huettinger, 1984]. We attempted to clarify the nature of the receptor involved in the binding of VLDL remnants (Chapter 2).

It was found that lipoprotein lipase-treated VLDL (LPL-VLDL), which serves as a model for VLDL-remnants, binds with high affinity to Hep G2 cells and that an increase in cholesterol/triglyceride ratio results in a gradual increase of the binding affinity. Factors responsible for this increase in binding affinity may either be the loss of apoC, which is known to inhibit the binding [Harkes, 1989; Windler, 1980], or conformational changes of apoE as a result of a different lipid composition of the particle [Bradley, 1984; Krul, 1985; Innerarity, 1986].

Competition experiments were performed in order to investigate whether a receptor other than the LDL receptor is involved in the binding of VLDL remnants. To this end we used, as unlabelled competitor, a high amount of unlabelled LDL (up to 30-fold) to ascertain maximal inhibition of LDL receptor-mediated association. From these competition experiments, we concluded that the binding of LPL-VLDL to Hep G2 cells was mediated exclusively by the LDL receptor and that no evidence could be obtained for the presence on Hep G2 cells, of an additional receptor, involved in the binding of LPL-VLDL. Preincubation experiments, with LDL, heavy HDL and insulin show that the binding of LPL-VLDL is regulated similarly to the binding of LDL. It has been reported that the apoE receptor is not influenced by interventions that affect the number of LDL receptors [Melchior, 1981; Thompson, 1983; Angelin, 1983; Arbeeny, 1984], so that this observation also argues against the presence of an additional receptor involved in the binding of LPL-VLDL in Hep G2 cells. Previous studies have shown that Hep G2 cells offer a suitable model system to study the metabolism of lipoproteins [Havekes, 1981, 1983, 1986]. However, whether the present data of LPL-VLDL uptake by Hep G2 cells holds true for the *in vivo* situation in the liver remains to be answered.

ApoE is thought to be the major determinant for regulating the metabolic fate of VLDL and VLDL remnant particles. In Chapter 3, it is shown that normal VLDL and lipolysed VLDL, taken as representative of VLDL remnants, once bound and taken up by the LDL receptor by Hep G2 cells, are poorly degraded as compared with LDL. This is due to the slower delivery of these particles to the late endosomal-lysosomal compartment. Similarly, VLDL and LPL-VLDL were found to be poor stimulators of the ACAT-activity, suggesting that the low amount of cholesterol liberated by the hydrolysis of LPL-VLDL and VLDL does not substantially lead to an increase in the cellular regulatory cholesterol pool [Xiang-Xi, 1991].

We suggest, as has recently been postulated for β-VLDL in mouse peritoneal macrophages [Tabas, 1991], that the polyvalent, high-affinity binding of VLDL and LPL-VLDL through apoE might be responsible for the observed retarded intracellular routing of these particles. One attractive hypothesis, concerning the mechanism behind this slower endocytic processing, is that the high-affinity polivalent ApoE binding to the LDL receptor produces a greater resistence to the acid-mediated release of the ligand from the receptor within the cell. If this is the case, the rate-limiting step would take place in the sorting endosomes, thus raising the question of the fate of the receptors bound to the ligand. Our data indicate that, though at a slower rate, a relevant portion of the internalised lipoprotein ends up in the lysosomes, presumably after being released from the receptor, which becomes available for recycling to the cell surface.

ApoE variants related to familial dysbetalipoproteinemia

Patients with familial dysbetalipoproteinemia (FD) mostly display E2E2 homozygosity and are characterized by an increased level of cholesterol in the chylomicron- and VLDL-remnant ($d < 1.019$ g/ml) lipoprotein fraction [Mahley, 1989]. The major underlying metabolic defect is a disturbance in the interaction of apoE2 with hepatic lipoprotein receptors.

The basic approach to dietary treatment of FD patients is to restrict caloric intake and to reduce cholesterol and saturated fat in the diet [10]. Previously, Innerarity et al. [1986] observed considerable weight loss in an E2E2 homozygous patient upon treatment by caloric restriction, concomitant with a dramatic reduction in the concentration of chylomicron- and VLDL-remnants. Strikingly, they found that this reduction is caused, at least partly, by an improved binding of the remnant lipoproteins to the LDL receptor. They hypothesized that this improved binding is the result of a conformational change of apoE2 due to a different micro-environment on the surface of the remnant particles. In a similar vein, Chappell and Lindgren [Chappell, 1989] found an increase of the binding affinity of $d < 1.006$ lipoproteins from three E2E2 homozygous FD patients upon significant reduction of the serum cholesterol concentration as a result of treatment with a low-calorie diet. We wondered whether the hypocholesterolemic effect of gemfibrozil in FD patients is also, at least in part, the result of an improved binding efficiency of VLDL and VLDL-remnant particles to the LDL receptor (Chapter 4). However, in our group of six E2E2 homozygous FD patients,

treatment with gemfibrozil does not lead to a consistent improvement in the binding of d < 1.019 lipoproteins to the LDL receptor as evaluated in *in vitro* binding experiments. Thus, our binding results are in contrast to the results obtained by Innerarity et al. in only one patient after severe dietary treatment. The reduction of the level of cholesterol in the d < 1.019 lipoprotein fraction did not lead to a significant reduction in the mean ratio of cholesterol to triglyceride in this lipoprotein fraction, nor did it affect plasma apoE levels. In addition, the d < 1.019 lipoproteins remained equally good substrates for LPL after treatment of patients with gemfibrozil. The absence of any effect of gemfibrozil treatment on these parameters confirms our observation that gemfibrozil has no effect on the ability of d < 1.019 lipoproteins to bind to the LDL receptor. The observation that in normolipidemic E2E2 homozygotes the clearance of chylomicron remnants is also delayed [Weintraub, 1987; Rubinsztein, 1990], supports our findings, and indicates that the defective binding of the d < 1.019 lipoprotein fraction from E2E2 homozygotes is not affected by plasma cholesterol levels. The reason for the discrepancy between our results and those of Innerarity et al. could be the difference in patients. In their experiment, treatment with caloric restriction was rather extreme and the patient underwent a considerable weight loss concomitant with a dramatic fall in plasma cholesterol level from severe hypercholesterolemia to hypocholesterolemia. Our patients were treated with gemfibrozil instead of caloric restriction; they did not lose weight and the reduction in plasma cholesterol level was much less dramatic. We conclude that, normalization of the serum cholesterol concentration in FD subjects by treatment with gemfibrozil does not consistently result in a change of the d < 1.019 lipoproteins in both lipid composition and the ability to bind to the LDL-receptor. Our results suggest, therefore, that gemfibrozil acts on the synthesis of VLDL rather than on its receptor-mediated clearance.

Heterozygosity for apoE2(lys146 → gln) is also associated with FD. In this case E2(lys146 → gln) behaves as a dominant trait in the expression of FD. Thus FD is expressed despite the presence of a normal apoE allele [Rall, 1983]. This is even more striking considering the fact that individuals heterozygous for apoE-deficiency, displaying less than half the normal amount of apoE in their plasma, have normal plasma lipoprotein levels and plasma lipoprotein distributions [de Knijff, 1991]. Thus, as FD cannot be caused by low concentrations of apoE in the plasma only, our results strongly suggest that the presence of the abnormal E2(lys146 → gln) variant itself is involved in the expression of FD. The studies described in Chapter 5 were performed to unravel the mechanism behind the dominant behaviour of apoE2(lys146 → gln) in the expression of FD.

The cholesterol to triglyceride ratio of the d < 1.019 lipoproteins of normolipidemic subjects increases upon treatment with LPL, leading to an enhanced binding efficiency to the LDL receptor [Mulder, 1991]. However, in the E2(lys146 → gln) heterozygous FD probands, and also in their relatives carrying the E2(lys146 → gln) allele, the d < 1.019 lipoproteins are less suitable as a substrate for LPL than the corresponding lipoproteins of their relatives who do not carry this apoE variant. This is particularly so when compared with the LPL-mediated lipolysis of the d < 1.019 lipoproteins of E2E2 homozygotes, which have previously been reported to be relatively resistant to lipolysis [Demant, 1991; Byung Hong

Chung, 1983; Ehnholm, 1984]. Therefore, in E2(lys146 → gln) allele carriers, the relative defect in the lipolysis of the d < 1.019 lipoproteins might be the direct cause of an increased level of cholesterol and triglyceride in this fraction.

ApoC2 is known to be an activator of LPL [for review see reference Saheki, 1991]. We found, however, that the d < 1.019 lipoprotein fraction of the E2(lys146 → gln) heterozygous FD probands, does contain a fairly normal amount of apoC2. Thus it is not apoC2 deficiency that renders these lipoproteins poor substrates for LPL.

It has been observed that the d < 1.019 lipoproteins of the E2(lys146 → gln) allele carriers contain a relatively high amount of apoE. We investigated the possibility that this amount of apoE2(lys146 → gln) present on the surface of triglyceride-rich lipoproteins might affect the susceptibility to LPL-mediated lipolysis. However, from the data obtained from the E2(lys146 → gln) allele carrying family members, a clear correlation between the apoE content of the d < 1.019 lipoprotein fraction and its susceptibility to lipolysis could not be detected. We conclude, therefore, that the dominant behaviour of apoE2(lys146 → gln) in the expression of FD is due to a retarded lipolysis of the d < 1.019 lipoprotein fraction, and, consequently, to a less efficient binding of these lipoproteins to the LDL receptor. The d < 1.019 lipoproteins displayed a large variation in their susceptibility to lipolysis, and the ability to interact with the LDL receptor also varied considerably. Therefore, we suggest that as well as the presence of the E2(lys146 → gln) allele, other, as yet unknown factors, are required to render relatively resistant to lipolysis the d < 1.019 lipoproteins in E2(lys146 → gln) allele carriers and, consequently, are poor ligands for interaction with the LDL receptor.

The effect of lipoprotein lipase on the processing of LDL and VLDL, in vitro and in vivo

It has been reported that LPL dramatically increases the cellular binding of apoE-containing lipoproteins, not because of its lipolytic activity but most probably because of its structural properties [Beisiegel, 1991]. Normally, only a small amount of LPL circulates in plasma, mainly in association with lipoproteins. *In vivo* the major part of circulating LPL was found to be associated with the LDL fraction [Villeli, 1991] *in vivo*, and we therefore questioned whether the stimulating effect of LPL on the cellular lipoprotein binding also holds true for LDL. Indeed, we found that the LPL-mediated enhancement of the cellular binding of LDL is of the same order of magnitude as that of VLDL. The stimulation of the binding of these lipoproteins is observed with Hep G2 cells, normal fibroblasts and with LDL receptor negative fibroblasts. The latter cell-line provides evidence against the possible involvement of the LDL receptor in the LPL-mediated stimulation of the binding. Further evidence for an LDL receptor-independent process was provided by the observation that modulation of the LDL receptor activity in Hep G2 cells and fibroblasts, did not affect the LPL-mediated binding of LDL and VLDL. The possibility of involvement of the LRP was excluded, because the LRP does not recognize apoE free LDL while the LRP requires calcium for its

binding, in contrast to the LPL-mediated binding of LDL and VLDL which was almost completely calcium-independent.

It has been shown that the high affinity binding of LPL to heparan sulphate proteoglycans of plasma membranes, is inhibited by treatment of the cells with heparinase [Cisar, 1989]. Since the same holds true for the LPL-mediated lipoprotein binding, we conclude that the major part of the binding of LDL and VLDL in the presence of LPL is caused by an LPL-mediated bridging between heparan sulphate proteoglycans on the plasma membrane and the lipoproteins, rather than by a stimulation of the binding to LRP and/or LDL receptor. This result was also confirmed by Williams and coworkers [1992]. At 37°C most of the LPL-mediated binding and LPL-mediated internalisation of LDL and VLDL could be inhibited by pretreatment of the cells with heparinase, indicating that the LPL-mediated internalisation is also dependent on the presence of heparan sulphate proteoglycans on the plasma membrane. Furthermore, our results show that the degradation of LDL and VLDL is lysosomal both in the absence and in the presence of LPL, which is similar to the results obtained by Williams et al. [1992], with respect to the LPL-mediated catabolism of LDL and Lp(a).

Rumsey et al. [1992] found that with receptor-negative fibroblasts also the addition of LPL resulted in a dramatic stimulation of the uptake of LDL, indicating that LPL increases lipoprotein uptake via a pathway which does not involve the LDL receptor. Although less pronounced, due to a shorter incubation time (4 instead of 8 hours) and a lower LPL concentration (3.4 instead of 10 μg/ml) we found that in LDL receptor-negative fibroblasts the internalisation of LDL and VLDL is also increased considerably upon incubation of the cells with LPL. However, if expressed in absolute amounts of LDL taken up per mg of cell protein, our data indicate that the receptor-negative fibroblasts are much less efficient than control fibroblasts in LPL-mediated uptake of lipoproteins. This led us to conclude that the major portion of the (LPL-mediated) heparan sulphate proteoglycan-bound lipoproteins are taken up via the LDL receptor, while only a small amount of the LPL-mediated binding of LDL is internalised independently of the LDL receptor. This statement is further sustained by the observation that the uptake of HSPG-bound LDL is suppressed in parallel with the down-regulation of the LDL receptor activity. Our results show that the internalisation of heparan sulphate proteoglycan-bound LDL, if expressed as ng of LDL/min/mg of cell protein, is as fast as the internalisation of LDL bound to the classical LDL receptor (in the absence of LPL).

From these studies, we conclude that LPL enhances the binding of LDL and VLDL to cells by bridging between the lipoproteins in the medium and HSPG on the plasma membrane. Thereafter, the HSPG-bound LDL and VLDL are internalised mainly via the rapid process of classical LDL receptor recycling system, if the LDL receptor is present. Simultaneously, the remaining portion of HSPG-bound lipoproteins may be internalised together at a much slower rate/or not with HSPG [Owens, 1991].

Although the lipase concentrations used in most of the *in vitro* experiments are more than 100 times the physiological concentration, we found that physiological concentrations of LPL also increase the binding of LDL to Hep G2 cells. This implies that LPL may also affect lipoprotein metabolism *in vivo*. As has been suggested by Williams at al., LPL may serve

as an atherogenic molecule in the arterial wall, by stimulating the uptake of apoB-rich lipoproteins by macrophages and smooth muscle cells, leading to foam cell formation. On the other hand, in the liver it would function anti-atherogenically by enhancing the uptake of apoB-rich atherogenic lipoproteins. The possible dual function of LPL *in vivo* may thus relate to its location.

In Chapter 8 the question is addressed whether LPL can influence the interaction of LDL with cells or tissues *in vivo* in the rat. Indeed, we found that the simultaneous administration of LPL with ^{125}I-LDL resulted in an apparent increase in the decay of LDL. By simultaneously analysing the liver-association, it could be verified that this effect is caused by an increased liver association of LDL in the presence of LPL. Under normal physiological conditions, the rat liver displays only little LDL receptor-activity. Therefore, the possible increased liver uptake of ^{125}I-LDL after its binding through LPL to the liver, was analysed in control rats and in rats pretreated with oestradiol. It is known that oestradiol treatment results in a selective upregulation of the number of LDL receptors in liver parenchymal cells [Harkes, 1983]. The enhancement of the liver association of ^{125}I-LDL by simultaneous injection of LPL in oestradiol-treated rats was comparable with that in the untreated animals if expressed in absolute terms. Both in control rats and after upregulation of the LDL receptor activity by oestradiol-treatment, LPL did not affect the serum concentration of ^{125}I-LDL at the later time points after injection, indicating that the catabolism of LDL is not affected by LPL. A possible explanation for the lack of an effect of LPL on the *in vivo* catabolism of LDL, might be caused by the intrahepatic cellular specificity of the effect of LPL. Oestradiol-treatment predominantly results in upregulation of the LDL receptor in the parenchymal cells [Harkes, 1983]. If *in vivo* LPL primarily affects the binding of LDL to endothelial- or Kupffer cells, the site for LDL uptake will reside at a different cell type. In agreement with this, we found that *in vitro* the increase in the binding of LDL as a result of the presence of LPL is about 5 times higher in endothelial and Kupffer cells than in parenchymal cells.

Studies with freshly isolated rat liver parenchymal, endothelial and Kupffer cells revealed that LPL enhances the binding of ^{125}I-LDL to all three cell types. The effect on parenchymal cells was much less pronounced than the effect on endothelial- and Kupffer cells. Only the LPL-mediated binding of LDL to parenchymal cells could be partly prevented by pretreatment of the cells with heparinase, indicating that HSPG play a role in the LPL-mediated binding of LDL to these cells. These results are in agreement with data obtained by Stow et al. [1985], who have localized membrane HSPG, by immunocytochemistry, predominantly to the sinusoidal plasmalemmal domain of rat liver hepatocytes.

This left us with the question, if not HSPG, what binding sites could be involved in the LPL-mediated binding of LDL to the endothelial and Kupffer cells. The LPL-mediated binding of LDL to parenchymal, endothelial and Kupffer cells was not affected by the presence of EGTA, indicating that this binding is calcium-independent. The binding sites involved, were found to be heparin sensitive, as the LPL-mediated binding of LDL was completely inhibited in the presence of heparin.

In conclusion, *in vivo* LPL enhances the association of LDL with the liver, in a

concentration dependent fashion. However, no evidence for an increased internalisation could be obtained. As LDL remains extracellularly associated with the liver, we considered the possibility that the LPL-mediated binding of LDL to the liver might allow lipid exchange with the liver. However, so far we were not able to detect any such function. Vilaro et al. [1988] have reported that exogenous LPL bound in the liver caused a dramatic increase in the utilization of a perfused triacylglycerol emulsion, with a rapid formation of free fatty acids and water-soluble metabolites. LDL is thought to be the end product of lipolysis, and it might be that LDL transfers the lipase to the liver, where it remains active untill it is finally taken up and degraded. This delivery to the liver, may prevent LPL for serving as an atherogenic molecule in the arterial wall, where it could stimulate the uptake of apoB-containing lipoproteins by macrophages and smooth muscle cells, leading to foam cell formation.

References

Angelin, B., Raviola, C.A., Innerarity, T.L., et al. (1983) J. Clin. Invest. 71, 816-831.

Arbeeny, C.M. and Rifici, V.A. (1984) J. Biol. Chem. 259, 9662-9666.

Beisiegel, U., Weber, W. and Bengtsson-Olivecrona, G. (1991) Proc. Natl. Acad. Sci. USA 88, 8342-8346.

Bradley, W.A., Hwang, S.-L.C., Karlin, J.B., Liu, A.H.Y., Prasad, S.C., Gotto, A.M. and Gianturco, S.H. (1984) J. Biol. Chem. 259, 14728-14735.

Byung, H.C. and Segrest, J.P. (1983) J. Lipid Res. 24, 1148-1159.

Chappell, D.A. and Lindgren, F.T. (1989) Arteriosclerosis 9, 728a.

Cisar, L.A., Hoogewerf, A.J., Cupp, M., Rapport, C.A. and Bensadoun, A. (1989) J. Biol. Chem. 264, 1767-1774.

Cortner, J.A., Coates, P.M., Le, N.-A., Cryer, O.R., Ragni, M.C., Faulkner, A. and Langer, T. (1987) J. Lipid Res. 28, 195-206.

Courtoy, P.J., Moguilevsky, N., Retegui, L.A., Castrancane, C.E. and Masson, P.L. (1984) Lab. Invest. 50, 329-334.

Dashti, N. and Wolfbauer, G. (1986) Biochim. Biophys. Acta 875, 473-486.

Demant, T., Bedford, D., Packard, C.J. and Shepherd, J. (1991) J. Clin. Invest 88, 1490-1501.

Van Dijk, M.C.M., Ziere, G.J. and van Berkel, T.J.C. (1992) Eur. J. Biochem. 205, 775-784.

Dorland, L., Haverkamp, B.L., Schut, B.L., Vliegenthart, J.F.G., Spik, G., Strecker, G., Fournet, B. and Montreuil, J. (1977) FEBS Lett. 77, 15-20.

Ehnholm, C., Mahley, R.W., Chappell, D.A., Weisgraber, K.H., Ludwig, E. and Witztum, J.L. (1984) Proc. Natl. Acad. Sci. USA 81, 5566-5570.

Eisenberg, S., Friedman, G. and Vogel, T. (1988) Arteriosclerosis 8, 480-487.

Goldberg, I.J., Kandel, J.J., Blum, C.B. and Ginsberg, H.N. (1986) J. Clin. Invest. 78, 1523-1528

Harkes, L. and Van Berkel, T.J.C. (1983) FEBS Lett 154, 75-80.

Harkes, L., Van Duijne, A. and Van Berkel, T.J.C. (1989) Eur. J. Biochem. 180, 241-248.

Havekes, L. (1981) Clin. Chim. Acta 116, 131-135.

Havekes, L., Van Hinsbergh V., Kempen, H.J. and Emeis, J. (1983) Biochem. J. 214, 951-958.

Havekes, L., Verboom, H., De Wit, E., Yap, S.H. and Princen, H.M.G. (1986) Hepatology 6, 1356-1360.

Huettinger, M., Corbett, J.R., Schneider, W.J., Willersar, J.T., Brown, M.S. and Goldstein, J.L. (1984) Proc. Natl. Acad. Sci. USA 81, 7594-7603.

Huettinger, M., Retzek, H., Hermann, M. and Goldenberg, H. (1992) J. Biol. Chem. 267, 18551-18557.

Innerarity, T.L., Hui, D.Y., Bersot, T.P. and Mahley, R.W. (1986) in Lipoprotein Deficiency Syndromes. (Angel, A. and Frohlich, J., eds.) pp. 273-288, Plenum Publishing, New York.

Kita, T., Goldstein, J.L., Brown, M.S., Watanabe, Y., Hornick, C.A. and Havel, R.J. (1982) Proc. Natl. Acad. Sci. USA 79, 5693-5697.

De Knijff, P., Van den Maagdenberg, A.M.J.M., Stalenhoef, A.F.H., Gevers Leuven, J.A., Demacker, P.N.M., Kuyt, L.P., Frants, R.R. and Havekes, L.M. (1991) J. Clin. Invest. 88, 643-655.

Krul, E.S., Tikkanen, M.J., Cole, T.G., Davie, J.M. and Schonfeld, G. (1985) J. Clin. Invest. 75, 361-369.

Mahley, R.W. and Rall, S.C. (1989) in The metabolic basis of inherited disease, 6th Edition. (Scriver C.R., Beaudet, A.L., Sly, W.S. and Valle, D., eds.) pp. 1195-1213, McGraw-Hill, New York.

McAbee, D.D. and Esbensen, K. (1991) J. Biol. Chem. 266, 23624-23631.

Melchior, G.W., Mahley, R.W. and Buckhold, D.K. (1981) J. Lipid Res. 22, 598-609.

Metz-Boutigue, M.-H., Jolles, J., Mazurier, J., Schoentgen, F., Legrand, D., Spik, G., Montreuil, J. and Jolles, P. (1984) Eur. J. Biochem. 145, 659-676.

Mulder, M., De Wit, E. and Havekes, L.M. (1991) Biochim. Biophys. Acta 1081, 308-314.

Owens, R.T. and Wagner, W.D. (1991) Arterioscler. Thrombos. 11, 1752-1758

Prieels, J.P., Pizzo, S.V., Glasgow, L.R., Paulson, J.C. and Hill, R.L. (1978) Proc. Natl. Acad. Sci. USA 75, 2215-2219.

Rall, S.C., Weisgraber K.H., Innerarity, T.L., Bersot T.P. and Mahley, R.W. (1983) J. Clin. Invest. 72, 1288-1297.

Rubinstein, D.C., Cohen, J.C., Berger, G.M. et al. (1990) J. Clin. Invest. 86, 1306-1312.

Rumsey, S.C., Obunike, J.C., Arad, Y., Deckelbaum, R.J. and Goldberg, I.J. (1992) J. Clin. Invest. 90, 1504-1512

Saheki, S., Takahashi, I., Murase, M., Takeuchi, N. and Uchida, K. (1991) Clin. Chim. Acta 204, 155-166.

Stow, J.L., Kjellen, L., Unger, E., Hook, M. and Farquhar, M.G. (1985) J. Cell Biol. 100, 975-980.

Tabas, I., Myers, J.N., Innerarity, T., Xiang-Xi, X., Arnold, K., Boyles, J. and Maxfield, F.R. (1991) J. Cell Biol. 115, 1547-1560.

Thompson, K.H. and Zilversmit, D.B. (1983) J. Nutr. 113, 2002-2010.

Vilaro, S., Llobera, M., Bengtsson-Olivecrona, G. and Olivecrona, T. (1988) Am. J. Physiol. 254, G711-G722.

Villeli, E. and Joven, J. (1991) Med. Sci. Res. 19, 111-112.

Wallinder, L., Peterson, J., Olivecrona, T. and Bengtsson-Olivecrona, G. (1984) Biochim. Biophys. Acta 769, 513-524.

Weintraub, M.S., Eisenberg, S. and Breslow, J.L. (1987) Eur. J. Clin. Invest. 80, 1571-1577.

Williams, K.J., Fless, G.M., Snyder, M.L., Brocia, R.W. and Swenson, T.L. (1992) J. Biol. Chem. 267, 13284-13292

Windler, E., Kovanen, P.T., Chao, Y.-S., et al. (1980) J. Biol. Chem. 255, 10464-10471.

Xu, X.X. and Tabas, I. (1991) J. Biol. Chem. 266, 1740-1748.

Ziere, G.J., Van Dijk, M.C.M., Bijsterbosch, M.K. and Van Berkel, T.J.C. (1992) J. Biol. Chem. 267, 11229-11235.

SUMMARY

VLDL are responsible for the transport of triglycerides from the liver to various tissues. After being secreted into the blood circulation, VLDL are lipolysed by the action of lipoprotein lipase (LPL), an enzyme attached to the endothelial cell lining of the capillaries. A large portion of the resulting VLDL remnants are rapidly removed from the circulation by the lipoprotein receptors present in the liver. The remainder are further converted into LDL. In the literature there is no consensus regarding the receptors involved in the removal of VLDL remnants by the liver. Hepatocytes, the liver cells responsible for the uptake of VLDL, possess not only the classical LDL receptor but also an apoE- or remnant receptor the nature of which has yet to be fully elucidated. To evaluate the hepatic receptor involved in the removal of VLDL remnants, we studied the binding of LPL-treated VLDL (LPL-VLDL), taken as representative for VLDL remnants, to Hep G2 cells (Chapter 2). We found that LPL-VLDL binds with high affinity to Hep G2 cells. Up- and down-regulation of the LDL receptor resulted in a stimulation and inhibition of the binding of LPL-VLDL. The results indicate that the binding of LPL-VLDL to Hep G2 cells is completely mediated by the LDL-receptor, and there was no evidence for the presence of an additional receptor involved in the binding of VLDL-remnants.

The intracellular pathway coupled to the binding of LPL-VLDL and VLDL to Hep G2 cells was compared with that of LDL, in order to verify whether ApoE present on LPL-VLDL and VLDL does indeed influence the endocytic routing of these particles (Chapter 3). It appears that after internalization through the LDL receptor, the transport of VLDL, as well as LPL-treated VLDL, to the late endosomal/lysosomal compartment is severely retarded as compared with that of LDL. As a consequence of this impaired transport to the lysosomes, VLDL and LPL-treated VLDL fail to stimulate ACAT activity. Therefore, the multivalent binding of ApoE in LPL-VLDL and VLDL to the LDL receptor might lead to a diminished or retarded release of LPL-VLDL and VLDL from the receptor in the sorting endosomes, which explains the retarded intracellular processing.

Since apoE is responsible for the receptor binding of VLDL and VLDL-remnants, a defect in apoE will result in impaired clearance of VLDL and VLDL remnants from the blood. This is the case in patients with Familial Dysbetalipoproteinemia (FD). It has been reported that the binding of apoE2 isolated from an E2E2 homozygous FD patient improved after dramatic reduction of the serum cholesterol concentration as a result of dietary treatment. Chapter 4 describes a study in which six E2E2 homozygous Familial Dysbetalipoproteinemic (FD) patients were treated with gemfibrozil (2*600 mg/day) for a period of four weeks. As a result of this treatment, serum cholesterol concentrations normalized in all six patients. In our study both the composition of the VLDL (cholesterol/triglyceride ratio) and the binding effeciency to the LDL receptor did not change upon treatment. Therefore, we suggest that gemfibrozil lowers serum cholesterol as the result

of affecting the synthesis of d < 1.019 lipoproteins rather than as a result of influencing the receptor-mediated clearance of these particles.

FD is not always associated with E2E2 homozygosity. Heterozygosity for E2(lys146 → gln) is also frequently associated with FD. E2(lys146 → gln) heterozygotes develop FD despite the presence of a normal apoE allele, indicating that it is a dominant trait in the expression of FD. The studies presented in Chapter 5 were performed in order to clarify the mechanism behind the dominancy of this apoE variant. In six families displaying the E2(lys146 → gln) variant, we found that the LPL-mediated lipolysis of the d < 1.019 lipoproteins of E2(lys146 → gln) allele carriers was significantly less efficient as compared with that of their relatives not carrying this apoE variant. Upon treatment with LPL, the cholesterol to triglyceride ratio of the d < 1.019 lipoproteins of the E2(lys146 → gln) carriers increased from 1.1 to 1.8, while in the controls this ratio increased from 0.7 to 1.6. In addition, high amounts of apoE per lipoprotein were observed in the d < 1.019 fraction of the carriers (five times as high as in the controls). This high amount of apoE present on the lipoproteins could not be related to the susceptibility of the lipoproteins to lipolysis. Neither could the lipolysis efficiency be related to the amount of free cholesterol per lipoprotein particle. From these studies, we concluded that the E2(lys146 → gln) allele, under certain conditions, predisposes to an impaired lipolysis of the d < 1.019 lipoprotein fraction, which consequently results in a defective binding of these lipoproteins to the LDL receptor. The impaired lipolysis could in such a way contribute to the dominant behaviour of the E2(lys146 → gln) variant in the expression of FD.

Earlier, it has been mentioned that VLDL bind more efficiently to the LDL receptor after being lipolysed by the action of LPL. It has also been reported that LPL, independent of its lipolytic activity, enhances the cellular binding of apoE-containing lipoproteins. We have studied the effect of LPL on the cellular binding and the subsequent processing of VLDL and LDL, in cultured Hep G2 cells and normal and LDL receptor-negative fibroblasts (Chapter 6 and 7). The presence of 1.7 μg/ml of LPL dramatically (up to 80-fold) enhances the binding of both VLDL and apoE free LDL to Hep G2 cells, and to normal and LDL receptor-negative fibroblasts. We found that the enhancement of the cellular binding of these lipoproteins in the presence of LPL was independent of the LDL receptor and the LRP, as the binding was calcium-independent and was not affected by up- and down-regulation of the LDL receptor activity. The LPL-mediated binding could be prevented by pretreatment of the cells with heparinase, indicating that heparan sulphate proteoglycans were involved. From these results we conclude that the LPL-mediated binding of LDL and VLDL is caused by a bridging of LPL between heparan sulphate proteoglycans present on the plasma membrane and the lipoproteins (Chapter 6). As described in Chapter 7, the formation of a complex of LPL with the lipoproteins is not a prerequisite of binding, as preincubation of the cells with LPL, followed by washing, prior to the binding experiment, increased the lipoprotein-binding to the same extent. The addition of heat-inactivated LPL also resulted in an enhanced uptake and subsequent lysosomal degradation of both LDL and VLDL, although the stimulatory factor was less than for the binding (25-fold, when measured after 5 hours at 37°C). Strikingly, LPL only marginally enhanced the uptake of LDL by LDL receptor-negative

fibroblasts. In addition, down-regulation of the LDL receptor activity of Hep G2 cells and fibroblasts resulted in a parallel decrease in the uptake of lipoproteins bound via LPL to heparan sulphate proteoglycans. From these results, we conclude that in LDL receptor-positive cells the LPL-mediated binding of LDL and VLDL to heparan sulphate proteoglycans was followed by internalisation of the lipoproteins mainly through the rapid process of the classical LDL receptor recycling system, whereas only a minor portion was internalised via the much slower process of HSPG uptake.

From the studies with cultured Hep G2 cells and fibroblasts, the question arose as to what the physiological consequences of the enhancing effect of LPL on the lipoprotein catabolism might be. The effect of LPL on the *in vivo* fate of LDL in the rat was studied (Chapter 8). We found that, *in vivo*, LPL enhances the association of LDL with the liver in a concentration dependent fashion. However, no evidence for an increased internalisation could be obtained. Upregulation of the LDL receptor activity by treating the rats with oestradiol resulted in a markedly increased interaction of LDL with the liver, which could be further increased by simultaneous administration of LPL.

In vitro studies with isolated rat liver endothelial-, parenchymal- and Kupffer cells showed that LPL enhances the interaction of LDL with all three cell types, although the magnitude of the effect of LPL on the binding of LDL to endothelial and Kupffer cells was five times higher than the effect on the binding to parenchymal cells. Only the LPL-mediated binding of ^{125}I-LDL to parenchymal cells could be inhibited by pretreating the cells with heparinase, indicating that in these cells heparan sulphate proteoglycans are involved. The LPL-mediated binding of ^{125}I-LDL to endothelial cells, Kupffer cells as well as the binding to parenchymal cells was calcium-independent. The LPL-mediated binding of ^{125}I-LDL to endothelial and Kupffer cells was inhibited completely by heparin, while that to parenchymal cells was lowered to about 10% of the maximal value.

These results indicate that LPL mediates the binding of ^{125}I-LDL to parenchymal cells mainly by attaching to heparan sulphate proteoglycans, while other proteoglycans structures or non-proteoglycan structures are involved in the LPL-mediated binding of LDL to endothelial- and Kupffer cells.

In vivo it appears that, in rats, LPL specifically enhances the association of ^{125}I-LDL to the liver. The lack of coupling to internalisation might allow further sinusoidal processing of LDL components.

SAMENVATTING

Voor het transport van in de lever gesynthetiseerde triglyceriden zijn zeer lage dichtheidslipoproteïnen (VLDL) verantwoordelijk. In de bloedcirculatie worden de triglyceriden in de VLDL gehydrolyseerd met behulp van lipoproteïne lipase (LPL), wat zich bevindt op het oppervlak van endotheelcellen die de bloedvaten bekleden. Het grootste deel van de op deze wijze gevormde VLDL-remnants (overblijfsels) worden vlug opgenomen door voornamelijk de levercellen. De overige remnants worden verder omgezet in lage dichtheids lipoproteïnen (LDL). Levercellen bezitten naast de klassieke LDL receptor nog een zogenaamde remnant- of apoE receptor, waarvan de aard nog niet is opgehelderd. In de literatuur bestaat geen eenduidigheid omtrent de receptor in de lever (de klassieke LDL receptor of een andere receptor) die verantwoordelijk is voor de opname van VLDL remnants. Om inzicht te krijgen in de receptor die betrokken is bij de opname van remnants hebben wij de binding van LPL behandeld VLDL (LPL-VLDL), als model voor VLDL remnants, aan Hep G2 cellen bestudeerd (hoofdstuk 2). Wij vonden dat LPL-VLDL met hoge affiniteit bindt aan Hep G2 cellen. Geen enkele aanwijzing kon worden gevonden voor aanwezigheid van een receptor anders dan de LDL receptor, die betrokken is bij deze binding.

In hoofdstuk 3 wordt de intracellulaire route van VLDL en LPL-VLDL na opname door Hep G2 cellen vergeleken met die van LDL, om na te gaan of de aanwezigheid van meerdere apoE moleculen per lipoproteïne-deeltje leidt tot het volgen van een andere intracellulaire verwerking van deze lipoproteïnen. Zowel VLDL als LPL-VLDL worden inefficiënt afgebroken na opname via de LDL receptor, en het transport van deze deeltjes naar het laat-endosomaal-lysosomale compartiment is aanzienlijk vertraagd. De multivalente interactie van apoE, wat geassocieerd is met VLDL en LPL-VLDL, met de LDL receptor zou kunnen leiden tot een vertraagde dissociatie van VLDL en LPL-VLDL van de receptor. Dit zou kunnen leiden tot de vertraagde intracellulaire processing van deze lipoproteïnen.

Omdat apoE verantwoordelijk is voor de receptorbinding van VLDL en VLDL remnants, leiden defecten in het apoE tot een gestoorde klaring van deze lipoproteïnen. Zo wordt verondersteld dat de defecte interactie van apoE2 met lipoproteïne receptoren het oorzakelijke metabole defect is bij familiale dysbetalipoproteïnemie (FD). Beschreven is dat de binding van apoE2 op β-VLDL geisoleerd uit plasma van een hyperlipidemische E2E2 homozygote FD patiënt verbeterde na een dramatische reductie van het plasma cholesterol als gevolg van een streng dieet en sterke gewichtsvermindering. In hoofdstuk 4 wordt een studie beschreven waarbij zes E2E2 homozygote FD patiënten gedurende 4 weken werden behandeld met gemfibrozil. Als gevolg hiervan normaliseerden de plasma cholesterol waarden van alle zes patiënten. Uit onze studie bleek dat na behandeling van de patiënten met gemfibrozil noch de samenstelling van de VLDL noch de bindings-efficiëntie aan de LDL receptor veranderd was. Deze resultaten suggereren dat gemfibrozil het plasma cholesterol verlaagt als gevolg

van een effect op de synthese van VLDL, en niet als gevolg van een effect op de receptor-gemedieerde klaring.

FD is niet altijd geassocieerd met E2E2 homozygotie. Ook heterozygotie voor het E2(lys146 → gln) allel is veelvuldig geassocieerd met FD. Omdat het E2(lys146 → gln) allel resulteert in FD ondanks de aanwezigheid van een normaal E3 allel, is er sprake van een dominant gedrag van het allel met betrekking tot de overerving van de ziekte. De studies beschreven in hoofdstuk 5, zijn uitgevoerd met de bedoeling enige opheldering te verkrijgen in het mechanisme achter het dominante gedrag van deze apoE variant. In zes families waarin de E2(lys146 → gln) variant voorkwam, werd een significant slechtere lipolyse gevonden van de d < 1.019 lipoproteïnen van de E2(lys146 → gln) dragers in vergelijking met familie leden die niet dragers waren van deze apoE variant. De d < 1.019 lipoproteïnen van de dragers bleken in vergelijking met de niet-dragers, relatief grote hoeveelheden apoE te bezitten. Er kon echter geen relatie worden gevonden tussen de hoeveelheid apoE aanwezig in de d < 1.019 fractie en de geschiktheid als substraat voor LPL. De resultaten duiden erop dat de aanwezigheid van apoE2(lys146 → gln) onder bepaalde omstandigheden leidt tot d < 1.019 lipoproteïnen die resistent zijn voor lipolyse, en als gevolg hiervan gestoord zijn in hun interactie met de LDL receptor. De gestoorde lipolyse zou op deze wijze kunnen bijdragen aan de dominantie van het E2(lys146 → gln) allel bij de expressie van FD.

In hoofdstuk 2 is beschreven dat als gevolg van lipolyse de efficiëntie waarmee VLDL aan de LDL receptor bindt, toeneemt. LPL kan echter ook onafhankelijk van enzymatische activiteit de cellulaire binding van apoE-bevattende lipoproteïnen verhogen. Wij hebben het effect van LPL op de cellulaire binding en vervolgens de opname en afbraak van VLDL en apoE-vrij LDL bestudeerd (hoofdstuk 6 en 7). Uit de resultaten blijkt dat LPL de binding van zowel VLDL als LDL aan Hep G2 cellen en normale- en LDL receptor-negatieve fibroblasten aanzienlijk stimuleert (tot 80 maal). Verder bleek de LPL-gemedieerde binding niet calciumafhankelijk, en ongevoelig voor verandering van de LDL receptor activiteit. Op grond van deze resultaten concluderen wij dat de LPL-gemedieerde binding van LDL en VLDL onafhankelijk is van de LDL receptor en het LDL-receptor-gerelateerde eiwit (LRP). Voorbehandeling van de cellen met heparinase had een sterke remming van de LPL-gemedieerde binding tot gevolg, wat duidt op een rol voor heparan sulfaat proteoglycanen. Wij concluderen dat de LPL-gemedieerde binding een gevolg is van de binding van LPL enerzijds aan heparan sulfaat proteoglycanen op het celoppervlak en anderzijds aan de lipoproteïnen (hoofdstuk 6). Zoals beschreven in hoofdstuk 7 is het niet nodig dat LPL een complex vormt met de lipoproteïnen voordat het in staat is de lipoproteïnebinding te stimuleren. Preïncubatie van de cellen met LPL, gevolgd door wassen, leidt eveneens tot een verhoging van de binding van de lipoproteïnen.

In aanwezigheid van LPL is eveneens de opname en de afbraak van VLDL en LDL verhoogd (hoofdstuk 7). Het effect van LPL op de opname en afbraak was echter minder dramatisch dan het effect op de binding. Opvallend genoeg had LPL bijna geen effect op de opname van LDL door LDL receptor negatieve cellen. Verder werd het effect van LPL op de opname geremd door inhibitie van de LDL receptor activiteit in Hep G2 cellen en normale fibroblasten. Op grond van deze resultaten wordt geconcludeerd dat de opname van de

lipoproteïnen, na binding via LPL aan heparan sulfaat proteoglycanen, hoofdzakelijk wordt gemedieerd door de LDL receptor.

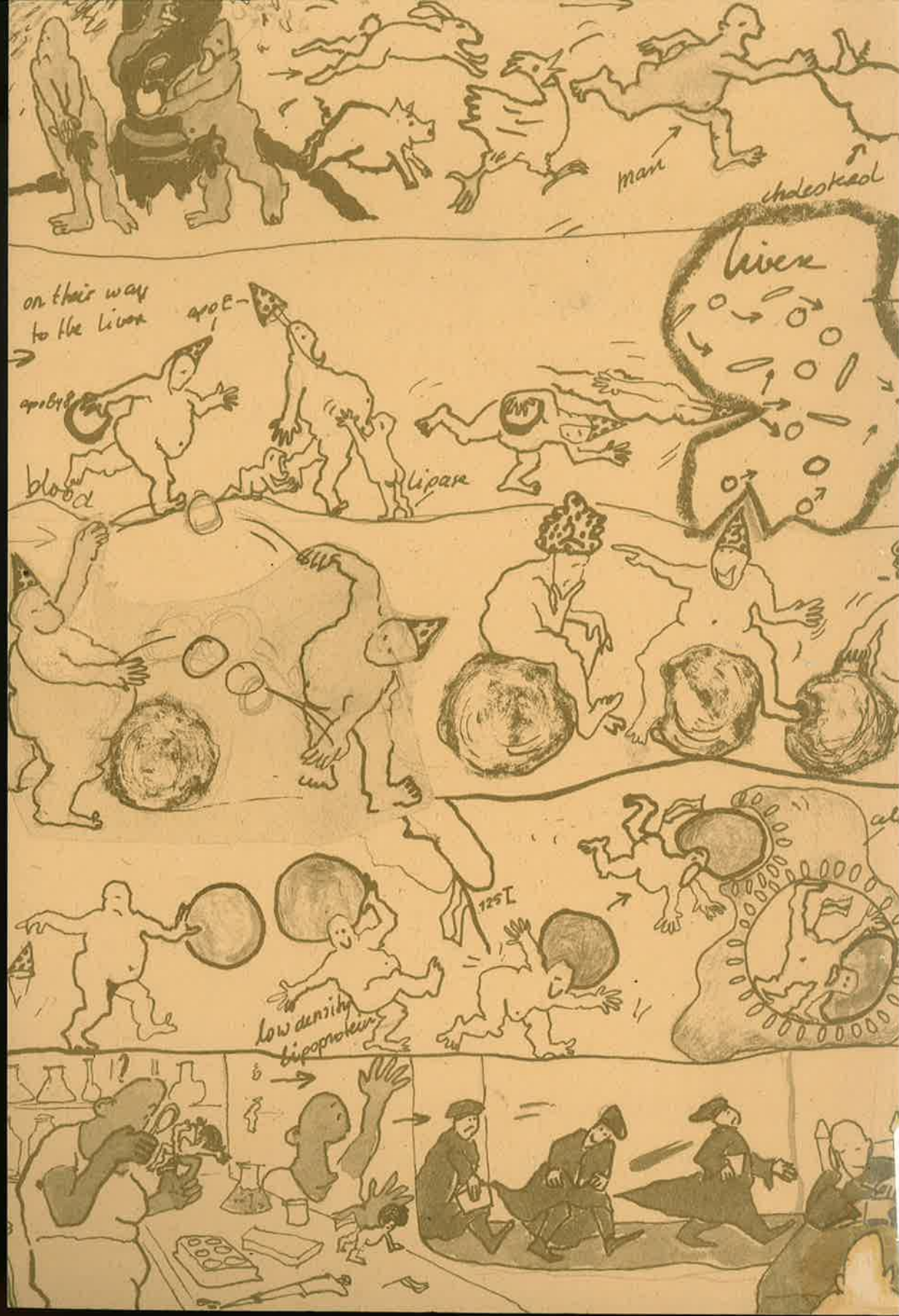
Uit bovenstaande observaties kwam vervolgens de vraag naar voren wat de fysiologische consequenties van het effect van LPL op de binding en opname van lipoproteïnen zouden kunnen zijn. In hoofdstuk 8 staan studies beschreven waarin het effect van LPL op het catabolisme van LDL *in vivo* is bestudeerd in de rat. Toediening van LPL tegelijk met ^{125}I-LDL had een verhoogde associatie van LDL met de lever tot gevolg. De verhoogde binding van LDL werd echter niet gevolgd door een verhoogde opname van LDL, zelfs niet na behandeling van de rat met oestradiol, waardoor de LDL receptor activiteit wordt gestimuleerd. Uit studies met geïsoleerde rattelever parenchym-, endotheel- en Kupffercellen kwam naar voren, dat LPL de binding van LDL aan alle drie celtypen verhoogt. Het effect van LPL op de binding aan endotheel- en Kupffercellen was minstens vijf maal zo groot als het effect op parenchymcellen. Alleen de LPL-gemedieerde binding van LDL aan parenchymcellen werd gedeeltelijk geremd door voorbehandeling van de cellen met heparinase. Het effect van LPL op de binding van LDL aan alle drie de cel-typen werd volledig teniet gedaan door heparine. Deze resultaten suggereren dat bij de LPL-gemedieerde binding van LDL aan parenchymcellen heparan sulfaat proteoglycanen betrokken zijn, terwijl andere proteoglycanen of structuren anders dan proteoglycanen betrokken zijn bij de LPL-gemedieerde binding aan endotheel- en Kupffercellen. *In vivo* lijkt LPL specifiek de associatie van LDL met de lever te stimuleren. Het uitblijven van een eveneens verhoogde opname zou een uitwisseling van LDL componenten met de lever mogelijk kunnen maken. Ook bestaat de mogelijkheid dat LDL zorg draagt voor de afgifte van LPL aan de lever, wat atherogene werking van LPL in de bloedvatwand zou kunnen verhinderen.

ABBREVIATIONS

apo	apolipoprotein
BMI	body mass index
BSA	bovine serum albumin
β-VLDL	β-migrating very low density lipoprotein
CE	cholesteryl ester
CM	chylomicron
d	density (g/ml)
EC	endothelial cells
ELISA	enzyme linked immunosorbent assay
EGTA	ethyleneglycol-bis-(2-aminoethyl)-tetraacetic acid
FC	free (unesterified) cholesterol
FCS	fetal calf serum
FD	familial dysbetalipoproteinemia
FFA	free fatty acid
FH	familial hypercholesterolemia
HDL	high density lipoprotein
HL	hepatic lipase
HSA	human serum albumin
HSPG	heparan sulphate proteoglycan
IDL	intermediate density lipoprotein
IEF	isoelectric focusing
KC	Kupffer cells
LDL	low density lipoprotein
Lp(a)	lipoprotein(a)
LPL	lipoprotein lipase
LPL-VLDL	lipoprotein lipase-treated very low density lipoprotein
LRP	low density receptor-related protein
PAGE	polyacrylamide gel electrophoresis
PC	parenchymal cells
PL	phospholipid
SD	standard deviation
SDS	sodium dodecyl sulphate
TC	total cholesterol
TG	triglyceride
Type III	type III hyperlipoproteinemia
VLDL	very low density lipoprotein

CURRICULUM VITAE

Monique Tietje Mulder werd op 5 september 1964 geboren te Emmen. In 1982 behaalde zij het Atheneum-B diploma aan de Gemeentelijke Scholengemeenschap te Emmen. Van 1982 tot 1988 studeerde zij biologie aan de Rijks Universiteit te Groningen, met als specialisatierichting medische biologie. Gedurende de doctoraalfase werd onderzoek verricht naar het cholesterol metabolisme in de lever van de rat met behulp van liposomen. Het tweede doctoraalonderwerp handelde over het effect van lithium op de afgifte van acetylcholine in de hersenen van de rat, in verband met de manische stoornis.
Van 1988 tot 1992 was zij als assistent in opleiding in dienst van de Nederlandse Hartstichting. Dit proefschrift beschrijft de resultaten van onderzoek uitgevoerd in de periode van juni 1988 tot april 1990 bij het Gaubius Instituut in Leiden. Na verhuizing van het instituut in april 1990 werd het onderzoek voortgezet bij het IVVO-TNO, Gaubius Laboratorium, eveneens in Leiden. In de periode van januari 1992 tot april 1992 volgde zij een beknopte cursus "management voor vrouwen" aan de Rijks Universiteit Leiden. Vanaf 1 mei 1993 is zij werkzaam op de afdeling Fibrinolyse en Proteolyse van het IVVO-TNO Gaubius Laboratorium.


man
cholesterol
liver
on their way to the liver
apoE
apoB
blood
lipase
125I
low density lipoprotein