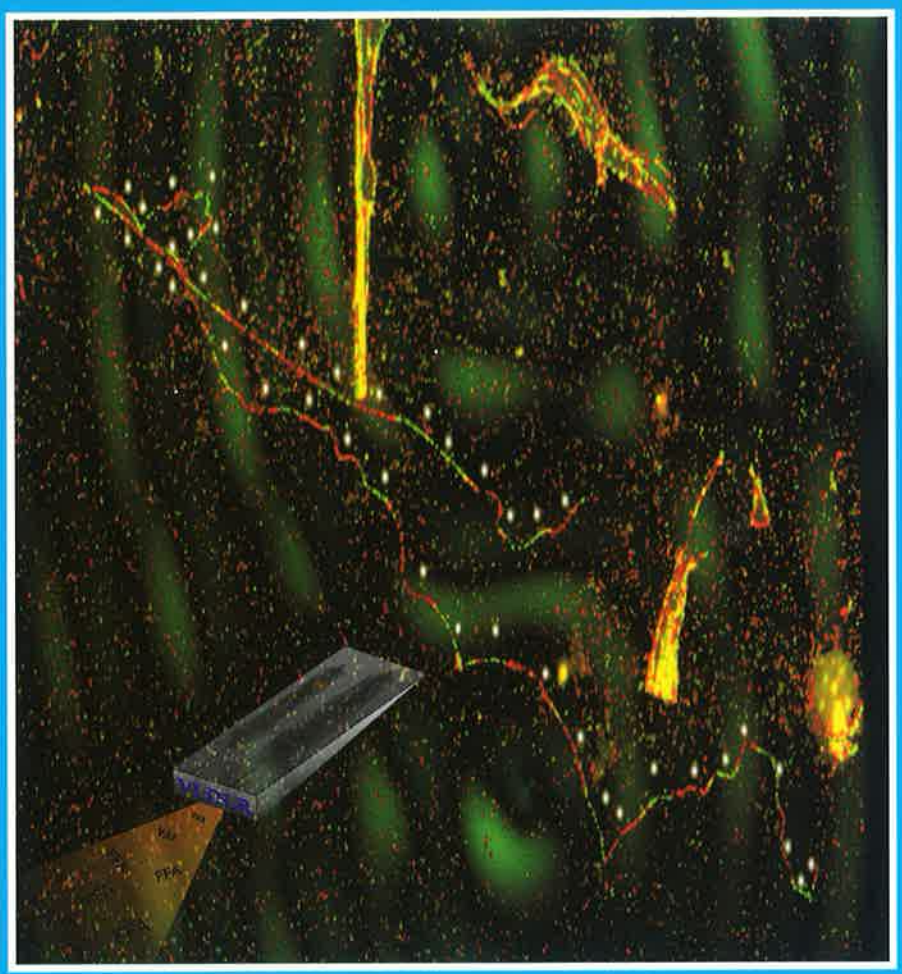
Role of Very Low Density Lipoprotein Receptor in Lipid Metabolism and Atherosclerosis



Paul J. Tacken

Role of Very Low Density Lipoprotein Receptor in Lipid Metabolism and Atherosclerosis

PROEFSCHRIFT

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Stellingen behorende bij het proefschrift:

"Role of very low density lipoprotein receptor in lipid metabolism and atherosclerosis"

1 De naam "apoE receptor 2" is toegewezen aan de receptor voordat deze voldoende gekarakteriseerd was *(dit proefschrift)*.

2 LDL receptor familieleden binden niet alleen meerdere liganden *in vitro,* ook uit *in vivo* data blijkt dat één receptor met meerdere liganden interacteert.

3 Evenals verstoringen in de vetmassa kunnen leiden tot veranderingen in het lipoproteïne-metabolisme, kunnen verstoringen in het lipoproteïne-metabolisme leiden tot veranderingen in de vetmassa.

4 Aangezien vetopslag afhankelijk is van zowel genetische als omgevingsfactoren geldt dat, ook al komt "ieder pondje door het mondje", niet ieder pondje voor eenieder tot dezelfde gewichtstoename zal leiden *(J. Friedman and J. Halaas, Nature, 395: 763-770, 1998)*.

5 Ondanks de vele hypotheses over de rol van LDL receptor familieleden bij atherosclerotische schuimcelvorming, is zo'n rol nog nooit onomstotelijk vastgesteld.

6 Het ontbreken van een duidelijk fenotype in een knock-out muis hoeft niet te betekenen dat het betreffende gen geen belangrijke fysiologische functie vervult.

7 De VLDL receptor transgene en knock-out muizen laten zien dat men, teneinde muismodellen goed te karakteriseren, de biologische parameters onder uiteenlopende omstandigheden dient te bestuderen *(dit proefschrift)*.

8 Voor de "restricted-ovulator" kip geldt: Je ei niet kwijt kunnen is slecht voor hart- en bloedvaten *(H. Bujo et al., Proc. Natl. Acad. Sci. USA, 92: 9905-9909, 1995)*.

9 Het is in deze drukke tijden goed te weten dat uit de relativiteitstheorie het volgende is af te leiden: als "de tijd vliegt", verloopt ze langzamer *(A. Einstein, Annalen der Physik, 17: 891-921, 1905)*.

10 Aangezien "gedachte" verleden tijd is, kan men in het heden onmogelijk een originele gedachte hebben.

11 Wetenschap is zoeken naar de waarheid, en het niet vertrouwen als je haar gevonden hebt.

Contents

Chapter 1

General introduction

1.1 Introduction

Cardiovascular disease is the major cause of death in Western society, accounting for about one half of the total number of annual deaths. The main cause of cardiovascular disease is atherosclerosis, a complex multi-factorial disorder characterized by an accumulation of lipid laden cells in the vessel wall. Elevated levels of cholesterol and triglycerides in the blood are important risk factors for the development of atherosclerosis. In order to be transported by the bloodstream, cholesterol and triglycerides are packaged into water-soluble lipoproteins. Synthesis, processing and catabolism of lipoproteins need to be tightly regulated to retain a correct distribution of lipids in the body.

1.2 Lipid metabolism

1.2.1 Lipoproteins and lipoprotein metabolism

Triglycerides and cholesterol are the most common dietary lipids. Cholesterol is essential for biosynthesis of cellular membranes, steroid hormones and bile acids. Triglycerides can be used for energy storage in adipose tissue and as an energy source in tissues like heart and skeletal muscle. Cholesterol and triglycerides are taken up from the diet by the intestine, or are synthesized by the liver. They are packaged into spherical macromolecular particles, called lipoproteins. The lipoproteins consist of a core of nonpolar lipids such as triglycerides and cholesterol ester, and a polar surface monolayer containing phospholipids, free cholesterol and several proteins, called apolipoproteins.

The plasma lipoproteins can be divided into five different classes, according to their density: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Apart from differing in density, these classes of lipoproteins also differ in electrophoretic mobility, lipid content and apolipoprotein composition.

Lipoprotein trafficking throughout the body can be divided in three pathways. The exogenous pathway mediates uptake of dietary lipids by the body. The endogenous pathway mediates distribution of lipoproteins produced by the liver to the periphery. The reverse cholesterol pathway mediates transport of cholesterol from the periphery to the liver.

1.2.2 The exogenous pathway

Dietary lipids are absorbed in the intestine and packaged into chylomicrons. These chylomicrons are large, triglyceride rich particles containing apolipoprotein (apo) B48, apoA1 and apoA4. They are secreted by the epithelial cells into the lymph and thus enter the circulation. Subsequently, the chylomicrons aquire apoE, apoC1, apoC2 and apoC3. While in circulation, the chylomicron triglycerides are hydrolyzed by lipoprotein lipase (LPL), which is present on the surface of the endothelial cells of the vessel wall (1, 2). Upon triglyceride hydrolysis, free fatty acids (FFA) are released that can be used as energy source for heart and skeletal muscle, or can be stored as triglycerides by adipocytes. Thus chylomicrons become enriched in cholesterol, and the residual particle is referred to as chylomicron remnant. The chylomicron remnants are rapidly removed from the circulation by the liver via receptor

mediated endocytosis. The hepatic receptors mediating remnant uptake are the LDL receptor (LDLR) (3, 4) and the LDL receptor related protein (LRP) (5-7).

1.2.3 The endogenous pathway

In the endogenous pathway, hepatic cholesterol and triglycerides are packaged into VLDL particles and secreted into the bloodstream (8). Nascent VLDL contains apoB100 and is enriched with apoE and apoC upon entering the circulation. Similar to chylomicrons, VLDL triglycerides are hydrolyzed by LPL, leading to the formation of IDL. The IDL is either removed by the liver, or is subjected to further lipolysis (9). Additional loss of triglycerides, phospholipids, apoC and apoE results in the formation of LDL, which is taken up via receptor mediated endocytosis in the liver.

1.2.4 The reverse cholesterol pathway

Cholesterol in peripheral tissues can be transported back to the liver by HDL particles. Cells can release cholesterol in the interstitial fluid, where HDL serves as an acceptor. Recently, the ABC1 gene has been identified as a key determinant of active cellular cholesterol efflux (10-12). In the circulation the cholesterol is converted into cholesterol ester by lecithin:cholesterol acyl transferase (LCAT). The HDL cholesterol can be delivered to the liver in three ways (13). First, cholesteryl ester transfer protein (CETP) can transfer cholesterol esters from HDL to VLDL, IDL and LDL. Second, HDL can acquire apoE in the circulation, allowing hepatic uptake via hepatic apoE receptors. Third, the scavenger receptor SR-B1 can bind HDL, leading to specific uptake of cholesterol esters (14, 15).

1.2.5 Lipid metabolism at the cellular level

Transcription factors like sterol regulatory element-binding proteins (SREBPs) and peroxisome proliferator-activated receptors (PPARs) play a central role in maintaining cellular lipid homeostasis. SREBPs are bound to membranes of the endoplasmatic reticulum and the nuclear envelope (16). In sterol-depleted cells, the N-terminal domain of SREBPs is released from the membrane and travels to the nucleus. In the nucleus, the SREBPs bind to sterol regulating elements (SREs) in the promoters of multiple genes involved in cholesterol, unsaturated fatty acid and triglyceride biosynthesis (reviewed in (17)). In addition, SREBPs mediate cellular cholesterol uptake by enhancing LDLR expression. Accumulation of sterols within cells blocks the proteolytic release of SREBPs from membranes. This results in a reduced transcription of target genes involved in fatty acid biosynthesis and completely aborts transcription of target genes involved in cholesterol biosynthesis (18, 19). Recent findings indicate that sterol sensing is mediated by SREBP cleavage-activating protein (SCAP). SCAP is involved in SREBP cleavage and becomes inactive when sterols overaccumulate in cells (20).

PPARs are ligand-dependent transcription factors. Three PPAR isotypes have been identified: α, β and γ. PPARα is mainly expressed in brown adipose tissue, liver kidney, heart and skeletal muscle. PPARβ is expressed in many tissues, including gut, kidney and heart. PPARγ is predominantly expressed in adipose tissue (21). PPARs exist as heterodimers with retinoic X receptors (RXRs) and bind to PPAR response elements (PPREs) within the promoter

regions of target genes. Upon binding of a ligand to either PPAR or RXR, the transcription of the target gene is activated (21). PPAR ligands shared by the three isotypes include polyunsaturated fatty acids and probably oxidized fatty acids. Ligands binding with high affinity to PPARα include linoleic acid, phytanic acid and several eicosanoids (22, 23), while the prostaglandin 15-deoxy-$D^{12,14}$-prostaglandin J_2 is the most potent natural ligand for PPARγ (24). The target genes of PPARα belong to pathways of lipid transport and catabolism, including fatty acid transport across membranes, fatty acid binding in cells, fatty acid oxidation and lipoprotein assembly and transport (25). PPARγ has been shown to be required for differentiation of adipose tissue *in vivo* (26). Furthermore, PPARγ affects transcription of genes implicated in lipogenic pathways, including LPL, adipocyte fatty acid binding protein (aP2), acyl CoA synthase and fatty acid transport protein (CD36). PPARγ expression itself has been shown to be regulated by insulin (27) and SREBP (28).

The cellular lipid metabolism needs to be tightly regulated, since imbalances in cellular uptake and expenditure of lipids can lead to disease. Examples of cells overaccumulating lipids are smooth muscle cells and macrophages in atherosclerosis and adipocytes in obesity.

1.2.6 Atherosclerosis

High levels of LDL and VLDL in the plasma are considered to be risk factors for the development of premature atherosclerosis. High plasma LDL and VLDL levels lead to accumulation of lipoproteins in the vessel wall, where they get oxidized. Various enzymes have been proposed to play a role in lipoprotein oxidation, including lipoxygenases (29-31), myeloperoxidase (32, 33), ceruloplasmin (34), haem proteins (35, 36) and NADPH oxidase (37). Oxidized lipoproteins attract monocytes and leukocytes, which infiltrate the vessel wall. Subsequently, the macrophages take up lipoproteins and become loaded with cholesterol. The appearance of these fat laden cells, also referred to as foam cells, is believed to be one of the first stages in the atherosclerotic process (38). In later stages, smooth muscle cells infiltrate the lesion and start secreting extracellular matrix components. This results in the formation of advanced fibrous plaques, which are covered by connective tissue and fibroblasts. In the core of these plaques, fat laden smooth muscle cells and macrophages can be found, together with T-lymphocytes, cholesterol crystals, mineralisation and necrotic debris (39). Rupture or fissure of the plaque causes hemorrhage, thrombosis and occlusion of the artery, and is the major cause of sudden death by myocardial infarcts.

1.2.7 Adipocyte triglyceride storage

In mammals, the major site of energy storage is the adipose tissue, where energy is stored in the form of triglycerides. Therefore, fatty acids are released from lipoproteins, taken up by the adipocyte and incorporated into triglycerides. Release of fatty acids from circulating chylomicrons and VLDL is mediated by LPL (40). Under normal circumstances, approximately half of the fatty acids released by LPL are trapped by the adipocyte, and half enter the general circulation (41). Transport of fatty acids across the cell membrane is mediated by fatty acid transporter proteins, such as CD36 and FATP (42-44). In the adipocyte, fatty acids are bound to

the adipocyte fatty acid binding protein (aP2) (45) and the keratinocyte fatty acid binding protein (mal1) (46). Until now, two pathways for triglyceride synthesis have been characterized biochemically: the glycerol-phosphate pathway and the monoacylglycerol pathway (reviewed in (47, 48)). The glycerol-phosphate pathway is believed to be present in all tissues (47), while the monoacylglycerol pathway has only been detected in small intestine enterocytes, rat suckling liver and rat adipocytes (49, 50). Acyl CoA:diacylglycerol acyltransferase (DGAT) is believed to catalyse the final step in both pathways for triglyceride synthesis. However, DGAT deficient mice are able to synthesize triglycerides, indicating that alternative mechanisms for triglyceride synthesis exist (51). Finally, fatty acids can be released from adipocyte triglyceride stores by triglyceride hydrolysis. Hormone sensitive lipase (HSL) has been identified as on of the enzymes mediating triglyceride hydrolysis in adipose tissue (52).

1.2.8 Obesity and insulin resistance

The mechanisms controlling adipose tissue mass are very complex. The most important control of adipose tissue mass is related to the balance between energy intake and energy expenditure. Energy intake can be controlled by regulation of appetite by a large number of peptides produced in brain and peripheral tissues (53-55). For example, leptin, which is produced by adipocytes, is part of a feedback mechanism to limit further food intake after prolonged feeding. Extreme obesity occurs in mice and humans with mutations in the genes encoding for leptin (56, 57) and the leptin receptor (58). Energy expenditure depends on energy used in exercise, basal metabolic rate and thermogenesis. In mammals, at least 20% of energy expenditure is due to an energy leak in the mitochondrial inner membrane that is controlled by uncoupling proteins (UCPs) (59, 60). Several polymorphisms in UCPs have been identified, of which some are associated with severe obesity (61).

The final site of regulation of adipose tissue mass is the adipocyte itself. Obesity may occur as a result of an increase in the number of adipocytes, an increase in the amount of triglycerides stored in the adipocyte, or both. Adipocyte number is believed to be dependent on the balance between the size of the precursor pool, rate and extent of differentiation, and the rate of cell loss through apoptosis (62). The rate and extent of adipocyte differentiation is controlled by transcription factors, including SREBP-1, C/EBPα and PPARγ (63).

The amount of triglycerides stored in the adipocyte can be controlled by regulation of triglyceride synthesis, storage and release. Acylation stimulating protein (ASP) is produced by adipocytes and stimulates uptake of fatty acids and their conversion to triglycerides (64, 65). Furthermore, ASP reduces HSL activity, thereby reducing fatty acid release by the adipocyte (65). The rate at which fatty acids become available for the adipocyte is determined by LPL and insulin, which is the principal hormone regulating LPL. Insulin is produced by β-cells in the pancreas, and its release is stimulated by a postprandial rise in blood glucose levels. Apart from facilitating triglyceride synthesis in both liver and adipose tissue, insulin facilitates tissue glucose absorption and the conversion of glucose to glycogen in liver and muscle (66). Obesity leads to resistance to the actions of insulin. Insulin resistance is a characteristic feature of most patients with type 2 diabetes mellitus, and is almost universal in obese type 2 diabetic patients. Insulin resistance leads to increased β-cell insulin secretion and hyperinsulinemia (67, 68). Type

2 diabetes occurs when increased insulin secretion fails to compensate for insulin resistance (69). Sensitivity for insulin in obese type 2 diabetic patients often increases when the body weight is reduced (66). Furthermore, thiazolidinediones (TZDs) improve insulin action and have recently been introduced as therapeutic agents for treatment of type 2 diabetes (70). TZDs functions as agonist for PPARγ, suggesting that TZDs exert their beneficial effects via PPARγ (70-72). Interestingly, adipocyte fatty acid binding protein aP2 deficient mice develop diet induced obesity, but, in contrast to wild type mice, do not develop insulin resistance (73). This suggests that cytoplasmic fatty acid binding proteins are involved in the generation of insulin resistance.

1.3 Receptors of the LDL receptor family

1.3.1 The LDL receptor

The first evidence that LDL is cleared from the bloodstream by a specific receptor came from the work of Brown and Goldstein (74-76). They demonstrated that defects in the receptor for LDL cause Familial hypercholesterolemia (FH). FH is an inheritable disorder, characterized by elevated levels of plasma LDL cholesterol. As a consequence, FH patients develop premature atherosclerosis and coronary artery disease. Further studies on the LDLR in fibroblasts derived from FH patients revealed multiple mutations that disrupted either transport or processing of the LDLR (77, 78). The cloning of the human LDLR gene (79) and the sequencing of its full-length cDNA (80) made it possible to identify the genetic basis for hundreds of FH-associated mutations. The generation of LDLR deficient mice produced a mouse model for FH (81). When fed a high fat diet, LDLR deficient mice develop hypercholesterolemia, resulting in formation of atherosclerotic lesions (82). Therefore, the LDLR deficient mouse is often used as a mouse model to study atherogenesis.

The LDLR binds lipoproteins via apoB100 and apoE. A single molecule of apoB100 is present on all LDL particles, and can interact with a single LDLR (83). ApoE mediates binding of IDL and VLDL remnants to the LDLR (84). The LDLR has a higher affinity for apoE than for apoB100. In addition, multiple apoE molecules present on a single lipoprotein particle can simultaneously interact with a single LDLR, or can mediate binding of the lipoprotein particle to multiple LDLRs (85). Particles internalized by the LDLR are degraded in lysosomes, in which the apoliproteins are degraded into amino acids while the lipids are released into the cytosol. The LDLR itself can either be degraded, or recycles back to the cell surface (86).

In the endogenous pathway, about two-third of the hepatic clearance of circulating LDL particles is mediated by the LDLR, while one-third is removed via extrahepatic receptors (87). Studies on the exogenous pathway in mice indicate that the LDLR is responsible for the clearance of 55-75% of the chylomicron remnants (88, 89). Thus the LDLR is a key factor in the regulation of cholesterol homeostasis by the liver.

1.3 .2 The LDLR family

The cloning of the LDLR gene triggered a search for homologous receptors that might play a role in lipoprotein metabolism. This resulted in the discovery of numerous receptors,

which share both functional and structural features, and together form the LDLR family. These receptors appear to originate from a common ancient ancestor. LDLR family members identified in nonmammalian species include the nematode *Caenorhabditis elegans* receptor (90), the fruit fly Y1 protein (91), the mosquito vitellogenin receptor (92), the migratory locust insect lipophorin receptor (93), the Xenopus LDL receptors (94) and the chicken vitelogenin (VTG) receptor (95). Receptors expressed in mammals include the LDL receptor itself (79), the VLDL receptor (96), the apolipoprotein E receptor 2 (apoER2) (97), the LDL receptor related protein (LRP) (98), megalin/gp330 (99), LRP3 to LRP7 (100-103) and LR11 (104).

All receptors of the LDLR family exhibit similar structural features. They carry an amino-terminal domain composed of cysteine-rich ligand binding repeats. Furthermore, all receptors carry an epidermal growth factor homology domain required for pH-dependent dissociation of ligands in endosomes (105). The receptors are anchored to the plasma membrane by a single transmembrane spanning region. Finally, all receptors carry a cytoplasmic tail required for clustering of the receptor into coated pits, followed by endocytosis (106).

Several LDLR family members depend on receptor-associated protein (RAP) in order to be expressed on the cell surface. RAP binds strongly to LRP, gp330/megalin and VLDLR, but weakly to the LDLR itself. RAP is required for proper folding and export of the receptors from the endoplasmatic reticulum by preventing premature binding of other co-expressed receptor ligands. Thus RAP acts as a chaperone for newly synthesized receptors, and protects for ligand induced aggregation and degradation of receptors in the endoplasmatic reticulum (107). RAP is a useful tool in both *in vivo* and *in vitro* receptor studies. Its high binding affinity for lipoprotein receptors prevents other ligands from binding, thereby blocking receptor function.

Receptors of the LDLR family can be divided into two subgroups, the "low molecular weight" and the "high molecular weight" receptors. The low molecular weight receptors carry only a single EGF precursor and a single ligand binding domain, while the high molecular weight receptors carry multiple EGF precursor and ligand binding domains. The low molecular weight receptors include the LDLR, VLDLR, apoER2, insect lipophorin receptor and chicken VTG receptor. The high molecular weight receptors include Y1, LRP, gp330/megalin, LR11 and the *C. elegans* receptor. Comparison of receptors by sequence alignment indicates that the two subgroups evolved independently from a common ancestor (108, 109).

Although many of the LDLR family members are reported to bind lipoproteins in *in vitro* binding assays, they also bind numerous other ligands. For several of the LDLR family members, *in vivo* evidence is found that confirms a role in lipoprotein metabolism. Several other family members are involved in physiological pathways unrelated to the lipoprotein metabolism. The characteristics of the LDL receptor family members will be described below.

1.3.3 Non-mammalian receptors, Y1 and VTG receptor

In both fruit fly (*Drosophila melanogaster*) and chicken, naturally occurring mutations in LDLR family members have been identified that cause female sterility. In both species this is due to the inability to produce egg yolk, the food source for the developing embryo. In the fruit fly, the female sterile mutant yolkless (*yl*) fails to transport yolk proteins, called VTGs, into the oocyte (110). The *yl* gene codes for the Y1 receptor, which is present in wild type oocytes and is

absent in *yl* mutants. Remarkably, the fruit fly VTGs exhibit sequence similarity to LPL, a ligand for several of the mammalian LDLR family members (91). Thus it seems likely that the Y1 receptor regulates VTG uptake via receptor mediated endocytosis.

In chicken, the major egg yolk components, VTG and triglyceride-rich VLDL, are imported into the oocyte by the chicken VTG receptor (111). Molecular characterization of the VTG receptor revealed that it is the chicken homologue of the mammalian VLDLR (95). Two splice variants of the VTG receptor have been found, one containing and one lacking the exon that codes for the O-linked sugar domain. The variant lacking the O-linked sugar domain is predominantly expressed in germ cells, while the variant containing the O-linked sugar domain is predominantly expressed in somatic cells (112, 113). Up until now, no functional differences between the two forms have been detected.

Hens of the mutant restricted-ovulator chicken strain are sterile due to a point mutation in the VTG receptor gene. Due to the mutation they lack a functional VTG receptor, resulting in impaired oocytic uptake of yolk precursor from the circulation. Therefore, lipoproteins accumulate in the plasma causing premature atherosclerosis (114).

1.3.4 The LDL receptor related protein

The LRP was the first receptor with sequence homology to the LDLR to be cloned (98). The LRP is expressed in hepatocytes (98), neurons and astrocytes in the central nervous system, epithelial cells of the gastrointestinal tract, smooth muscle cells, fibroblasts, Leydig cells, granulosa cells in ovary, dendritic interstitial cells of kidney and monocyte-derived cells in liver, lung and lymphoid tissues (115). The LRP binds and internalizes lipoproteins containing apoE (116), and binding is enhanced by LPL (116). Apart from lipoproteins, the LRP binds numerous other ligands, including α_2-macroglobulin (116), LPL (117), RAP (118), thrombin (119), thrombospondin-1 (120, 121), free urokinase plasminogen activator (uPA), uPA/urokinase plasminogen activator inhibitor (PAI)-1 complex (122) and several other proteinase/serpin complexes (123).

Although the LDLR mediates hepatic chylomicron remnant uptake, humans and rabbits with LDLR gene defects display normal chylomicron remnant clearance (124, 125). Thus it seems likely that a second receptor is involved in hepatic chylomicron remnant clearance. To investigate whether the LRP mediates clearance of chylomicron remnants, LRP deficient mice have been generated (126). LRP deficient mice are not viable, and die in utero. The reason for this embryonic lethality remains unclear (126). To circumvent the problem of embryonic lethality, inducible LRP knockout mice were generated. Upon inducing the LRP gene defect in the liver of mice that are also deficient for the LDLR, chylomicron remnants accumulate in the plasma (7). It is estimated that in wild type mice, the LRP is responsible for clearance of approximately 20-25% of the chylomicron remnants (88, 89).

Apart from its role in lipoprotein metabolism, the LRP is reported to mediate cell migration *in vitro* in a process dependent on uPA (127, 128). Both uPA and the uPA receptor (uPAR) are described to play a role in cell migration (129). The uPAR has no internalization sequence and its catabolism is regulated by the LRP. Together these findings led to the hypothesis that LRP mediates cell migration by regulating uPAR catabolism.

1.3.5 Megalin/gp330

Megalin, also known as gp330, is the largest known mammalian LDLR family member (Mr = 330 kDa). It has been identified initially as the major autoantigen in an induced glomular nephritis model in rat, Heymann nephritis (130). Megalin binds many of the ligands that also bind to the LRP, including apoE rich lipoproteins (131), RAP (118), LPL (132), thrombin (119) and uPA-PAI-1 complex (131). In contrast to LRP, megalin was described to mediate internalization of LDL via apoB100 (133). Furthermore, LRP and megalin differ in their respective expression patterns. Megalin is expressed in absorptive epithelia of the intestine, the proximal tubules of the kidney, type II pneumocytes of the lung and ventricular system of the brain (134, 135).

Megalin deficient mice are born alive, but most die perinatally from respiration insufficiency. Megalin deficient mice manifest abnormalities epithelial tissues including lung and kidney. Furthermore, they are characterized by an abnormal formation of the forebrain and forebrain derived structures, including incomplete eye development, lack of olfactory bulbs and corpus callosum, a fused ventricular system and incomplete separation of forebrain hemispheres (136). Megalin deficient mice that survive to adulthood are unable to retrieve carrier-bound retinol and 25-(OH) vitamin D3 from the glomerular filtrate in kidney proximal tubules, indicating that megalin plays an important role in vitamin A and D homeostasis (137, 138).

There are several indications that the forebrain abnormalities of megalin deficient mice are caused by lack of cholesterol due to an inability in lipoprotein uptake from the extraembryoinic fluid. First, administration of the drug AY9944 to pregnant rats results in a similar phenotype as seen in megalin deficient mice (139). AY9944 inhibits 7-dehydrocholesterol-Δ7-reductase, an enzyme in the cholesterol biosynthetic pathway. Defects in this enzyme lead to Smith-Lemli-Opitz syndrome in humans, which is also characterized by forebrain abnormalities (140). Second, apoB100 deficient mice die during midgestation and display failure of neural development (141). The yolk sac repackages cholesterol and fat-soluble vitamins taken up from the maternal circulation in lipoproteins containing apoB100 (142, 143). Thus megalin could mediate both internalization of maternal lipoproteins by the yolk sac and internalization of newly synthesized apoB100 containing lipoproteins by the embryonic neuroepithelium.

1.3.6 The apolipoprotein E receptor 2

The apoER2, LDLR and VLDLR together constitute the subclass of mamalian "low molecular weight receptors". The position of their exon/intron boundaries are almost identical (see figure 1). However, the apoER2 contains a unique insertion sequence of 59 amino acids in the cytoplasmic domain. The apoER2 is expressed in brain, placenta and testis, and binds both apoE rich β-VLDL and reelin (97) (144). While the non-primate apoER2 contains 8 ligand binding repeats, the primate apoER2 receptor contains only 7. This is due to the skipping of a pseudo exon found in primate apoER2 (145).

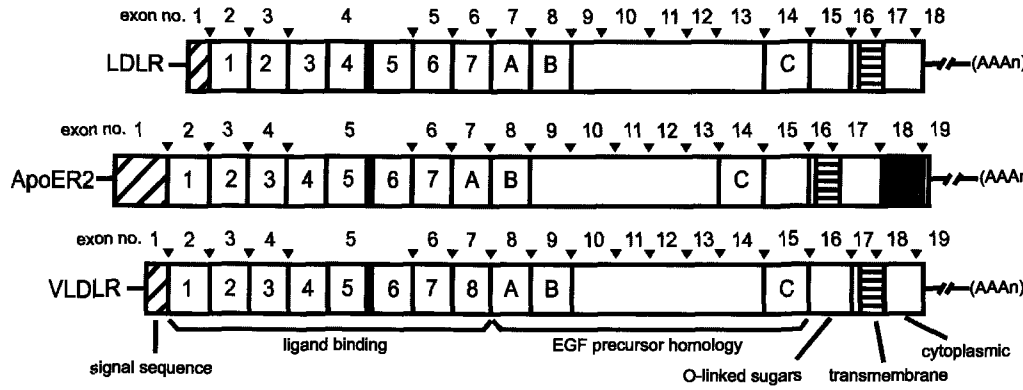

Figure 1. Exon/intron boundaries of the LDLR, apoER2 and VLDLR
Comparison of the exon/intron structure of the VLDLR, apoER2 and VLDLR. The functional domains of the receptors are labeled in the lower portion of the figure. The ligand binding repeats are numbered 1 to 6. The growth factor repeats are lettered A to C. The positions at which introns interrupt the coding region are indicated by arrowheads. Exon numbers are shown between the arrowheads. This figure was adapted from Kim et al, J Biol Chem *272*: 8498-4, 1997 with permission from the editor.

Several splice variants have been described for the apoER2. A study by Soutar et al. suggests that in human, besides the full length mRNA, the major mRNA species are variants lacking exon 5, 15 or 18. Although exon 5 codes for three ligand binding domains, deletion of this exon does not affect apoER2 β-VLDL binding affinity (146).

The apoER2 was only recently discovered, and its lipoprotein binding characteristics have not yet been studied extensively in *in vitro* binding assays. Whether the apoER2 performs a function in lipoprotein trafficking *in vivo* remains to be determined. ApoER2 deficient mice have been reported to display male fertility problems and minor changes in brain morphology (147).

1.3.7 The VLDL receptor

The VLDLR displays approximately 95% amino acid conservation between human (148-151), mouse (152, 153), rabbit (96) and rat (154), and 84% amino acid conservation between human and its chicken homologue, the VTG receptor (95). Thus compared to the LDLR, which displays 76% amino acid conservation between human and rabbit, the VLDLR is very well conserved throughout evolution.

The VLDLR is most abundantly expressed in heart, skeletal muscle and adipose tissue, but not in liver (149, 151-153, 155). VLDLR expression is localized in endothelial and smooth muscle cells of arteries and veins (156). Since heart and skeletal muscle use fatty acids as an

energy source, and adipose tissue uses fatty acids for energy storage, the VLDLR was hypothesized to play a role in the delivery of fatty acids to these peripheral tissues.

The *in vitro* binding characteristics of the VLDLR partly overlap with those of the other LDL receptor family members. The VLDLR binds apoE rich VLDL (96, 148), chylomicrons (157), LPL (158), receptor associated protein (RAP) (159), reelin (144), thrombospondin-1 (160), uPA-PAI-1 complex (161) and several other proteinase/serpin complexes (123). The VLDLR in itself binds VLDL with low affinity in *in vitro* binding assays. However, enrichment with apoE and the presence of LPL both markedly increase VLDLR binding affinity for VLDL (158). Furthermore, the VLDLR is capable of reversing hypercholesterolemia in LDLR deficient mice when ectopically expressed in liver. This implies the VLDLR is capable of mediating lipoprotein uptake *in vivo* (162).

Similar to the chicken VTG receptor, two different splice variants of the VLDLR have been described, one containing and one lacking the O-linked sugar domain (148, 151). The variant with the O-linked sugar domain is preferentially expressed in heart and skeletal muscle, while the variant without the O-linked sugar domain is predominantly expressed in non-muscle tissues (163, 164). Furthermore, bovine aortic endothelial cells exclusively express the variant without the O-linked sugar domain, suggesting differential splicing is cell type specific (163). It remains to be determined whether the differential expression of two splice variants reflects differences in VLDLR function.

Several studies have investigated the role of diet in VLDLR expression. A study in rats by Jokinen et al. (154) shows no effect of fasting and refeeding on VLDLR mRNA and protein levels. In contrast, a study in mice by Kwok et al. (165) reveals an increase in heart and a decrease in epididymal fat VLDLR protein levels after fasting. A study by Tiebel et al (155) examines the effect of feeding an atherogenic diet on VLDLR mRNA expression levels in wild type, *LDLR-/-*, *apoE-/-* and *LDLR-/-;apoE-/-* mice. It shows downregulation of the VLDLR in heart and skeletal muscle upon feeding the atherogenic diet in LDLR-/- mice, but not in the other mouse models. In contrast, VLDLR mRNA expression is shown to be upregulated upon feeding of the atherogenic diet in adipose tissue of all mouse models, except *LDLR-/-;apoE-/-* mice. Thus the effect of the diet on VLDLR expression seems to depend upon several factors, like the species studied and the presence or absence of other lipoprotein receptors and apoE.

VLDLR deficient mice display normal plasma cholesterol, triglyceride and lipoprotein levels and show no severe abnormalities. However, they display 15-20% reduction in body weight due to reduced adipose tissue mass (166). Thus the VLDLR might play a role in delivery of fatty acids to adipose tissue. The relatively mild phenotype of VLDLR receptor deficient mice could imply that the VLDLR is not a key player in lipoprotein metabolism. However, backup mechanisms (other receptors?) could compensate for its absence.

Similar to apoER2 deficient mice, VLDLR deficient mice display minor changes in brain morphology. However, apoER2 VLDLR double knockout mice display profound malformation of cerebral cortex, hippocampus and cerebellum, and die a few weeks after birth (147). The phenotype of apoER2 VLDLR double knockout mice is almost identical to the phenotype of both reelin and mDAB1 deficient mice (147). Reelin is a large extracellular matrix protein that is produced by Cajal-Retzius neurons in specific regions of the developing brain. Reelin controls

neuronal migration and positioning in the developing brain (reviewed in (167)). *Reeler* mice have a defective reelin gene, resulting in malformation the cerebral cortex, hippocampus and cerebellum (168-170). The mammalian Disabled gene (mDAB1) is a cytoplasmatic adaptor protein that functions downstream of reelin, and is predominantly expressed in neurons. mDAB1 deficient mice develop a phenotype indistinguishable from *reeler* mice (171-173). Binding of reelin to the VLDLR and to the apoER2 is reported to induce phosphorylation of mDAB1 (144). These results indicate that the apoER2 and the VLDLR are the cell surface receptors that mediate the transmission of the Reelin signal across the neuronal cell membrane.

In addition to regulating neuronal migration via reelin *in vivo*, the VLDLR has been reported to mediate cell migration *in vitro* via a uPA dependent pathway, as was reported before for the LRP. Like the LRP, VLDLR regulates uPAR catabolism (174). This implies that the VLDLR mediates cell migration via the same pathway as discussed before for the LRP (see section "The LDL receptor related protein"). Studies on smooth muscle cell migration using RAP to block LRP and VLDLR function show either inhibition (128, 175) or no effect (176) on migration. In contrast, studies using other cell types show increased cellular migration when LRP and/or VLDLR are either blocked or absent (127, 174, 177). Thus, the effect of the receptors on uPA dependent migration and the underlying mechanisms are still poorly understood.

1.3.8 Lipoprotein receptors and atherosclerosis

As discussed before for the chicken VTG receptor and the LDLR, lipoprotein receptors can indirectly affect atherosclerosis by regulating plasma lipoprotein levels. Additionally, lipoprotein receptors could affect atherosclerotic plaque formation directly. Receptors of the LDLR family have been detected in smooth muscle and endothelial cells of both healthy and atherosclerotic arteries, and in the macrophage derived foam cells of the atherosclerotic plaque.

In humans, LDLR expression is limited to the outer media, and is not detected in the intima of healthy or atherosclerotic arteries (178, 179). However, wild type mice whose macrophage population was replaced by LDLR deficient macrophages via bone marrow transplantation display reduced atherosclerotic lesion formation. These mice show normal plasma lipid levels, implying the macrophage LDLR mediates macrophage cholesterol uptake and foam cell formation (180). In contrast, transplantation of wild type bone marrow into LDLR deficient mice does not affect atherosclerotic lesion formation (181, 182). These seemingly contrasting results can be explained by LDLR expression regulation. LDLR expression is downregulated by high cellular cholesterol levels (183). Therefore, the LDLR might be downregulated in the wild type macrophages upon transplantation into the hypercholesterolemic LDLR deficient mice. These data indicate that the LDLR can directly affect foam cell formation in mice under conditions of modestly high serum cholesterol levels.

The VLDLR, LRP and the recently discovered LR11 are expressed in the vessel wall. Expression levels of VLDLR, LRP and LR11 in rabbit aorta markedly increase upon feeding of an atherogenic high cholesterol diet (104, 184). LRP expression is induced threefold by the atherogenic diet, while VLDLR expression levels are increased up to a factor 100 (184). In plaques, LR11 expression is limited to smooth muscle cells (104), while the LRP is expressed in

both smooth muscle cells and macrophages (178, 185, 186). The VLDLR is present in endothelial cells, smooth muscle cells and macrophages of the atherosclerotic plaque (184).

In contrast to the LDLR, the LRP and the VLDLR are not downregulated by intracellular cholesterol levels (148, 187, 188). Chinese hamster ovary cells overexpressing the VLDLR accumulate lipids and transform into foam cells upon incubation with β-VLDL (188). In a similar fashion, both the VLDLR and the LRP might contribute to lipid loading of smooth muscle cells and macrophages in the atherosclerotic plaque. In addition, both LRP and VLDLR could modulate the cellular composition of the plaque by affecting smooth muscle cell migration (see sections "The LDL receptor related protein" and "The VLDL receptor"). Finally, the VLDLR has been hypothesized to promote binding, uptake and/or transcytosis of lipoproteins by endothelial cells (189). Thus the VLDLR could modulate lipid influx into the vessel wall.

Figure 2 shows possible mechanisms by which LR11, LRP and VLDLR could affect atherosclerotic plaque formation. Future research on *in vivo* models will have to elucidate the relative roles of these receptors in the atherosclerotic process.

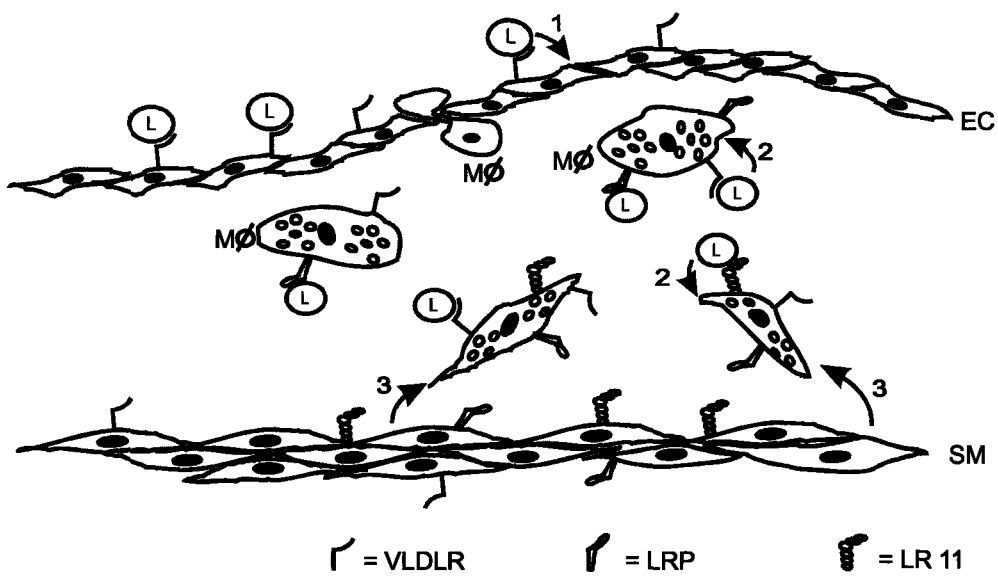

Figure 2. Possible mechanisms for involvement of lipoprotein receptors in atherosclerotic plaque formation

Lipoprotein receptors of the LDLR family could affect atherosclerotic plaque formation by mediating transport of lipids and lipoproteins into the plaque (1), foam cell formation (2) and smooth muscle cell migration (3).

L= lipoprotein particle, EC= endothelial cell, MΦ= macrophage

1.4 Apolipoproteins

Circulating lipoproteins carry specific apolipoproteins on their surface. Apolipoproteins play an important role in distribution and transport of lipoproteins. LDLR, VLDLR, LRP and megalin/gp330 are reported to interact with lipoproteins via apoE, while LDLR and megalin/gp330 can also bind lipoproteins via apoB100. ApoC1, apoC2 and apoC3 are reported to modulate binding of lipoproteins to lipoprotein receptors and LPL. The ApoEs and apoCs will be discussed in the next paragraphs.

1.4.1 Apolipoprotein E

ApoE is expressed by liver parenchymal cells, astrocytes and macrophages. Furthermore, apoE expression is detected in several organs, including spleen, lung, adrenal, ovary, kidney and muscle (190-193). ApoE is associated with circulating chylomicrons, chylomicron remnants, VLDL, IDL and HDL.

The human apoE gene has been shown to be polymorphic (194, 195). The apoE3 allele is the most common (70-80%), followed by the apoE4 (12-18%) and the apoE2 allele (3-12%) (196, 197). The apoE isoforms differ in their association with lipoproteins (198, 199), binding affinity for lipoprotein receptors (200-203) and interaction with HSPG (204, 205). ApoE deficiency and several rare dominant apoE mutations have been reported to cause a lipoprotein disorder called Familial dysbetalipoproteinemia (FD) (206-210). FD is characterized by accumulation of chylomicron and VLDL remnant lipoproteins in the plasma (211). Most FD patients are homozygous for the apoE2 allele, although only a small percentage of the homozygous apoE2 carriers eventually develop FD (212).

In addition to apoE genotype, apoE content is an important determinant of receptor binding properties of lipoproteins (213, 214). Lipoprotein apoE content has also been reported to affect the rate of triglyceride hydrolysis by LPL (215, 216). Thus regulation of apoE expression seems imperative for correct lipoprotein trafficking.

1.4.2 Apolipoprotein C

The human apoCs (apoC1, apoC2, apoC3 and apoC4) are regarded as members of a family because they all are low molecular weight proteins associated with chylomicrons, VLDL and HDL. All apoCs are expressed by the liver, while apoC2 and apoC3 are also expressed in intestine (217-221). Low apoC1 expression levels have also been found in lung, skin, testis and spleen (217). The apoCs are secreted in the circulation, where they rapidly exchange between HDL and chylomicrons/VLDL (222, 223).

Several apoC mutations, especially in the apoC2 gene, have been described, which are associated with altered plasma lipid concentrations. The clinical phenotype of apoC2 deficiency is similar to that of LPL deficiency, and is characterized by hypertriglyceridemia and high plasma chylomicron levels (224). ApoC2 has been reported to act as a cofactor for LPL mediated hydrolysis of triglycerides (225, 226).

Enrichment of chylomicrons and VLDL with a mixture of apoCs inhibits uptake by the liver (227-230). *In vitro* studies suggest that this is due to impaired binding of the apoC-enriched lipoprotein to their receptors. Lipoprotein binding to the LDLR is impaired by apoC1, apoC2

and apoC3 (231, 232). In addition, apoC1 and apoC2 also inhibit lipoprotein binding to the LRP (233).

In line with the previous data, apoC1 overexpression in mice results in increased plasma VLDL, triglyceride and cholesterol levels (234-236). The elevated lipid levels are due to impaired VLDL clearance by the liver (235-237). At least part of the effect of apoC1 on VLDL clearance can be attributed to inhibition of VLDL clearance by the LRP (238). In addition, apoC1 overexpression mice exhibit diminished abdominal adipose tissue stores and a complete deficiency of subcutaneous fat (239). In this respect it is interesting that apoC1 inhibits lipoprotein binding to the VLDLR (240), which plays a role in adipose tissue accretion (166). This suggests that apoC1 overexpression diminishes adipose tissue stores by inhibiting the binding of lipoproteins to the VLDLR.

1.5 Lipoprotein lipase

LPL is the principal enzyme responsible for the hydrolysis of triglycerides of circulating lipoproteins. Free fatty acids liberated by triglyceride hydrolysis enter either storage (241) or oxidative (242) pathways. Adipocytes, skeletal muscle cells and heart muscle cells are the major sites of LPL production. The LPL is secreted and transported to the luminal side of the vascular endothelium, where it is bound to heparan sulphate proteoglycans (HSPG) (243). LPL expression at relatively low levels is detected in a wide variety of tissues, including kidney, brain, aorta, spleen, lung, uterus intestine, mammary gland and diaphragm (244). Furthermore, LPL is expressed in smooth muscle cells and macrophages in the atherosclerotic plaque (245, 246).

In addition to HSPG binding, LPL contains a carboxy-terminal binding site for lipoprotein receptors of the LDLR family. LPL is reported to bind to LRP (116, 247-249), LDLR (250, 251), VLDLR (158) and megalin (132), thereby enhancing binding and internalization of lipoproteins. The mechanism behind LPL facilitated lipoprotein uptake by the lipoprotein receptors remains to be determined. It is proposed that LPL mediates lipoprotein binding to HSPG, which in turn transfers the LPL-lipoprotein complex to the lipoprotein receptor. However, Beisiegel et al. proposed that LPL promotes binding directly by bridging the lipoprotein particle to the receptor (116).

In humans, homozygous LPL deficiency is characterized by extreme hypertriglyceridemia and recurrent pancreatitis. While homozygous LPL deficiency is uncommon, heterozygous LPL deficiency is estimated to be present in 3-7% of the general population. Mutations in the LPL gene are reported to be the cause of familial chylomicronemia and premature atherosclerosis (252). Heterozygous LPL deficiency is associated with increased triglyceride and decreased HDL cholesterol levels (253-255).

LPL deficient mice display severe hypertriglyceridemia and die soon after birth. LPL heterozygous knockout mice are mildly hypertriglyceridemic, and display a decrease in VLDL catabolic rate (256). Muscle specific expression of catalytically inactive human LPL does not rescue LPL deficient mice from neonatal death. However, on an LPL heterozygous knockout background, muscle specific expression of catalytically inactive LPL lowers both plasma triglyceride and cholesterol levels. Furthermore, muscle uptake of radiolabeled VLDL

triglycerides and apolipoproteins is increased, implying LPL bridging of lipoproteins to lipoprotein receptors and/or HSPG occurs *in vivo* (257).

Specific expression of catalytically active human LPL in heart muscle causes lowering of plasma triglycerides in wild type mice, and rescues LPL deficient mice from neonatal death (258). LPL deficient mice expressing catalytically active human LPL in heart and skeletal muscle, but not adipose tissue, develop normally with regard to weight gain and body mass composition. However, the chemical composition of the adipose tissue of these mice indicates that adipocytes compensate for LPL deficiency by marked increase of *de novo* fatty acid synthesis (259). The role of adipose tissue LPL on profound weight gain has been assessed by crossbreeding mice with adipose tissue LPL deficiency with a mouse model for morbid obesity, the *ob/ob* mouse. The *ob/ob* mouse displays an increased appetite due to a deficiency in leptin, a protein produced by adipose tissue (260). Leptin acts in the hypothalamus to regulate neuronal activity and the production of neuropeptides that help to coordinate appetite and metabolism (54, 261, 262). Adipose tissue LPL deficiency results in decreased weight and fat mass in *ob/ob* mice, implying that LPL mediates adipose tissue triglyceride storage during profound weight gain (259).

The effect of LPL on atherosclerosis has been studied in transgenic mice. Transgenic mice expressing high levels of LPL in several tissues, excluding vessel wall macrophages, display high plasma LPL levels. The LPL transgenic mice have low serum cholesterol and triglyceride levels and display reduced atherosclerotic lesion formation. LPL heterozygote knockout mice also display reduced atherosclerotic lesion formation, despite an increase in cholesterol and triglyceride levels (263). These data suggest that plasma LPL can protect from atherosclerosis by lowering plasma lipoprotein levels. In contrast, LPL production by vessel wall macrophages could increase atherosclerotic lesion formation by inducing foam cell formation. This is supported by the finding that mice deficient for macrophage LPL display reduced atherosclerotic lesion size (264). Thus the contribution of LPL to atherosclerosis can be both pro- and anti-atherogenic, depending on the site of its expression.

1.6 Outline of this thesis

The studies presented in this thesis investigate the two receptors most closely related to the LDLR: the VLDLR and the apoER2. The role of the VLDLR in lipoprotein metabolism has been poorly defined. Studies in mice deficient for hepatic LRP and LDLR have suggested an extrahepatic, RAP-sensitive pathway for the metabolism of triglyceride rich lipoproteins (265). The VLDLR is present on vascular endothelium, and is expressed in tissues active in free fatty acid metabolism. Therefore, the VLDLR has been hypothesized to play a role in peripheral free fatty acid delivery. In addition, the expression of VLDLR in endothelial cells, smooth muscle cells and macrophages of atherosclerotic lesions could suggest a role for the VLDLR in atherosclerosis.

To examine the VLDLR *in vivo*, we have generated two transgenic mouse models that overexpress the human VLDLR. Chapter 2 discusses the generation of VLDLR transgenic mice by coinjection of two overlapping genomic human VLDLR fragments into murine zygotes (VLDLR-Tg mice). Human VLDLR expression in these mice is controlled by endogenous

promoter sequences present in the construct. Chapter 3 discusses the generation of VLDLR transgenic mice that overexpress the human VLDLR under control of the preproendothelin-1 promoter, which drives expression mainly in endothelial cells (PVL mice).

The role of the VLDLR in lipoprotein metabolism has been investigated in both PVL and VLDLR deficient mice (chapter 3). The effect of VLDLR deficiency and endothelial overexpression have been studied under conditions of impaired liver lipoprotein clearance. Therefore, both PVL and VLDLR deficient mice have been studied on an LDLR deficient background. Furthermore, dietary stress has been applied by feeding of a high fat diet, and/or prolonged fasting.

VLDLR deficient mice display a moderate reduction of adipose tissue mass (166). Chapter 4 discusses the effect of the VLDLR on obesity and obesity induced insulin resistance. Therefore, adult VLDLR deficient mice and wild type littermates have been fed a high fat/high caloric diet, which was reported before to induce obesity and insulin resistance in wild type mice. As a second model for obesity, VLDLR deficient mice have been studied on an *ob/ob* background.

Chapter 5 discusses the role of the VLDLR in atherosclerosis. First, the role of the VLDLR in smooth muscle cell migration has been determined *in vivo*. Smooth muscle cell migration is thought to be important in atherosclerotic neointima formation. We studied intimal thickening after vascular injury in VLDLR deficient mice as a model for smooth muscle cell migration. Second, the hypothesis has been tested that the VLDLR directs lipoproteins to the vessel wall, thereby affecting atherosclerosis. Therefore, atherosclerosis was studied in PVL mice on an LDLR deficient background. Third, the effect of VLDLR deficiency on atherosclerosis was determined in VLDLR deficient mice on an LDLR deficient background.

Finally, the lipoprotein binding characteristics of the recently discovered apoER2 have been investigated *in vitro*. Chapter 6 discusses the effect of different apoE variants and apoE deficiency upon VLDL binding to the apoER2. In addition, the effect of LPL on VLDL binding by the apoER2 has been determined.

Our studies clearly demonstrate involvement of the VLDLR in lipid metabolism. The results of our studies will be discussed in chapter 8.

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CHAPTER 2

Effective generation of VLDL receptor transgenic mice by overlapping genomic DNA fragments: high testis expression and disturbed spermatogenesis

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Abstract

The generation of functional transgenes via microinjection of overlapping DNA fragments has been reported previously to be successful, but is still not a widely applied approach. Here we show that the method is very reliable, and should be considered, in case a single large insert clone of the desired gene is not available. In the present study, two large DNA fragments consisting of overlapping cosmids, together constituting the human very low density lipoprotein receptor (VLDLR) gene (35 kb), were used to generate VLDLR transgenic (VLDLR-Tg) mice. Three transgenic founders were born, of which two (strain #2 and #3) generated transgenic offspring. Using Fiber-FISH analysis, the integration site was shown to contain at least 44 and 64 DNA fragments in mouse strains #2 and #3, respectively. This copy number resulted in integration sites of 1.5 and 2.5 megabase in size. Notably, over 90% of the fragments in both mouse strains #2 and #3 were flanked by their complementary fragment. In line with this observation, Southern blot analysis demonstrated that the correct recombination between fragments predominated in the transgenic insertion. Human VLDLR expression was detected in testis, kidney and brain of both mouse strains. Since this pattern did not parallel the endogenous VLDLR expression, some crucial regulatory elements were probably not present in the cosmid clones. Human VLDLR expression in testis was detected in germ cells up to the meiotic stage by *in situ* mRNA analysis. Remarkably, in the F1 generation of both VLDLR-Tg mouse strains the testis was atrophic and giant cells were detected in the semineferous tubuli. Furthermore, male VLDLR-Tg mice transmitted the transgene to their progeny with low frequencies. This could imply that VLDLR overexpression in the germ cells disturbed spermatogenesis.

Introduction

Particularly in cases where it is not possible to isolate a fragment that contains the entire gene of interest, the use of smaller overlapping DNA fragments, followed by co-injection into murine zygotes, is a convenient and efficient technique to generate transgenic mice. Previously, two independent studies showed that co-injection of 2-3 overlapping DNA fragments resulted in the correct reconstitution and expression of a transgene (1, 2). In the study by Pieper, a lambda library was used to isolate three relatively small (7, 13 and 17 kb) overlapping fragments, together constituting the human serum albumin gene. Co-injection of these fragments was reported to lead to reconstitution of the gene in 74% of the transgenic mice generated. Migchielsen used 2 cosmid clone inserts (29 and 39 kb) to successfully generate transgenic mice expressing the human adenosine-deaminase gene. In the present study, the technique of co-injecting overlapping DNA fragments was applied because no large insert clones were available for the gene of interest, the human very low density lipoprotein (VLDL) receptor (VLDLR). Furthermore, our detailed analysis of the integration site using Southern blot and fiber-FISH analysis showed, that the homologous recombination, which is thought to mediate the reconstitution of the VLDLR gene, is a highly efficient process.

The VLDLR is a member of the low density lipoprotein receptor (LDLR) family (3), that has been reported to bind multiple ligands. The VLDLR binds apoE rich VLDL (3, 4), receptor associated protein (RAP) (5), lipoprotein lipase (6), urokinase-type plasminogen activator (uPA) plasminogen activator inhibitor (PAI-1) complexes (7) and several other serpin proteinase/serpin complexes (8). However, the physiological importance of these ligand-binding capacities remains to be elucidated. Homozygous VLDLR knockout mice show a modest decrease in body weight, body mass index, and adipose tissue mass (9). This relatively mild phenotype could be explained by the presence of many LDLR family members, that show distinct, but overlapping ligand binding characteristics. Mice that are deficient for both the VLDLR and the closely related apolipoprotein E receptor 2 were reported to display disturbed neuronal migration during fetal brain development (10). To complement the VLDLR knockout model, our aim was to study the VLDLR in an overexpression mouse model.

The expression pattern of the human transgene partly overlapped with the endogenous expression pattern of the mouse, and was characterized by high-level testis expression in germ cells. Interestingly, the early generations of the VLDLR-Tg mice showed atrophy of the testis, and a reduced transmission of the transgene. This was observed in two independent mouse strains, and suggests that the development of germ cells overexpressing the VLDLR was disturbed.

Methods

Generation of transgenic mice

A human cosmid library, generated by partially digesting human DNA with *MboI* and subcloning the fragments in SupercosI, was screened for the VLDLR gene. Therefore, the complete human VLDLR cDNA (pHV58), a kind gift of dr. Lawrence Chan, was used as a probe. Positive cosmid clones were analyzed by Southern blot analysis, making use of a series of small cDNA probes spanning the VLDLR gene. Cosmid clone 3.1 spanned a region of 15 kb 5' of the gene to exon 15. Cosmid clone 10.9 spanned a region of exon 4 to 23 kb 3' of the gene. To separate the inserts from the vector, both cosmids were digested with *NotI*, precipitated, and resuspended in TE (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA) buffer. The DNA solution was then layered onto a continuous linear gradient of 10 to 40% sucrose in 1 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and centrifuged at 130,000 *g*. Gradient fractions were collected to determine which fractions only contained the insert, and these were pooled. Gradient medium was removed by five successive rinses with TE in a Centricon 100 microconcentrator (Amicon, Danvers, U.S.A.). Both fragments were diluted in TE to a concentration of 7 ng/ml. This mixture was injected into B:CBA zygotes to generate transgenic mice according to standard procedures (11).

To screen for mice carrying correctly reconstituted human VLDLR genes, mouse liver DNA was digested with *StuI*. The region of overlap between the inserts of the two cosmids is flanked by *StuI* restriction sites. This *StuI* fragment of approximately 9 kb is therefore diagnostic for correct integration of the transgene by homologous recombination between the two

overlapping DNA fragments (figure 1). Detection of the diagnostic fragment was performed by Southern blot analysis. An exon 6 to 14 spanning *HincII* restriction fragment of the cDNA, pHV58.H, was used as probe. Founder offspring was characterized by PCR on tailtip DNA with forward primer U19 (CCTTTGAGGTCTAAACAAAT) and reverse primer L19 (TTTACAGATGGCCTATACAA), both localized in exon 19 and specific for the human VLDLR gene. Transgenic offspring was bred onto a C57BL/6 background.

Fiber-FISH

DNA fibers were prepared from mouse lymphocytes according to the halo technique (12) as modified by Florijn et al (13). Cosmids 3.1 and 10.9 were labelled by standard nick-translation with digoxigenin-11-dUTP or biotin-11-dUTP. The hybridization solution consisted of 3 ng probe per µl for each cosmid. Probes were dissolved in 30% formamide, 10% dextran sulphate, 2xSSC, 50 mM sodium phosphate pH 7.0, 5 mM EDTA containing a 10 times excess of Cot1 DNA. Probe mixes were directly applied to slides containing DNA fibers and denatured for 2 min at 80°C. Hybridization was overnight at 37°C in a moist chamber. Post hybridization washes consisted of 3 x 10 min washes with 2xSSC at 37°C. Bi-colour immunofluorescence detection of digoxigenin and biotin in FITC and Texas Red, respectively, was accomplished as described in Florijn et al. (14). Also fluorescence microscopy, imaging and analysis was performed according to Florijn et al. (14). Three slides of an F3 male of both mouse strain #2 and strain #3 were analyzed.

Quantitative analysis of mouse versus transgene expression

A semi-quantitative RT-PCR method was used to examine the level of transgene expression relative to endogenous mouse VLDLR expression, as described before (see Chapter 3). RNA of different mouse tissues was isolated with RNAzol™ (Biotecx Laboratories, USA), and RT-PCR was performed with the use of Superscript™ II RNase H Reverse transcriptase (GibcoBRL, USA), both according to the manufacturer's protocol.

Splice variant specific PCR

Relative expression levels of the two VLDLR variants that include or lack exon 16, coding for the O-linked sugar domain, were determined by PCR with forward primer VR4 (AATATACCTGTTCCTGTCCC, bp 2305 to 2324 in human) and reverse primer VR1 (TTCAAGTACACAGGATTGTC, bp 2610 to 2629 in human). Both primers show 100% sequence identity to mouse and human VLDLR sequences.

Northern blot analysis

mRNA was isolated from aorta, heart, brain, adrenal, muscle, testis, kidney, liver, spleen and adipose tissue of transgenic and wild type mice. Northern blot analysis was performed using the pHV58 cDNA as probe. Membranes were reprobed with a rat GAPDH probe (15).

Histological analysis

Organs from transgenic mice and wild type littermates were obtained and fixed in formalin. After fixation, samples were embedded in paraffin and 5 μm sections were cut. Sections were stained with hematoxylin/eosin.

RNA in situ hybridization

In the F2 generation of both mouse strains, testes from VLDLR transgenic mice and wild-type littermates were dissected, fixed in 4% paraformaldehyde and embedded in paraffin (Stemcowax, Adamas Instruments BV, Amerongen, The Netherlands). Five-μm sections were mounted on RNAse free 3-aminopropyl triethoxysilane-coated slides (Sigma Chemical Co, St Louis, MO) and dried at 42°C. Sections were de-waxed and fixed in 4% paraformaldehyde, treated with 20 μg/ml proteinase K (Roche Diagnostics Nederland B.V., Almere, The Netherlands) for 20 min and fixed again. To avoid non-specific binding of probe to amino acid residues, sections were treated with acetic anhydride in 0.1 M triethanolamine HCl (pH 8.0) for 10 min. Sections were dehydrated till 100% EtOH and air dried.

DIG labeled human VLDLR probes (bp 2305 to 3217), both sense and antisense, were prepared by using the DIG-mRNA labeling kit (Roche Diagnostics Nederland B.V.). Probes were quantified and dissolved in hybridization mix (50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl, 5 mM EDTA (pH8.0), 10% dextrane sulphate, 1x Denhardt's solution, 0.5 mg/ml tRNA) at 0.1 ng/μl. Probe was heated at 80°C for 2 min before pipetting onto the sections. Hybridization was performed at 55°C, for 16 h. Sections were washed in 5X SSC at 55°C and 50% formamide, 2X SSC at 65°C for 30 min, subsequently. Sections were extensively washed in NTE buffer (0.5 M NaCl, 10 mM Tris-HCl, 5 mM EDTA) at 37°C. Unbound probe was degraded by RNase treatment in 20g/ml RNase A in NTE buffer at 37°C. Finally, sections were washed in 2X SSC at 65°C, and 0.1X SSC at room temperature.

To visualize bound DIG-labeled probe, sections were incubated in PBS, containing 10% horse serum for 1 h at RT, to block non-specific binding of antibodies, where after sections were incubated with a monoclonal mouse anti-DiG antibody (Roche Diagnostics Nederland B.V.), 1:200 in PBS containing 5% horse serum, in a humidified chamber at room temperature. After extensive washing steps in PBS, slides were incubated for 60 min with a secondary biotinylated anti-mouse IgG (Elite ABC-peroxidase staining kit, Vector Laboratories Inc., Burlingame, CA), diluted 1:200 in PBS including 5% normal horse serum, in a humidified chamber at room temperature. The avidin-biotin complex reaction was performed according to the manufacturer's protocol. To visualize bound antibodies, sections were washed in PBS and covered with 0.3 μg/μl 3, 3' diaminodibenzene (DAB, Dako Corp., Carpintera, CA) in PBS, to which 0.03% H_2O_2 was added. Sections were counter-stained with Mayer's hematoxylin.

Results

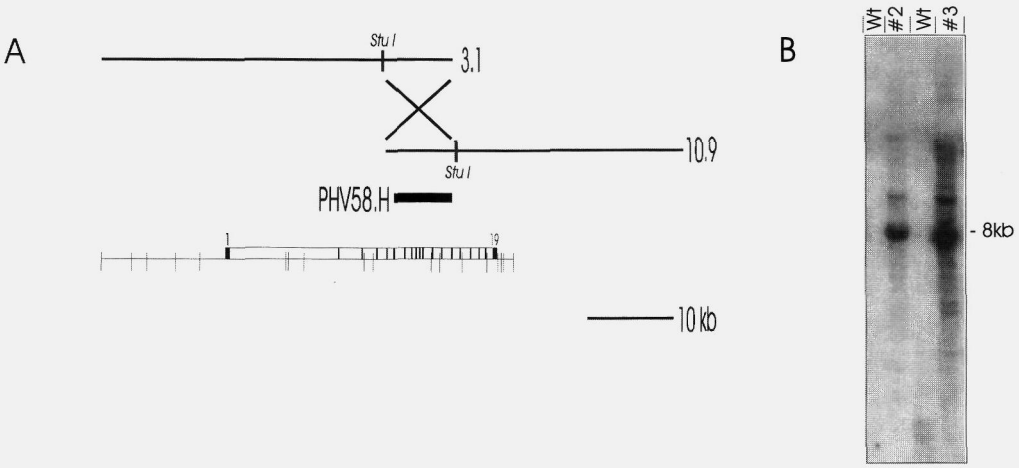

Figure 1: Generation and Southern blot analysis of VLDLR-Tg mice
Schematic representation of the inserts of cosmids 3.1 and 10.9. StuI sites flank the diagnostic fragment. Probe pHV58.H spans exon 6 to 14 within the diagnostic fragment. Boxed area represents the human VLDLR gene, which contains 19 exons. Bottom line represents an EcoRI restriction map of the human VLDLR region (A). Genomic DNA of mice from mouse strain #2 and mouse strain #3 and their wild type littermates (Wt) was digested with *StuI* and subjected to electrophoresis. The human VLDLR was detected by Southern blot analysis, using the pHV58.H probe (B).

Generation and characterization of transgenic mice

The screening of the cosmid library, using PHV58 as a probe, resulted in the isolation of 6 VLDLR positive clones. Cosmids 3.1 (40 kb insert) and 10.9 (35 kb insert) were used to generate transgenic mice, since they spanned the largest genomic region surrounding the VLDLR. Together they comprised the entire human VLDLR gene, including 15 kb of 5' and 23 kb of 3' sequences (figure 1A). The DNA was injected into murine zygotes, which were implanted into pseudo-pregnant foster mothers. The offspring was characterized by PCR. Three transgenic founders were identified, of which two (strain #2 and #3) generated transgenic offspring. Strain #2 and strain #3 DNA was further analyzed by Southern blotting, using the pHV58.H probe. This cDNA probe, which spans exon 6 to 14 of the human VLDLR, hybridizes to an 8 kb *StuI* fragment that is diagnostic for correctly reconstituted transgenes. Figure 1B shows that in both mouse strains the diagnostic 8 kb fragment was the predominant restriction fragment recognized by the pHV58.H probe. However, in both mouse strains several larger bands were found. This could be attributed to border fragments and fragments that did not recombine correctly.

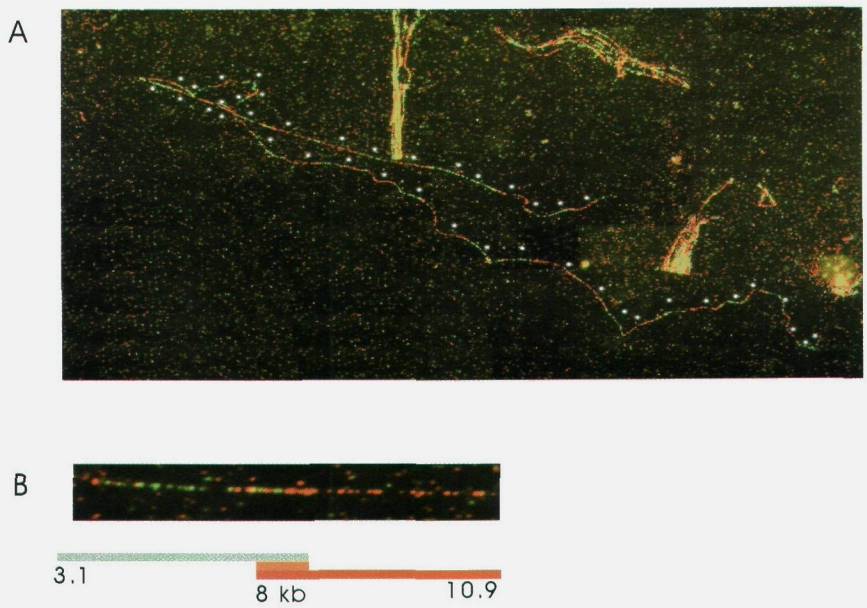

Figure 2: Structure of transgene integration site
Panel (A) shows Fiber-FISH analysis of the transgene integration site in mouse strain #2, using cosmids 3.1 (green) and 10.9 (red) as probe. White dots mark each individual integrated DNA fragment. Panel (B) shows recombination between fragment 3.1 and 10.9 in detail together with a schematic representation. Overlapping region between cosmids 3.1 and 10.9 stains yellow.

Fiber-FISH analysis of transgene integration site

Fiber fish analysis of lymphocyte DNA of mice of the F3 generation of strains #2 and #3 revealed that the transgenic DNA fragments were integrated as large tandem repeats. Many, or perhaps all of the integration sites that were visualized were only partly representing the transgenic insertion, because of limitations to the maximum size of fibers that can be demonstrated with the fiber-FISH technique. Analysis of the fibers indicated that in both mouse strain #2 and #3 there is a single integration site, since the same pattern of alternating fragments recurred in all fibers analyzed in each strain. The largest intact fiber found in mouse strain #2 carried 44 integrated transgenic DNA fragments, implying an integration site of at least 1.5 megabase (figure 2A). The largest intact fiber found in strain #3 mice showed an integration site containing 64 transgenic DNA fragments, implying an integration site of at least 2.5 megabase. Figure 2B shows recombination between fragment 3.1 and 10.9 in detail. In strain #2 mice, 98% of the fragments that integrated were flanked by their complementary fragment at either side (figure 2A). In strain #3 mice 93% of the fragments were flanked by their complementary fragment at either side (data not shown).

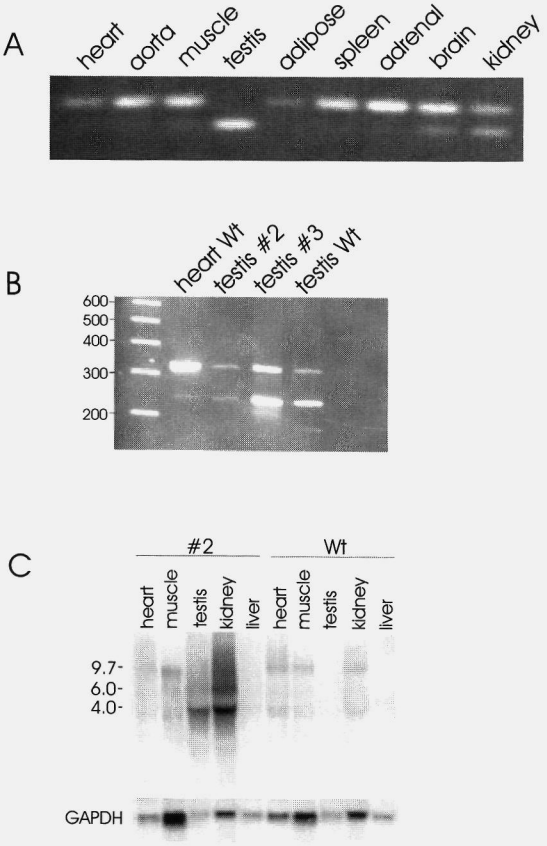

Figure 3: Expression of human VLDLR transgene

To determine human versus mouse VLDLR RNA expression levels, semi-quantitative RT-PCR was performed on total RNA isolated from various VLDLR-Tg strain #2 mouse tissues. PCR products were separated by electrophoresis on an ethidium stained 3% agarose gel (A). Relative expression levels of VLDLR splice variants were determined by PCR in testis of wild type (Wt), strain #2 (#2) and strain #3 (#3) mice and heart of Wt mice as described in Methods. The 325 kb band represents the splice variant containing, while the 241 kb band represents the variant lacking the O-linked sugar domain (B). Northern blot analysis of VLDLR-Tg strain #2 (#2) and wild type (Wt) RNA was performed with the ^{32}P-labeled pHV58 probe. The membranes were re-probed with a ^{32}P-labeled GAPDH cDNA probe (C).

Analysis of transgene expression

The semi-qantitative PCR method was used to determine the relative amount of human versus endogenous mouse VLDLR expression (see chapter 3). This method is based on the competition of 2 primers, one specific for mouse and one specific for human VLDLR sequences, for a common third primer. Mouse strain #2 and #3 showed identical expression patterns. In testis, human VLDLR expression levels were very high when compared to endogenous mouse VLDLR levels (figure 3A). In kidney and in brain expression levels were comparable to those of the endogenous VLDLR gene. In the other tissues tested, human VLDLR expression levels were below the detection level of the semi-quantitative PCR. This means human VLDLR expression levels were at least 16 times lower than endogenous VLDLR expression levels.

In both mice and humans, two splice variants of the VLDLR are expressed, one lacking and one containing exon 16, which codes for the O-linked sugar domain. The relative amount of expression of both variants is tissue dependent. In mouse heart, the variant containing exon 16 is the most abundant, while in testis the variant lacking exon 16 is the most abundant. To examine if both VLDLR splice variants are expressed correctly in the transgenic mice, RT-PCR was performed with primers spanning exon 15 to 18. Figure 3B shows that both variants were

expressed in the testis of both transgenic mouse strains, and that their relative amounts of expression did not differ significantly from those in the wild type mouse.

Northern blot analysis of mouse mRNA was reported to reveal a 9.7 kb and 4 kb band (16), while that of human mRNA reveals a 4 and a 6 kb band (17). Figure 3C shows that there was cross-reaction between the human pHV58 cDNA probe and the mouse mRNA. However, both testis and kidney show higher VLDLR expression levels in transgenic as compared to wild type mice. Both 4 and 6 kb bands were detected, indicating that the human VLDLR transgene is transcribed correctly.

Transgene inheritance

Transgenic founder mice are often chimeric, resulting in low numbers of transgenic offspring in the F1 generation. Remarkably, both mouse strains #2 and #3 also showed significantly less transgenic than wild type offspring in the F2 generation when male F1 hemizygous VLDLR-Tg mice were used for breeding (table 1). When female F1 hemizygous VLDLR-Tg mice were used for breeding, transgene inheritance followed Mendelian rules (table 1). This abnormal transgene inheritance pattern was also found in the F3 generation of strain #3, but not of strain #2, VLDLR-Tg mice and was also absent in subsequent generations.

Table I: Efficiency of transgene transmission to progeny

Generation of breeding parents	Strain	Number of VLDLR-Tg male breeding parents	Offspring of male VLDLR-Tg breeding parent Transgenic/total (%)	Number of VLDLR-Tg female breeding parents	Offspring of female VLDLR-Tg breeding parent Transgenic/total (%)
F1	#2	2	8/37 (22%)*	4	21/39 (54%)
	#3	3	26/88 (29%)*	5	33/68 (49%)
F2	#2	3	26/52 (50%)	4	28/53 (53%)
	#3	3	17/56 (30%)*	5	26/58 (45%)

Male and female VLDLR-Tg mice were bred with non-transgenic C57BL/6 mice to generate transgenic offspring. * = significantly different from 50%, using the chi-square test ($P<0.05$).

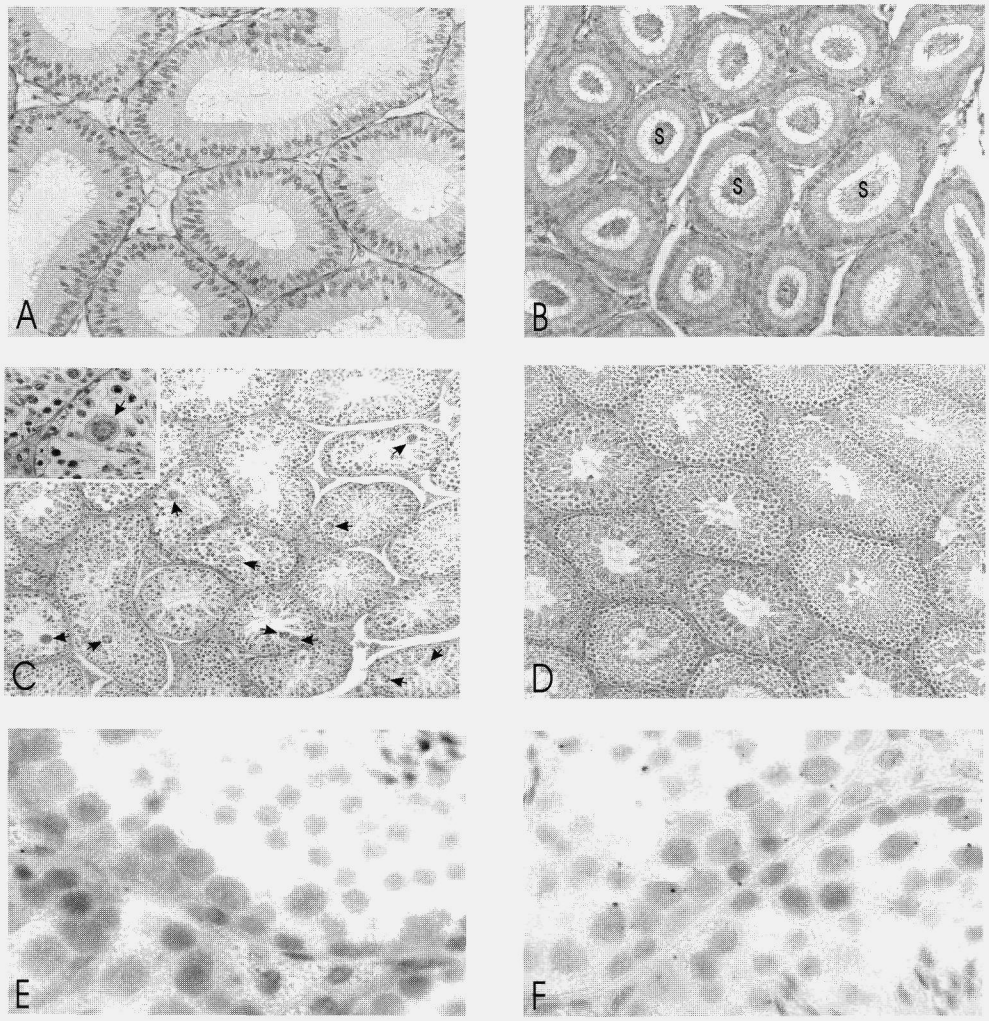

Figure 4: Histological analysis of VLDLR-Tg mice
Epididymis of strain #2 harboured no sperm cells (A), while large numbers of sperm cells (s) were observed in epididymis of wild type littermates (B). The seminiferous tubules in strain #2 testis showed atrophy with multinucleated giant cells (arrows); inset shows a multinucleated giant cell in detail (C). In testis of wild type littermates no abnormalities were observed (D). Panels (E) and (F) show human VLDLR mRNA *in situ* hybridisation on seminiferous tubules of strain #2 and wild type littermates, respectively. Positive staining indicates human VLDLR expression in early developmental stages of strain #2 germ cells.

Histological analysis of transgenic mice

Histological analysis of VLDLR-Tg mice did reveal gross abnormalities in the testis of mice in the F1 generation of mouse strain #2 and #3. In mouse strain #2, but not strain #3, these

abnormalities were also detected in the F2 generation. The epididymis of these mice harbored no or only small numbers of mature sperm cells (figure 4A and 4B). The seminiferous tubules showed signs of atrophy, and large amounts of giant cells were present (figure 4C and 4D). In the F2 generation of both mouse lines, analysis of human VLDLR expression in testis by RNA in situ hybridisation revealed that expression was detected in Sertoli cells (not shown) and in germ cells up to the meiotic division (figure 4E and 4F). Expression of human VLDLR mRNA in Leydig cells was difficult to establish due to high background staining. However, Leydig cell staining was often more intense in VLDLR-Tg mice than in wild type littermates. Thus the transgene appeared to be expressed in both somatic as well as germ cells.

Discussion

The present study describes the efficient generation of VLDLR-Tg mice using two overlapping DNA fragments. Southern blot analysis revealed that most fragments were recombined correctly by homologous recombination to reconstitute intact human VLDLR genes. Fiber-FISH analysis was used to study the structure of the transgenic insertion more closely. The cosmid fragments were shown to be integrated in very large arrays in both mouse strains, thereby generating integration sites of at least 1.5 and 2.5 megabase. Furthermore, fiber-FISH analysis revealed that more than 90% of the integrated cosmid inserts were flanked by their complementary fragment. The large number of correctly reconstituted VLDLR genes in the transgenic insertion could be explained by the fact that extrachromosomal (homologous) recombination (ECR) is very active in fertilized oocytes. This process is followed by integration of the recombined molecule into the genome. In cultured mammalian cells, the frequency of ECR between cotransferred plasmids varies widely (18). However, ECR frequencies observed upon stable transfection are higher than those observed in transient transfection assays. This has led to the hypothesis that ECR is coupled to subsequent integration of the recombinant molecule (19). Therefore, the number of successful integrations in founders #2 and #3 does not necessarily reflect ECR frequencies in murine zygotes.

Northern blot analysis on VLDLR-Tg mouse tissues revealed that the size of the human VLDLR transcripts was correct. However, the expression pattern of the VLDLR transgene only partly overlapped the natural expression pattern of the VLDLR. In human, as well as in mouse, the VLDLR is expressed in heart, skeletal muscle, kidney, adipose tissue, ovary, testis and brain (16, 17, 20-22). In both VLDLR-Tg mouse strains, human VLDLR expression was only detected in testis, kidney and brain. Most likely, not all regulatory elements necessary to obtain a natural expression pattern lie within 15 kb 5' and 23 kb 3' of the human VLDLR gene. It is not exceptional for regulatory elements to be located at large distances from the genes they regulate. Genes that were reported before to have regulatory regions that are located at a distance of more than 15 kb up- or downstream of the coding region include apoE, human growth hormone and beta-globin (23-26).

When male F1 transgenic mice of both mouse strains were mated with wild type mice, the majority of the F2 offspring consisted of non-transgenic mice. This effect on transgene

inheritance was not seen when female F1 transgenic mice were mated with wild type mice. The skewing in progeny of F1 generation male transgenic mice coincided with abnormalities in their testis. The testis showed signs of atrophy and large amounts of giant cells were observed. Giant cells are thought to be a marker for disturbed germ cell development. Presumably these cells are formed upon a blockade in sperm maturation, causing the cells to fuse. Taken together, these data suggest that the VLDLR transgene makes the sperm cell more prone to a blockade in its development. The effect of the VLDLR transgene on germ cell viability could be due to either the physical presence of the VLDLR transgene in the mouse genome or to its expression. Unfortunately we could not establish why the phenotype disappeared in later generations. We could not detect changes in transgene copy number and global expression levels between subsequent generations (data not shown). In addition, no differences were detected in the F2 generation between cell type specific transgene expression in testes of both mouse strains. Since the testes of F2 generation strain #2 mice showed signs of atrophy while those of strain #3 mice appeared normal, it seems unlikely that the disappearance of the phenotype is related to a change in cell type specific transgene expression. This is supported by the observation that the relative expression levels of the two VLDLR splice variants, which are differently expressed in a cell type specific manner (27-29), remained the same in the F1 to F4 generation (data not shown).

Human VLDLR expression in VLDLR-Tg mouse germ cells was confirmed by *in situ* mRNA analysis. It revealed that the human VLDLR was expressed in developing germ cells up to the meiotic stage and in Sertoli cells. This resembles the situation in the chicken, where the chicken homologue of the VLDLR was also reported to be expressed in the developing germ cells. In chicken however, expression is confined to the late post-meiotic stage of spermatogenesis (27).

The high VLDLR expression levels in VLDLR-Tg germ cells could interfere with germ cell migration. During their development, germ cells migrate from the basal side inward towards the luminal side of the seminiferous tubules. The VLDLR was described before to regulate cell migration, both via the reelin-signaling pathway (10) and via catabolism of the uPA receptor (uPAR) (30). Interestingly, fertility problems were reported before in male mice that are deficient for another member of the LDLR family, the apolipoprotein E receptor 2 (10).

In the present study both independently generated VLDLR-Tg mouse strains showed a similar phenotype. Thus it seems unlikely that the integration site itself is the cause of the disturbed germ cell development. Furthermore, in our experience with the generation of transgenic mice using large YACs, we never detected aberrations in germ cells or skewing in the numbers of transgenic offspring. This is supported by other reports on YAC transgenic mice, in which a normal 50% transmission of the transgene to the progeny was found (31, 32).

In conclusion, we have shown that the use of two overlapping DNA fragments resulted in large integration sites, containing a high percentage of reconstituted, transcriptionally active transgenes. This makes it a useful and convenient technique for generating transgenic mice.

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CHAPTER 3

LDL receptor deficiency unmasks altered VLDL triglyceride metabolism in VLDL receptor transgenic and knockout mice

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Abstract

The very low density lipoprotein receptor (VLDLR) has been proposed to play a role in the delivery of fatty acids to peripheral tissues. However, despite reduced adipose tissue mass in VLDLR deficient (*VLDLR-/-*) mice this has been difficult to substantiate. In the present study, VLDLR deficient and VLDLR overexpressing (PVL) mice were crossbred onto a low density lipoprotein receptor knockout (*LDLR-/-*) background to study the VLDLR under conditions of relatively high serum VLDL and triglyceride levels. Absence of the VLDLR resulted in a significant increase in serum triglyceride levels (1.9 fold) when mice were fed a high fat diet. In contrast, overexpression of the VLDLR resulted in a significant decrease in serum triglyceride levels (2.0 fold) under similar conditions. When kept on a chow diet, a period of prolonged fasting revealed a significant increase in serum triglyceride levels in *VLDLR-/-;LDLR-/-* mice (2.3 fold) as compared with *LDLR-/-* controls. This could not be attributed to altered apolipoprotein B and VLDL triglyceride production rates. Furthermore, no major differences in nascent VLDL triglyceride content were found between *VLDLR-/-;LDLR-/-* mice and *LDLR-/-* controls. However, the triglyceride content of circulating VLDL of *VLDLR-/-;LDLR-/-* mice (63%) was relatively high as compared with *LDLR-/-* controls (49%). These observations suggest that the VLDLR affects peripheral uptake of VLDL triglycerides. In conclusion, under conditions of LDLR deficiency in combination with high fat feeding or prolonged fasting the effect of the VLDLR on VLDL triglyceride metabolism was revealed.

Introduction

The VLDLR is a member of the low density lipoprotein receptor (LDLR) family (1). It has been described to bind apolipoprotein E (apoE) rich very low density lipoprotein (VLDL) (1, 2) and lipoprotein lipase (LPL) (3), suggesting a role for the VLDLR in lipid metabolism. However, other ligands such as receptor-associated protein (RAP) (4) and urokinase-type plasminogen activator plasminogen activator inhibitor complexes (5) were also described to bind to the VLDLR. In addition, the VLDLR was found to play a role in transmission of extra-cellular signals to intra-cellular signaling processes, in a molecular pathway that regulates neuronal migration (6).

The VLDLR is expressed in blood vessels, where expression is located in endothelial and smooth muscle cells (7). Furthermore, expression levels are high in heart, skeletal muscle and adipose tissue, but expression is absent from liver. Heart, skeletal muscle and adipose tissue are active in fatty acid metabolism. Together with the observation that the VLDLR can bind apoE rich VLDL this has led to the hypothesis that the VLDLR is involved in delivery of fatty acids to extra-hepatic organs. This hypothesis is supported by observations in mice showing upregulation of VLDLR mRNA levels in heart and downregulation in adipose tissue after prolonged periods of fasting (8). In addition, *LDLR-/-* mice fed an atherogenic diet show downregulation of VLDLR mRNA levels in heart and upregulation in adipose tissue (9). However, plasma cholesterol and triglyceride levels were not affected in *VLDLR-/-* mice. So far the only *in vivo*

evidence implying a role for the VLDLR in lipid metabolism is the observation that *VLDLR-/-* mice show reduced adipose tissue mass (10).

In wild type as well as in *VLDLR-/-* mice, high density lipoprotein (HDL) is the predominant lipoprotein in plasma, while plasma levels of VLDL are relatively low. Thus differences in VLDL metabolism between wild type and *VLDLR-/-* mice are difficult to detect. We chose to study the VLDLR in the *LDLR-/-* mouse model, which displays relatively high serum VLDL and triglyceride levels (11). Both the *VLDLR-/-* mouse and a newly generated transgenic mouse model that expresses the human VLDLR in endothelial and smooth muscle cells (PVL mouse) were used. These mice were fed either a chow or a high fat diet, and serum lipid levels were studied both after 4 hours and after overnight fasting.

LDLR deficiency in combination with a high fat diet or overnight fasting led to elevated serum triglyceride levels in the absence of the VLDLR. In contrast, overexpression of the VLDLR led to lowering of serum triglyceride levels. While there was no major difference in nascent VLDL triglyceride content between *VLDLR-/-;LDLR-/-* mice and *LDLR-/-* controls, *VLDLR-/-;LDLR-/-* mice showed a relatively high circulating VLDL triglyceride content. This suggests the VLDLR has an effect on the peripheral uptake of VLDL triglycerides.

Methods

Generation of transgenic mice

To express an additional VLDLR in the endothelium, an expression construct was generated using the human VLDLR cDNA in combination with the preproendothelin-1 promoter. This promoter was previously shown *in vivo* to express a luciferase reporter gene mainly in endothelial cells, and to a lesser extent in smooth muscle cells of the vessel wall of transgenic mice (12). The pHV58 plasmid, carrying the human VLDLR cDNA, was generously provided by L. Chan (13). The HL3 reporter plasmid, carrying the firefly luciferase gene under control of the 5.9 kb promoter region of the endothelin-1 gene, was described before as p5.9mPPET-LUC (12). The HL3 reporter plasmid was digested with *NotI* to remove the luciferase reporter gene. The pHV58 plasmid was digested with *NotI* to isolate the human VLDLR cDNA, that was subsequently subcloned in the *NotI* digested HL3 reporter plasmid, generating the PVL plasmid containing the human VLDLR cDNA under control of 5.9kb of the preproendothelin-1 promoter region (see figure 1).

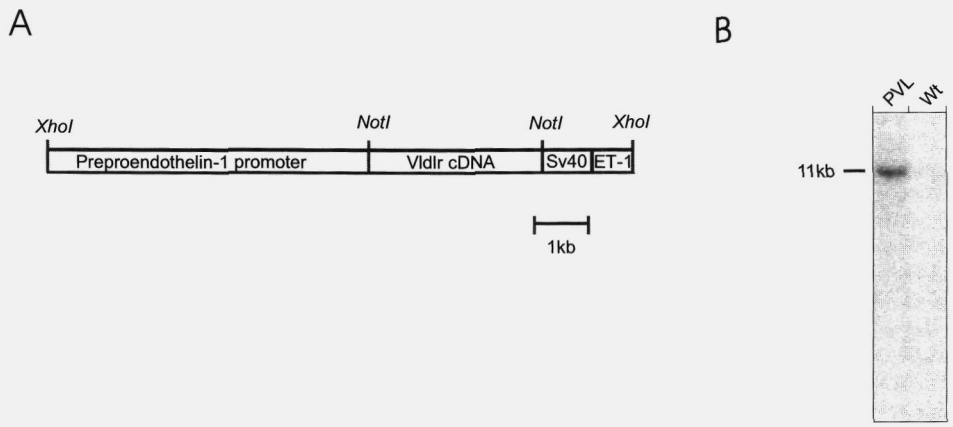

Figure 1. Generation and Southern blot analysis of PVL transgenic mice
Schematic representation of the generation of the PVL construct (A). Genomic DNA of wild type and PVL mice was digested with *XhoI* and subjected to electrophoresis. PVL construct was detected using the ^{32}P-labeled pHV58.H probe (B).

The PVL plasmid DNA was digested with *XhoI* to obtain the insert, and subsequently was layered onto a continuous linear gradient of 10 to 40% sucrose in 1.0 M NaCl, 10.0 mM Tris HCl, pH 8.0, 1 mM EDTA, and centrifuged at 35000 rpm. Gradient fractions were collected to determine which fractions only contained the PVL insert, and these were pooled. Gradient medium was removed by five successive rinses with injection buffer (10 mM Tris-HCl, pH7.5, 0.1 mM EDTA) in a Centricon 100 microconcentrator (Amicon, Danvers, Massachusetts, USA.). The PVL insert was diluted in injection buffer to a concentration of 6 ng/ml. This mixture was injected into B:CBA zygotes to generate transgenic mice according to standard procedures (14).

To screen for transgenic mice, offspring was characterized by PCR on tailtip DNA with forward primer U19 (CCTTTGAGGTCTAAACAAAT) and reverse primer L19 (TTTACAGATGGCCTATACAA), both localized in exon 19 of the human VLDLR. The size of the inserted construct was verified by digesting tailtip DNA with *XhoI,* followed by Southern blot analysis using an exon 6 to 14 spanning *HincII* restriction fragment of the pHV58 cDNA, pHV58.H, as a probe (figure 1B). Eight out of 40 offspring that were characterized by PCR were shown to carry the transgene (data not shown). Six of the founder mice produced transgenic offspring. All experiments in the present paper were performed with the mouse line that showed the highest human versus mouse VLDLR expression as was determined by semi-quantitative RT-PCR (data not shown).

Semi-quantitative analysis of human versus mouse VLDLR expression.

To measure the level of transgene expression relative to endogenous mouse VLDLR expression, a semi-quantitative RT-PCR method was developed. Reverse primer VR1

(TTCAAGTACACAGGATTGTC) shows 100% sequence identity to the human (bp 2610 to 2629) and mouse (bp 2678 to 2697) VLDLR cDNA sequence. Forward primer MVR1 (ACAGTGAGACAAAAGATATC, bp 2454 to 2473) is specific for mouse, and forward primer HVR5 (AACTCAACAGAAATTTCCAGC, bp 2406 to 2425) specific for human VLDLR cDNA sequence. Both the MVR1 and HVR5 primer will compete for the VR1 primer in a PCR. MVR1 and VR1 amplify 244 bp of mouse VLDLR sequence, while HVR5 and VR1 amplify 224 bp of human VLDLR sequence. Calibration of the RT-PCR reaction, using human and mouse VLDLR cDNA mixed in different relative amounts, proved that both amplifications were equally efficient (data not shown). Semi-quantitative RT-PCR was performed on total RNA isolated from various mouse tissues for 34 cycles at 52 °C. PCR products were separated by electrophoresis on an ethidium stained 3% agarose (agarose MP, Boehringer Mannheim, Mannheim, Germany) gel. The gel was photographed using the ImaGo^{tm} (B&L Systems, Maarssen, The Netherlands) and the relative amount of human vs. mouse product was calculated with ImageQuant software (Molecular dynamics, Sunnyvale, California, USA).

Northern blot analysis of total 10 μg of total heart RNA of PVL mice and wild type littermates was performed, using pHV58.H as a probe. Membranes were re-probed with rat GAPDH cDNA (15).

RNA of mouse tissues was isolated with RNAzol^{TM} (Biotecx Laboratories, Houston, Texas, USA), and RT-PCR was performed with the use of Superscript^{TM} II RNase H Reverse transcriptase (GibcoBRL, Gaithersburg, Maryland, USA), both according to the manufacturer's protocol.

Antibody

The rabbit polyclonal anti-human antibody, raised against amino acids 1-724 of the human VLDLR, was generously provided by H. Hobbs. The antibody has also been shown to recognize mouse VLDLR protein (16).

Western blot analysis

Membrane proteins fractions of mouse heart were isolated. Therefore, hearts were frozen in liquid nitrogen, homogenized with a tight fitting pestle in a solution of 0.25 M sucrose, 50 mM Tris (pH 7.4) and complete^{TM} EDTA free mini protein inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). Subsequently the suspension was centrifuged at 600 g, followed by centrifugation of the supernatant at 10000 g. Finally, the supernatant was centrifuged at 130000 g. The resulting pellet containing the membrane proteins was resuspended in TBS (10 mM Tris, 150 mM NaCl, 2 mM $CaCl_2$ and 2 mM $MgCl_2$). Protein concentration was determined with the Pierce BCA protein assay (Pierce, Rockford, Illinois, USE) according to the manufacturer's protocol. Eighty microgram of protein was subjected to SDS-PAGE on a 6% polyacridamidegel. Proteins were blotted onto Hybond^{TM} ECL^{TM} nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). The membrane was washed extensively in wash buffer, containing 0.1% (v/v) Tween-20 (Sigma, St. Louis, Missouri, USA) in phosphate buffered saline (PBS). Subsequently the membrane was blocked with blockbuffer, containing 0.1% (v/v) Tween-20 and 5% nonfat milk (w/w) in PBS, followed by incubation with rabbit polyclonal anti-human VLDLR antibody in blockbuffer. The membrane was then extensively

washed in blockbuffer, followed by incubation with a peroxidized anti rabbit IgG antibody (NIF824, Amersham Pharmacia Biotech, Little Chalfont, UK). Finally the membrane was washed with wash buffer, followed by washing with PBS and ECL™ detection according to the manufacturers protocol (Amersham Pharmacia Biotech, Little Chalfont, UK).

Mice

VLDLR-/- mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA) and were bred to *LDLR-/-* mice to produce *VLDLR+/-;LDLR+/-* mice. *VLDLR+/-;LDLR+/-* mice were subsequently bred to *LDLR-/-* mice to produce *VLDLR+/-;LDLR-/-* mice. *VLDLR+/-;LDLR-/-* mice were bred to produce *VLDLR-/-;LDLR-/-* and *LDLR-/-* littermates. PVL mice were crossed back onto a C57bl6/J background, and generation N3 mice were used. PVL mice were bred to *LDLR-/-* mice to produce PVL;*LDLR+/-* mice. PVL;*LDLR+/-* mice were bred to *LDLR-/-* mice to generate PVL;*LDLR-/-* mice. PVL;*LDLR-/-* mice were bred to *LDLR-/-* mice to produce PVL;*LDLR-/-* and *LDLR-/-* littermates. For protein expression analysis, PVL mice were bred onto a *VLDLR-/-* background using a similar strategy as was used for generating PVL;*LDLR-/-* mice.

All studies in this paper were performed using 10 to 20 weeks old females. Littermates were housed in groups. Mice were fed either a regular breeding chow diet or a high fat diet. The chow diet contained 6.2% fat (Hope Farms, Woerden, The Netherlands). The high fat diet contained 15% cacao butter, 0.25% cholesterol, 40.5% sucrose, 10% cornstarch, 1% corn oil and 5.95% cellulose (w/w) (Hope Farms, Woerden, The Netherlands).

Serum cholesterol and triglyceride analysis

Mice were fasted for 4 hours (9 am to 1 pm) or overnight (5 pm to 9 am), and blood was collected in Microvette CB 300 tubes (Sarstedt, Nuernbrecht, Germany), that were immediately placed on ice. Tubes were spun in an eppendorf centrifuge at 4 °C at 4000 rpm. Serum cholesterol and triglyceride levels were determined using commercially available kits (kit no. 236691 from Boehringer Mannheim and kit 337-B from Sigma, respectively).

Serum FPLC analysis

For the analysis of the distribution of cholesterol and triglycerides over the different lipoprotein fractions pooled samples of mouse serum were analyzed on a Smart-system (Pharmacia, Uppsala, Sweden) with a Superose 6 column as described previously (17).

VLDL production

VLDL triglyceride production rate was determined essentially as described before (18). Groups of 6 *VLDLR-/-;LDLR-/-* mice and 6 *LDLR-/-* control mice fed a chow diet were fasted overnight. Tran ^{35}S-label™ (ICN Pharmaceuticals, Irvine, California, USA) was dissolved in PBS and 100 μCi was injected into the tail vein. After 30 minutes, Triton WR1339 (Sigma) was injected into the tail vein. At 0, 30, 60 and 90 minutes after Triton injection blood samples were collected in Microvette tubes (Sarstedt), and were placed on ice. Serum triglyceride levels were measured as described above, and a curve fit was performed using GraphPad Prism (GraphPad

Software, San Diego, California, USA). VLDL was isolated by ultracentrifugation (d<1.006 g/ml) from blood samples collected 90 minutes after Triton injection. Part of the VLDL was used to determine the apoB production rate. ApoB was specifically precipitated by isopropanol precipitation (19). The pellet was dissolved at 60°C in a 20% sodium dodecyl sulphate solution, and radioactivity of the sample was determined.

VLDL lipid analysis

To determine the lipid composition of circulating and nascent VLDL, VLDL lipid content was measured both before and 90 minutes after Triton treatment. Analysis of mouse serum that was incubated with Triton showed that Triton treatment does not affect lipid composition of the different lipoprotein fractions (data not shown). Therefore, the lipid composition of nascent VLDL can be calculated from the difference between VLDL lipid content before and after Triton treatment. VLDL total cholesterol content was determined as described above. VLDL triglyceride, free cholesterol and phospholipid content were determined with enzymatic assay kits (701904 and 310328 from Boehringer Mannheim) and the analytical kit B (Wako Chemicals GmbH, Neuss, Germany) respectively. VLDL cholesterol ester content was calculated by subtracting free cholesterol from total cholesterol content.

Results

Characterization of VLDLR transgenic mice (PVL)

To overexpress the VLDLR in mouse endothelium, transgenic mice (PVL) were generated using the human VLDLR cDNA under control of the preproendothelin-1 promoter. A semi-quantitative RT-PCR was developed to determine the level of human VLDLR expression relative to endogenous mouse VLDLR expression in PVL mice. Semi-quantitative RT-PCR performed on RNA isolated from PVL mouse tissues revealed that mouse and human VLDLR expression levels were in the same range in heart, aorta and kidney. In skeletal muscle, human VLDLR expression levels were lower than endogenous mouse VLDLR expression levels, whereas the opposite was true for testis and liver (figure 2A). Northern blot analysis of PVL mouse heart RNA, using the pHV58.H probe, revealed expression of a 3.8 kb message (figure 2B). To assess the expression of the human VLDLR protein, heart membrane protein fractions were isolated from wild type, PVL, *VLDLR-/-* and PVL;*VLDLR-/-* mice. Western blot analysis using a rabbit polyclonal anti-human VLDLR antibody revealed two bands in wild type mice that were absent in the *VLDLR-/-* mouse. These two protein bands, which have been described before (10), presumably represent the precursor and the mature form of the VLDLR. Expression of the human VLDLR protein was detected in PVL;*VLDLR-/-* mice, and revealed two protein bands of the same size as those of the endogenous VLDLR. The intensity of the bands detected in the PVL mice was stronger than those in wild type littermates (figure 2C), implying that total VLDLR expression is higher in PVL than in wild type mice.

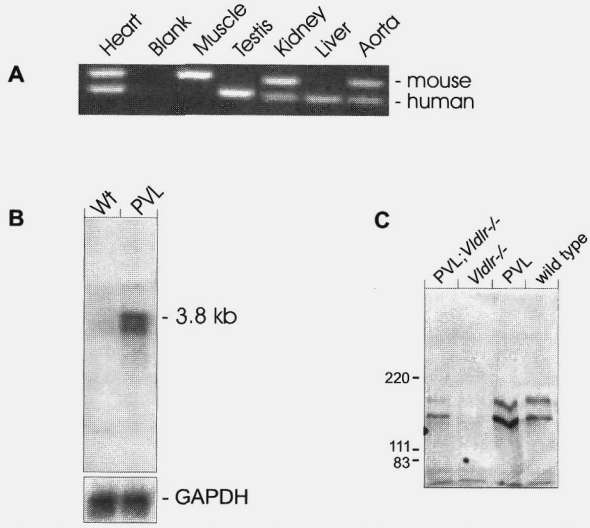

Figure 2. Expression analysis of PVL transgenic mice

To determine human versus mouse VLDLR RNA expression levels, semi-quantitative RT-PCR was performed on total RNA isolated from various PVL mouse tissues. PCR products were separated by electrophoresis on an ethidium stained 3% agarose gel (A). Northern blot analysis of PVL and wild type (Wt) RNA was performed with the ^{32}P-labeled pHV58.H probe. Membranes were re-probed with a ^{32}P-labeled GAPDH cDNA probe (B). Membrane protein fractions were isolated from PVL;*VLDLR-/-*, *VLDLR-/-*, PVL and Wt mouse heart. Eighty microgram of protein was subjected to SDS-PAGE on a 6% polyacrylamidegel. VLDLR protein was detected using a rabbit polyclonal anti-human VLDLR antibody (C).

Serum lipid levels of VLDLR-/- and PVL mice on LDLR-/- background

When fed a standard chow diet, PVL (data not shown) and *VLDLR-/-* (10) mice show normal serum lipid levels. To study the VLDLR under conditions of relatively high serum VLDL and triglyceride levels, both PVL and *VLDLR-/-* mice were first crossed onto an *LDLR-/-* background. Subsequently, serum lipid levels were studied on chow and high fat diet, both after 4 hours and after overnight fasting (table 1). *VLDLR;LDLR-/-* mice showed a significant elevation of serum triglyceride levels (2.3 fold) on a chow diet after overnight fasting when compared with LDLR-/- controls. When fed a high fat diet for 6 weeks, *VLDLR-/-;LDLR-/-* mice showed significantly elevated serum triglyceride levels both after 4 hours (1.9 fold) and after overnight fasting (2.4 fold) when compared with controls. In contrast, after 4 hours fasting, PVL;*LDLR-/-* mice displayed a significant decrease in serum triglyceride levels both on chow (1.2 fold) and on high fat diet (2.0) when compared with *LDLR-/-* controls. No significant differences in serum triglyceride levels were found between PVL;*LDLR-/-* mice and *LDLR-/-* controls on a chow diet after overnight fasting. No significant differences in total serum cholesterol levels were found between *VLDLR-/-;LDLR-/-* mice and *LDLR-/-* controls or between PVL;*LDLR-/-* and *LDLR-/-* controls under all circumstances studied.

Table I. Serum cholesterol and triglyceride levels of *VLDLR-/-;LDLR-/-* mice, PVL;*LDLR-/-* mice and *LDLR-/-* controls

mousemodel	diet	n	4 hour fast		overnight fast	
			cholesterol (mM)	triglycerides (mM)	cholesterol (mM)	triglycerides (mM)
VLDLR-/-;LDLR-/-	chow	15	6.22 ± 1.78	0.73 ± 0.27	8.44 ± 1.32	4.18 ± 1.73 *
LDLR-/-[a)]	chow	19	5.25 ± 1.18	0.54 ± 0.22	7.88 ± 1.41	1.79 ± 0.63
VLDLR-/-LDLR-/-	W	18	44.76 ± 14.04	5.47 ± 2.51 *	42.76 ± 14.04	1.23 ± 0.16 *
LDLR-/-[a)]	W	20	38.57 ± 14.79	2.91 ± 1.59	38.57 ± 14.79	0.52 ± 0.11
PVL;*LDLR-/-*	chow	16	5.33 ± 0.54	0.57 ± 0.15 *	6.52 ± 0.89	1.53 ± 0.66
LDLR-/-[a)]	chow	20	5.70 ± 1.09	0.71 ± 0.25	6.44 ± 0.69	1.72 ± 0.42
PVL;*LDLR-/-*	W	9	47.16 ± 10.28	1.74 ± 0.55 *	n.d.	n.d.
LDLR-/-[a)]	W	13	49.92 ± 10.07	3.47 ± 1.70	n.d.	n.d.

Serum cholesterol and triglyceride concentrations were measured in *VLDLR-/-;LDLR-/-* mice and *LDLR-/-* littermates and in PVL;*LDLR-/-* mice and *LDLR-/-* littermates. All littermates were housed in groups. Lipid levels were measured in mice that were fasted for 4 hours or were fasted overnight. Mice were kept on a chow diet or on a high fat diet (W). [a)] Note that *LDLR-/-* control groups for PVL and *VLDLR-/-* mice differ in their genetic background. Values shown are means ± SD. n.d. = not determined, * = significantly different from *LDLR-/-* littermates ($P<0.05$), using the Student's t-test.

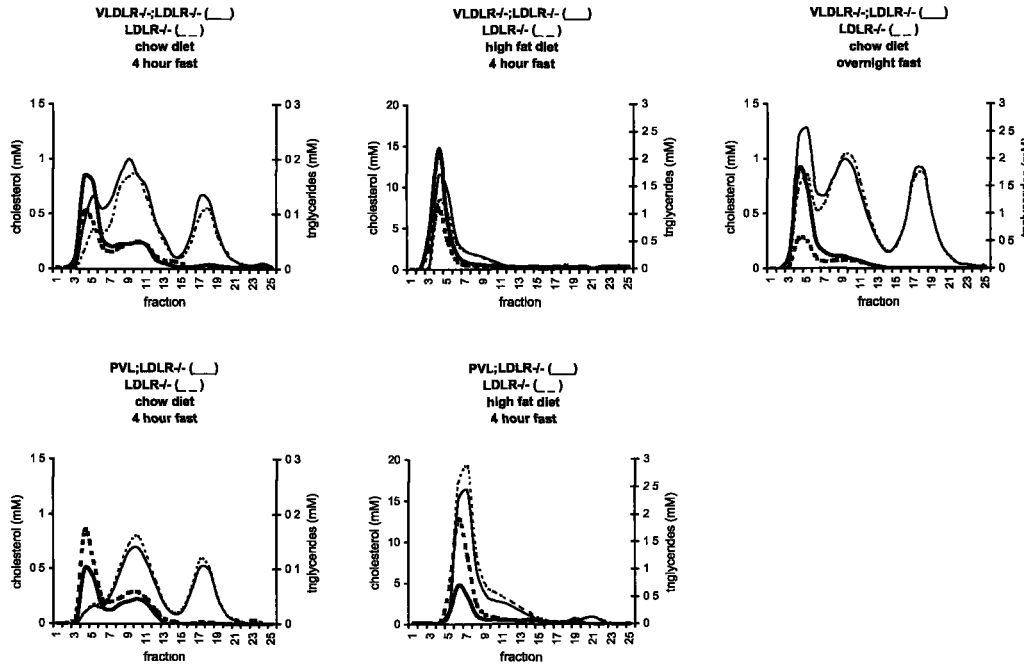

Figure 3. Serum FPLC profiles of *VLDLR-/-;LDLR-/-* mice, PVL;*LDLR-/-* mice and *LDLR-/-* controls

Mouse sera isolated from various mouse models under different dietary conditions were subjected to FPLC fractionation. Cholesterol (thin lines) and triglycerde (thick lines) levels of each fraction were determined as described in Methods. Pooled sera were analyzed from 15 *VLDLR-/-;LDLR-/-* and 19 *LDLR-/-* littermates that were kept on a chow diet and fasted for 4 hours or overnight, 18 *VLDLR-/-;LDLR-/-* and 20 *LDLR-/-* littermates that were kept on a high fat diet and fasted for 4 hours, 16 PVL;*LDLR-/-* and 20 *LDLR-/-* littermates that were kept on a chow diet and fasted for 4 hours and 9 PVL;*LDLR-/-* and 13 *LDLR-/-* mice that were kept on a high fat diet and fasted for 4 hours.

Serum FPLC analysis showed that the effect of the VLDLR on serum triglyceride levels was mainly due to an effect on VLDL triglycerides. While *VLDLR-/-;LDLR-/-* mice show elevated VLDL triglyceride levels, PVL;*LDLR-/-* mice showed a lowering of VLDL triglyceride levels when compared with their controls. The extent of the effect of the VLDLR on VLDL triglycerides depended on the diet or the duration of the fasting period (figure 3).

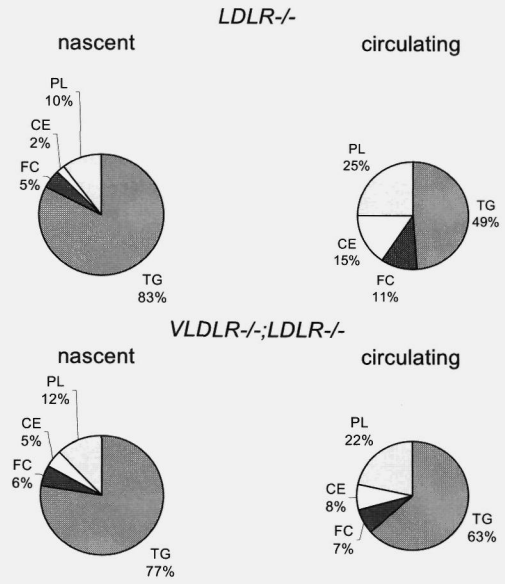

Figure 4. VLDL lipid content of VLDLR-/-;LDLR-/- mice and LDLR-/- controls
Serum was collected from the overnight fasted mice in the VLDL production experiments (see Methods), both before and 90 minutes after Triton WR1339 injection. VLDL was isolated from pooled serum by ultracentrifugation (d<1.006 g/ml). VLDL phospholipid (PL), cholesterol ester (CE), free cholesterol (FC) and triglyceride (TG) content were determined. Composition of nascent VLDL was determined by subtracting the amount of VLDL lipids before Triton treatment from those 90 minutes after treatment. Composition of nascent and circulating plasma VLDL were determined in *LDLR-/-* mice and *VLDLR-/-;LDLR-/-* mice.

VLDL production

To determine whether the relatively high serum triglyceride levels in *VLDLR-/-;LDLR-/-* mice were due to enhanced VLDL production, apoB and triglyceride production rates were determined in *VLDLR-/-;LDLR-/-* and *LDLR-/-* controls that were fed a chow diet and were fasted overnight. Mice were injected with Triton WR1339 to impair lipolysis and VLDL clearance, and the accumulation of triglycerides and the radiolabeled apoB was determined in the serum over a period of 90 minutes. No significant differences were found between *VLDLR-/-;LDLR-/-* mice and *LDLR-/-* controls for both triglyceride (76.1 ± 9.4 and 62.4 ± 18.5 µmol/h/kg) and apoB (1.39 ± 0.34 and 1.00 ± 0.44 arbitrary units/h/kg) production rates. Furthermore, nascent VLDL triglyceride content of *VLDLR-/-;LDLR-/-* mice (77%) was comparable to that of *LDLR-/-* controls (83%). However, circulating serum VLDL of *VLDLR-/-;LDLR-/-* mice contained 63% triglycerides, compared with 49% in *LDLR-/-* controls (figure 4). This implies that the clearance of triglycerides from circulating VLDL is disturbed in *VLDLR-/-;LDLR-/-* mice.

Discussion

The purpose of this study was to gain insight into the role of the VLDL receptor in lipoprotein metabolism. The *in vivo* evidence that supports a role for the VLDLR in lipid metabolism is limited to the observation that *VLDLR-/-* mice show reduced adipose tissue mass (10). Thus far, *VLDLR-/-* mice were not reported to display altered serum lipid levels. However, mice have very low serum VLDL and triglyceride levels. To study the VLDLR under conditions

of relatively high serum VLDL and triglyceride levels, both VLDLR deficient and VLDLR overexpressing mice were crossbred onto an LDLR-/- background. Absence of the VLDLR was associated with elevated serum triglyceride levels under conditions of dietary stress. In contrast, overexpression of the VLDLR under similar conditions was associated with lowering of serum triglyceride levels. Thus the variation in serum triglyceride levels is consistent with the variation in VLDLR expression in both mouse models.

In *VLDLR-/-;LDLR-/-* mice, the increase in serum triglycerides was not accompanied by a significant increase in VLDL production rate when compared with *LDLR-/-* controls. In addition, no significant differences were found in serum cholesterol levels between *VLDLR-/-;LDLR-/-* mice and *LDLR-/-* controls. Therefore it seems not likely that the VLDLR influences serum triglyceride levels by affecting clearance of whole lipoprotein particles by the liver. Analysis of VLDL lipid composition showed that the triglyceride content of circulating VLDL in *VLDLR-/-;LDLR-/-* mice is relatively high as compared with *LDLR-/-* controls. This implies that the clearance of triglycerides from circulating VLDL is disturbed in *VLDLR-/-;LDLR-/-* mice.

The effect of the VLDLR on VLDL triglyceride metabolism could be explained by an effect on lipolysis. The principal enzyme responsible for the hydrolysis of triglycerides of circulating lipoproteins is lipoprotein lipase (LPL). It has been described to perform a bridging function between extra-cellular heparan sulphate proteoglycans and lipoprotein particles (20, 21). In addition, LPL has been reported to bind to receptors of the LDLR family, including the VLDLR, thereby enhancing their affinity for lipoproteins (3, 5). We hypothesize that the VLDLR and LPL together promote retention of circulating VLDL on the vessel wall. Subsequently, LPL could exert its lipolytic effect on triglycerides residing in the VLDL particle without affecting VLDL cholesterol. Thus VLDLR overexpression would lead to an increase, while VLDLR deficiency would lead to a decrease in the rate of lipolysis of VLDL triglycerides.

In contrast to our present results, studies expressing the VLDLR ectopically in the liver of *LDLR-/-* mice fed a high cholesterol diet showed no effects on serum triglyceride levels, while serum cholesterol levels were reduced by 50%. Furthermore, VLDLR expression in liver affected intermediate density lipoprotein (IDL) and LDL instead of VLDL fractions, and was shown to facilitate the clearance of IDL particles (10). Apparently, when expressed in liver, the VLDLR can be engaged in uptake of IDL/LDL via lipoprotein receptor mediated endocytosis. Thus VLDLR mediated binding and processing of lipoproteins seems to depend on the site of VLDLR expression.

The *VLDLR-/-* mouse was previously reported to have normal serum lipid levels (10). In the present paper we show that the VLDLR does affect serum triglyceride levels when specific conditions are applied. LDLR deficiency in combination with conditions of fasting, or feeding a high fat diet, revealed the effect of the VLDLR on VLDL triglyceride metabolism. Fasting and high fat feeding were previously reported to affect the regulation of VLDLR expression. VLDLR expression in mice was shown to increase in heart and decrease in adipose tissue under conditions of prolonged fasting (8). In contrast, feeding a high fat diet was reported to downregulate VLDLR mRNA expression in heart and upregulate expression in adipose tissue in *LDLR-/-* mice (9). These observations are in line with the hypothesis that the VLDLR is

involved in the delivery of fatty acids to peripheral tissues. Upon fasting, fatty acid demand in tissues like the heart will increase, while there will be less fatty acid storage in adipose tissue. Upon feeding a high fat diet, the reverse will be true. We hypothesize that in the present study the effect of the VLDLR on serum triglyceride levels was revealed by the effect of dietary status on fatty acid fluxes. Apparently, triglyceride metabolism is tightly regulated, and backup mechanisms can compensate for VLDLR deficiency. Only when specific dietary conditions are applied do these backup mechanisms fail to compensate for VLDLR deficiency or VLDLR overexpression.

Similar to the effect of the VLDLR on VLDL triglyceride content, the reduction in adipose tissue mass observed in *VLDLR-/-* mice (10) could be explained by impaired hydrolysis of VLDL triglycerides. A second mouse model that exhibits reduced adipose tissue mass is the apolipoprotein C1 (apoC1) transgenic mouse (22). Interestingly, apoC1 was reported to strongly inhibit lipoprotein binding to the VLDLR (23), while it does not impair LPL lipolysis activity *in vitro* (24). RAP was also reported to inhibit lipoprotein binding by the VLDLR. Intriguingly, adenovirus mediated overexpression of RAP into the circulation of mice that lacked both the LDLR and the LRP in their livers showed an increase of serum triglycerides. Adenovirus mediated RAP expression led to high serum levels of RAP. The effect of RAP on the metabolism of triglyceride-rich lipoproteins could not be attributed to RAP-binding proteins in the liver (25). Considering the results of the present study, it could be hypothesized that the extra-hepatic effect of RAP is due to blocking of lipoprotein binding to receptors like the VLDLR in peripheral tissues.

In summary, our results suggest a role for the VLDLR in peripheral uptake of VLDL triglycerides. Apparently, backup mechanisms can compensate for VLDLR deficiency or overexpression. Only under stressed conditions of LDLR deficiency in combination with high fat feeding or prolonged fasting periods, is the effect of the VLDLR on VLDL triglyceride metabolism revealed.

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CHAPTER 4

Protection from obesity in mice lacking the VLDL receptor

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(Submitted)

Abstract

It has previously been reported that mice lacking the very low density lipoprotein receptor (*VLDLR-/-*) exhibit normal plasma lipid levels and a modest decrease in adipose tissue mass [*Frykman et al. (1995) Proc. Natl. Acad. Sci. 92, 8453-8457*]. In the present study, the effect of VLDLR deficiency on profound weight gain was studied in mice. Obesity was induced either by feeding a high fat, high caloric (HFC) diet or by crossbreeding mice onto the genetically obese *ob/ob* background. After 17 weeks of HFC feeding, *VLDLR-/-* mice remain lean, whereas their wild-type littermates (*VLDLR+/+*) become obese. Similarly, weight gain of *ob/ob* mice is less profound in the absence of the VLDLR. Moreover, VLDLR deficiency in these two obesity-prone backgrounds induces marked hypertriglyceridemia. The protection from obesity in *VLDLR-/-* mice involves decreased peripheral uptake of dietary fatty acids, since *VLDLR-/-* mice exhibit a significant reduction in the whole body free fatty acid uptake, with no clear differences in food intake and fat absorption. These observations are supported by a strong decrease in average adipocyte size seen in *VLDLR-/-* mice of both obesity models, implying reduced adipocyte TG storage in the absence of the VLDLR. To our knowledge, these results represent the first demonstration that the VLDLR may directly contribute to the entry of fatty acids into tissues, and suggest that selective inhibition of the VLDLR may offer a therapeutic target for treatment of obesity.

Introduction

The very low density lipoprotein receptor (VLDLR) is a member of the low density lipoprotein receptor (LDLR) family (1). The most striking features that distinguish the VLDLR from the LDLR are i) eight ligand-binding repeats, instead of seven and ii) its expression pattern among tissues. The VLDLR is highly expressed in skeletal muscle, heart and adipose tissue, and only in trace amounts in the liver, whereas the LDLR is abundantly expressed in the liver (1, 2).

A role for the VLDLR in lipoprotein metabolism was suggested by *in vitro* experiments showing that the VLDLR binds and internalizes particles that are rich in apolipoprotein (apo) E, such as VLDL, Intermediate-Density-Lipoproteins (IDL) and chylomicrons (1, 3). The binding of these lipoprotein particles to the VLDLR was stimulated by lipoprotein lipase (LPL) (3, 4), and inhibited by a 39-kDa protein named the receptor-associated protein (RAP) (5). In addition to lipoproteins, the VLDLR has been shown to bind several other ligands including urokinase complexed to its inhibitor, plasminogen activator inhibitor type I (6), and thrombospondin-1 (7).

Based on its binding characteristics, endothelial localization (8) and tissue expression pattern, it is hypothesized that the VLDLR facilitates the binding of triglyceride (TG)-rich particles in the capillary bed and subsequent delivery of free fatty acids (FFA) to tissues active in fatty acid metabolism (3, 9, 10). In line with this hypothesis, it was shown in mice that VLDLR mRNA levels are upregulated in heart and downregulated in adipose tissue after prolonged fasting (11). Reciprocally, VLDLR mRNA levels were downregulated in heart and upregulated in adipose tissue of LDLR-deficient mice fed an atherogenic diet (12).

To directly investigate a role for the VLDLR in lipid metabolism, mice were generated lacking the VLDL receptor by gene-targeting (13). *VLDLR-/-* mice exhibited no differences in plasma lipoproteins and the sole abnormality detected was a modest decrease in body weight (15-20%) (13). Recently, *VLDLR-/-* mice were cross-bred with ApoE receptor 2 (ApoER2)-deficient mice (14). These double knockout mice exhibited a striking disorganization of neurons in the cerebellum, indicating that the presence of the VLDLR in the brain, in combination with the ApoER2, is required in a signaling pathway that regulates neuronal migration (14).

Thus, in addition to its function in the brain, a clear role for the VLDLR in lipid metabolism remains to be established. Since it has been hypothesized that the VLDLR plays a role in the delivery of FFA to peripheral tissues, we investigated whether under conditions of severe dietary stress, the absence of the VLDLR may introduce a rate-limiting step in the delivery of free fatty acids to peripheral tissues. Therefore, *VLDLR-/-* mice and wild-type littermates (*VLDLR+/+*) were fed a high fat, high caloric (HFC) diet to enhance the intake of fatty acids. Previous studies have shown that this HFC diet induces obesity and insulin resistance in normal C57BL/6J mice (15). In addition, *VLDLR-/-* mice were cross-bred with the genetically-determined obese *ob/ob* mice. In our hands, *VLDLR-/-* mice are fully protected from HFC diet-induced obesity and insulin resistance. Also in the more severe genetic model of obesity, *VLDLR-/- ob/ob* mice gained significantly less weight as compared to their *ob/ob* wild-type littermates. The protection from obesity upon VLDLR-deficiency is most likely due to an impaired uptake of fatty acids by adipose tissue, since whole body FFA uptake was strongly decreased in *VLDLR-/-* mice with no significant differences in food intake and fat absorption.

Methods

Animals

VLDLR-/- mice were obtained from the Jackson Laboratories. Non-transgenic littermates (*VLDLR+/+*) were used as controls. Heterozygous *ob/-* mice (Jackson Laboratories) were crossbred with VLDLR +/- mice to obtain *VLDLR-/-* mice on a homozygous *ob/ob* background (*VLDLR-/- ob/ob*). *Ob/ob* littermates (*VLDLR+/+ ob/ob*) were used as controls. All mice in the study were males on the C57BL/6J background and were housed individually under standard conditions with free access to water and food. Food was withdrawn at 9:00 a.m. and the experiments were either performed at 1:00 p.m. (4 hours fasting) or at 9:00 a.m. the next day (24 hours fasting).

Diet and dietary treatment

After weaning, mice were fed a standard-rat-mouse chow diet. After 6 months of age, 10 *VLDLR-/-* and 12 *VLDLR+/+* mice were given a high-fat, high-caloric (HFC) diet (46.2% of the calories as fat) for a period of 17 weeks. The HFC diet contained 24% corn oil, 24% casein, 20% cerelose, 18% cornstarch and 6% cellulose, by weight (Hope Farms, Woerden, the Netherlands). 7 *VLDLR-/- ob/ob* and 8 *VLDLR+/+ ob/ob* mice were fed standard-rat-mouse chow diet (17.2% of the calories as fat) and were followed in time. Food intake was monitored individually.

Histological analysis of adipose tissue

Adipose tissues after formalin fixation were cut in sections (3 μm) and stained with hematoxylin phloxin saffron. For the quantification of adipocyte size, the sectional areas of adipose tissues were analyzed using Leica Qwin image analysis software.

Plasma lipid and lipoprotein analysis

In all experiments, blood was collected by tail bleeding in chilled paraoxonized capillary tubes (16). These tubes were placed in ice and immediately centrifuged at 4 °C. Levels of total plasma cholesterol, plasma TG (without free glycerol) and free fatty acids (FFAs) were determined using enzymatically available kits #236691 (Boehringer Mannheim GmbH), #337-B (Sigma GPO-Trinder kit, St. Louis, MA, USA) and a Nefa-C kit (Wako Chemicals GmbH, Neuss, Germany), respectively. For fast protein liquid chromatography (FPLC) fractionation of lipoproteins, 50 μl pooled plasma from at least 8 mice per group was injected onto a Superose 6 column (3.2 x 30 mm, Smart-system, Amersham Pharmacia), and eluted at a constant flow rate of 50 μl/min with PBS (pH 7.4, containing 1 mmol / L EDTA). Fractions of 50 μl were collected and assayed for total cholesterol and TG levels as described above.

Plasma glucose and insulin assays

Levels of plasma glucose were determined using the enzymatically available kit, #315-500 (Sigma). Plasma insulin levels were measured using a radioimmunoassay kit (Sensitive Rat Insulin Assay; Linco Research Inc., St. Charles, Missouri, USA).

Glucose tolerance test

Glucose tolerance tests were performed in 7 *VLDLR-/-* and 7 *VLDLR+/+* mice fed a HFC diet for a period of 17 weeks, in 5 *VLDLR-/-* and 5 *VLDLR+/+* mice on a chow diet and in 7 *VLDLR-/- ob/ob* and 7 *VLDLR+/+ ob/ob* mice. Animals were fasted for 4 hours and a basal blood sample was collected from the tip of the tail (t = 0 minutes). Mice were subsequently injected i.p. with glucose (2 g/kg), and additional blood samples were collected at 15, 30, 60 and 120 min. All blood samples were spun and the separated plasma was measured for glucose as described above.

Fat absorption

Fat absorption was measured by comparing the amount and kind of fatty acids present in the HFC diet with that in the feces. Feces were collected from metabolic cages and food intake was measured over a period of 3 days. After freeze-drying and mechanically homogenizing, aliquots (15 mg) of HFC diet and feces were extracted (methanol-hexane 4:1 (v/v) with 0.01% butylated hydroxytoluene), hydrolyzed and methylated (17). Heptadecanoic acid (C17:0) was added to all samples as an internal standard before the extraction and methylation procedure. Fatty acid methyl esters were separated and quantified by gas liquid chromatography on a Varian gas chromatograph (model 3800) equipped with a CP-Sil88column (50 m x 0.25 mm [inner diameter]) and a flame ionization detector. Dietary and fecal fatty acids were quantified by peak area comparison with the internal standard.

^{3}H-FFA turnover experiments

To study the *in vivo* fatty acid turnover, *VLDLR-/-* and *VLDLR+/+* mice after 4 weeks on HFC diet, were fasted (4 hours) and anesthetized with an intraperitoneal injection of 0.5 mL/kg hypnorm (Janssen Pharmaceutica) and 12.5 mg/kg midazolam (Roche, Netherlands). Body temperature was maintained at 37 °C by use of heat lamps. An indwelling catheter was then inserted in the right jugular vein and ^{3}H-labeled fatty acids were infused at a rate of 200 µl/h (0.08 µCi/min [9,10(n)-^{3}H] palmitic acid (Amersham) dissolved in a BSA solution (2 mg/ml in sterile saline). At t = 0 a bolus (1.25 µCi palmitic acid) was injected into the tail vein of the mice. A blood sample (100 µl) was collected from the tip of the tail at 1 hour after the start of infusion, when steady state has been reached. The whole body uptake of ^{3}H-FFA in plasma was determined as previously described (18, 19). Briefly, basal plasma FFA concentrations were measured enzymatically as described above. The amount of plasma radioactivity in the FFA and TG fraction was determined after lipid extraction and separation from the other lipid components by thin layer chromatography (TLC) on silica gel plates (Merck) by using hexane/diethylether/acetic acid (83:16:1, vol/vol/vol) as resolving solution. [^{14}C]-trioleate and [^{14}C]-palmitic acid (Amersham) were used as internal standards. ^{3}H-palmitate oxidation was assessed by measuring ^{3}H-H_2O (19). This was done by subtracting the dpm in an aliquot (10 µl) of plasma, which had been evaporated to dryness and then resolved in H_2O, from the dpm of an unevaporated aliquot. The amount of storage (esterfication) of FFA was calculated by subtracting the FFA oxidation from the FFA turnover (19).

Statistical analysis

The Mann-Whitney nonparametric test for 2 independent samples was used to define differences between *VLDLR-/-* and *VLDLR+/+* mice. The criterion for significance was set at $P<0.05$. All data are presented as means ± SD.

Results

To investigate the effects of VLDLR deficiency under conditions of severe dietary stress, *VLDLR-/-* and *VLDLR+/+* mice were fed a HFC diet for a period of 17 weeks. During this period, male *VLDLR-/-* mice gained little body weight upon HFC feeding, whereas the body weight of the male *VLDLR+/+* mice continued to increase (Fig. 1 and Fig. 2*A*). Similar findings were observed with females (results not shown). The difference in body weight between *VLDLR-/-* and *VLDLR+/+* mice was reflected by a marked reduction in both subcutaneous (~80%) and visceral (~70%) adipose tissue mass in *VLDLR-/-* mice (Fig. 2*B*), indicating that *VLDLR-/-* mice are strongly protected from diet-induced obesity. Histological analysis of epididymal fat pads (Fig. 2*C*) revealed that adipocytes from *VLDLR-/-* mice were significantly smaller than those from *VLDLR+/+* mice (3679±453 vs 7889±572 µm2, P=0.009) under condition of HFC feeding. Similar differences were found for subcutaneous fat as well (not shown).

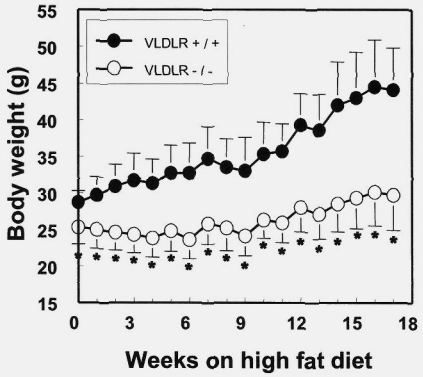

Figure 1 Growth curves of *VLDLR-/-* and *VLDLR+/+* mice on a HFC diet

At 6 months of age, mice were fed a HFC diet for a period of 17 weeks and were weighed weekly. Values represent the mean ± SD of 10 *VLDLR-/-* and 12 *VLDLR+/+* mice. **P*<0.05 at all time points, indicating the difference between *VLDLR-/-* and *VLDLR+/+* mice, using nonparametric Mann-Whitney tests.

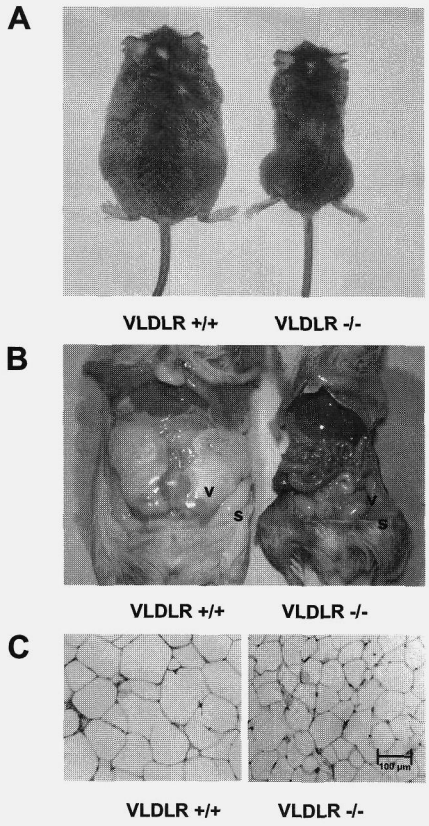

Figure 2: Gross appearance and adipose tissue of *VLDLR-/-* and *VLDLR+/+* mice after 17 weeks of HFC feeding

(2*A*) Typical male littermates, (2*B*) Subcutaneous (s) and visceral (v) adipose tissue pads, and (2*C*) Paraffin-embedded sections of white adipose tissue.

Diet-induced obesity is frequently associated with insulin resistance, so we next investigated whether depletion of the VLDLR would affect insulin sensitivity. Plasma glucose and insulin levels were significantly reduced in *VLDLR-/-* mice compared to *VLDLR+/+* mice after 17 weeks of HFC diet feeding (Table 1). These results indicate that *VLDLR-/-* mice remain insulin sensitive, whereas *VLDLR+/+* mice have indeed become insulin resistant after high fat feeding. This is sustained by an intraperitoneal glucose tolerance test (Fig. 3*A*). *VLDLR-/-* mice on a HFC diet were much more efficient in the clearance of an intraperitoneal injected bolus of glucose than *VLDLR+/+* mice, demonstrating that the absence of the VLDLR significantly hampers the development of insulin resistance upon introducing diet-induced obesity.

Table 1: Comparison of biochemical parameters in *VLDLR-/-* and *VLDLR+/+* mice on a HFC diet and on the obese *ob/ob* background

Genotype	n	Glucose (mmol/L)	Insulin (ng/mL)	TC (mmol/L)	TG (mmol/L)	FFA (mmol/L)
Diet-induced obesity						
VLDLR+/+	12	9.8 ± 1.7	4.6 ± 2.5	3.2 ± 0.5	0.5 ± 0.2	0.8 ± 0.1
VLDLR-/-	10	6.6 ± 3.7*	1.8 ± 0.5*	3.3 ± 0.3	1.2 ± 0.2*	0.9 ± 0.1
Genetically-induced obesity						
VLDLR+/+ ob/ob	7	14.6 ± 4.2	6.5 ± 2.2	5.0 ± 0.9	0.7 ± 0.2	0.8 ± 0.1
VLDLR-/- ob/ob	7	12.9 ± 5.7	5.5 ± 0.8	5.5 ± 1.0	1.9 ± 0.7*	1.1 ± 0.1*

In the diet-induced obesity group *VLDLR-/-* and *VLDLR+/+* mice were fed a HFC diet for 17 weeks. The genetically-determined obese *VLDLR-/- ob/ob* and *VLDLR+/+ ob/ob* mice were compared at 17 weeks of age. Plasma glucose, insulin, cholesterol (TC), triglyceride (TG) and free fatty acid (FFA) levels were measured after 24 hours fasting as described in the methods section. Values represent the mean ± SD. *$P<0.05$, indicating the difference between *VLDLR-/-* and *VLDLR+/+* mice, using nonparametric Mann-Whitney tests.

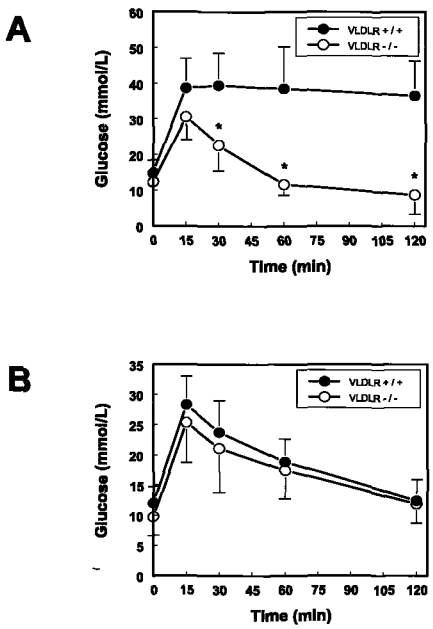

Figure 3: Glucose tolerance tests in *VLDLR-/-* and *VLDLR+/+* mice on both a HFC (3*A*) and a chow diet (3*B*)

After 17 weeks of HFC or chow feeding *VLDLR+/+* and *VLDLR-/-* mice were fasted for 4 hours and then intraperitoneal injected with a glucose bolus (2g/kg). Changes in plasma glucose concentration were monitored in time. Values represent the mean ± SD of 7 mice per group on a HFC diet and 5 mice per group on a regular chow diet. *$P<0.05$, indicating the difference between *VLDLR-/-* and *VLDLR+/+* mice, using nonparametric Mann-Whitney tests.

We next wanted to investigate whether the protection from insulin resistance in *VLDLR-/-* mice was either directly due to the absence of the VLDLR, or indirectly related to the less severe obesity in these mice. Therefore, we performed a glucose tolerance test in *VLDLR-/-* and *VLDLR+/+* mice on a regular chow diet that were exactly matched for body weight. It is shown in Figure 3*B* that there were no obvious differences in glucose tolerance between *VLDLR-/-* and *VLDLR+/+* when matched for body weight, indicating that improved insulin sensitivity in *VLDLR-/-* mice is directly related to reduced obesity.

To investigate whether the protection from diet-induced obesity and insulin resistance upon VLDLR deficiency was associated with changes in plasma lipid metabolism, several lipid parameters were measured in plasma after 17 weeks of HFC feeding. Plasma TG levels in *VLDLR-/-* mice were significantly higher than in *VLDLR+/+* mice (Table 1). In contrast, no significant differences were found in plasma FFA levels. The elevated levels of plasma TG in *VLDLR-/-* mice were mainly due to an accumulation of TG in the VLDL-sized fractions (compare Fig. 4*B* with Fig. 4*A*). The VLDL fraction contained also more cholesterol in *VLDLR-/-* mice compared to *VLDLR+/+* mice, although there were no significant differences in plasma total cholesterol levels (Table 1).

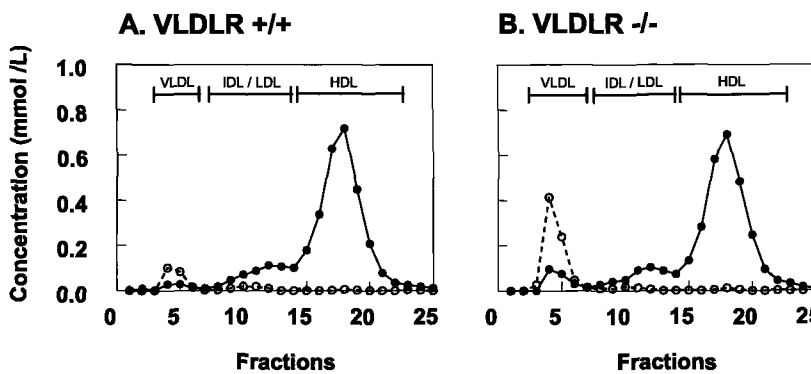

Figure 4: Lipoprotein profiles of *VLDLR+/+* (4*A*) and *VLDLR-/-* (4*B*) mice fed a HFC diet
Plasma of *VLDLR-/-* and +/+ mice was pooled and separated on the base of size by FPLC. The total cholesterol (TC, closed symbols) and triglyceride (TG, open symbols) content of each individual fraction was measured enzymatically, as described in the methods section.

Table 2: Food intake and fat absorption in *VLDLR-/-* and *VLDLR+/+* mice fed a HFC diet

Genotype	Food intake	Fat absorption (%)		
	g / 24 h	C16:0	C18:1	C18:2
VLDLR+/+	3.3 ± 0.9	98.8 ± 0.7	99.7 ± 0.2	99.8 ± 0.2
VLDLR-/-	2.7 ± 0.9	99.2 ± 0.3	99.8 ± 0.1	99.9 ± 0.1

Food intake was monitored individually and represents the average at 17 weeks of HFC feeding. Feces were collected and food intake was measured over a period of 3 days to calculate the fat absorption by determining the kind and amount of fatty acids in both the HFC diet and the feces by GC analysis. Fat absorption is calculated as the amount of fatty acids (%) from the diet that did not appear in the feces. Values represent the mean ± SD of 10 *VLDLR-/-* and 11 *VLDLR+/+* mice (food intake) and 5 *VLDLR-/-* and 7 *VLDLR+/+* mice (fat absorption).

To investigate the mechanisms underlying the protection from HFC diet-induced obesity in *VLDLR-/-* mice, food intake, intestinal fat absorption and whole body FFA uptake were determined. Average food intake was similar in *VLDLR-/-* mice compared to *VLDLR+/+* mice (Table 2). As shown in Table 2, *VLDLR-/-* mice show almost complete absorption of the main fatty acids in the HFC diet, comparable to *VLDLR+/+* mice. Turnover studies in *VLDLR-/-* and *VLDLR+/+* mice with continuous infusion of ^{3}H-FFA to estimate whole body FFA uptake, showed a significant reduction in the whole body FFA uptake and peripheral FFA oxidation in *VLDLR-/-* mice, indicative for a decreased flux of fatty acids into peripheral tissues (Fig. 5*A*). No significant differences in FFA whole body storage were found between *VLDLR-/-* and *VLDLR+/+* mice (Fig. 5*A*). Furthermore, the appearance of ^{3}H-FFA into plasma TG was significantly increased in *VLDLR-/-* mice compared to *VLDLR+/+* mice (Fig. 5*B*).

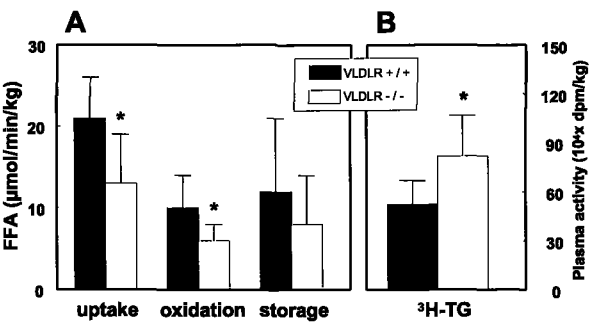

Figure 5: Whole body free fatty acid (FFA) turnover (5*A*) and appearance of FFA into plasma triglycerides (TG) (5*B*) in *VLDLR-/-* and *VLDLR+/+* mice

After 4 weeks on the HFC diet, *VLDLR+/+* and *VLDLR-/-* mice were continuously infused with ^{3}H-FFA via the jugular vein (0.08 µCi/min) for 1 hour. The amount of radioactivity in plasma in the FFA and TG fraction was determined after lipid extraction and the FFA uptake, peripheral oxidation and storage were calculated as described in the methods section. Values represent the mean ± SD of 10 *VLDLR-/-* and 7 *VLDLR+/+* mice. *$P<0.05$, indicating the difference between *VLDLR-/-* and *VLDLR+/+* mice, using nonparametric Mann-Whitney tests.

We wondered whether the protection from development of obesity in *VLDLR-/-* mice on the HFC diet also holds true for *VLDLR-/-* on a genetic *ob/ob* background. The growth curve (Fig. 6) and gross appearance (Fig. 7*A*) of these mice clearly show that *VLDLR-/- ob/ob* mice gained less weight as compared to *VLDLR+/+ ob/ob* mice. These effects on weight gain were reflected by both a reduction in subcutaneous (~60%) and in visceral (~55%) adipose tissue mass in *VLDLR-/- ob/ob* mice (Fig. 7*B*). Histological analysis of epididymal fat pads (Fig. 7*C*) revealed that similar to HFC feeding, adipocytes from *VLDLR-/- ob/ob* mice were significantly smaller than those from *VLDLR+/+ ob/ob* mice (5682±177 vs 9860±1348 μm2, P=0.014). Analysis of subcutaneous fat also showed a smaller average adipocyte area for *VLDLR-/- ob/ob* mice as compared to *VLDLR+/+ ob/ob* mice (results not shown).

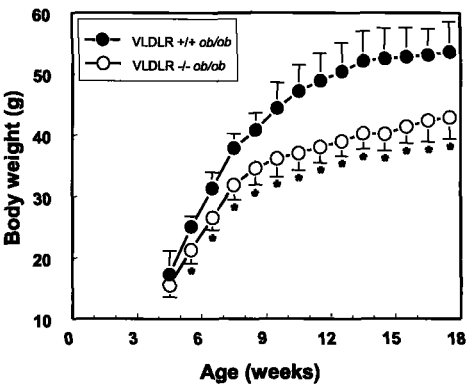

Figure 6: Growth curves of *VLDLR-/-* and *VLDLR+/+* mice on an *ob/ob* background
7 *VLDLR-/- ob/ob* and 8 *VLDLR+/+ ob/ob* mice were fed a regular chow diet and were weighed weekly from week 4,5 to week 17,5 after birth. Values represent the mean ± SD. **P*<0.05, indicating the difference between *VLDLR-/- ob/ob* and *VLDLR+/+ ob/ob* mice, using nonparametric Mann-Whitney tests.

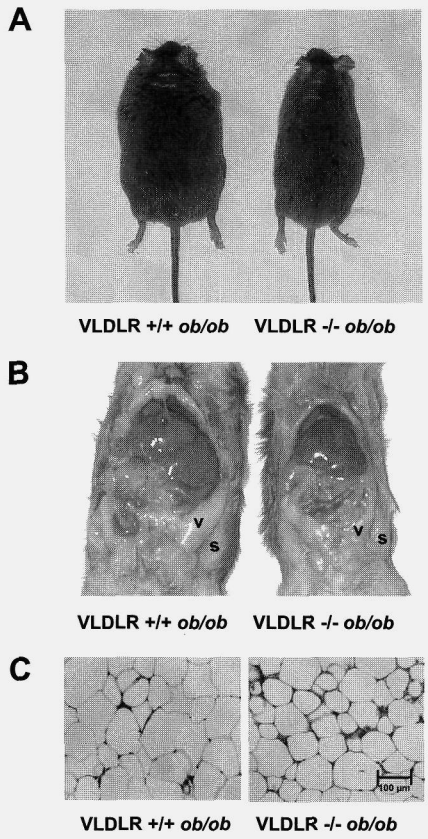

Figure 7: Gross appearance and adipose tissue of *VLDLR-/- ob/ob* and *VLDLR+/+ ob/ob* mice
(7*A*) Typical male littermates, (7*B*) Subcutaneous (s) and visceral (v) adipose tissue pads, and (7*C*) Paraffin-embedded sections of white adipose tissue.

In line with the observations in the *VLDLR-/-* mice on a HFC diet, *VLDLR-/- ob/ob* mice exhibited increased plasma TG levels compared to *VLDLR+/+ ob/ob* mice (Table 1), mainly due to an accumulation of TG in the VLDL-sized fractions (compare Fig. 8*B* with Fig. 8*A*). The VLDL fraction contained also more cholesterol in *VLDLR-/- ob/ob* mice compared to *VLDLR+/+ ob/ob* mice (Fig. 8). In contrast with the observations in the *VLDLR-/-* mice on a HFC diet, *VLDLR-/- ob/ob* mice had significantly higher FFA levels. No differences in plasma glucose and insulin levels were observed in *VLDLR-/- ob/ob* mice as compared to *VLDLR+/+ ob/ob* mice (Table 1). A glucose tolerance test in *VLDLR-/- ob/ob* and *VLDLR+/+ ob/ob* mice showed also no significant differences in glucose clearance (Fig. 9). Furthermore, no differences in food intake could be detected between *VLDLR-/- ob/ob* mice and *VLDLR+/+ ob/ob* mice (6.9 ± 1.7 vs 6.6 ± 1.2 g/24 h, respectively).

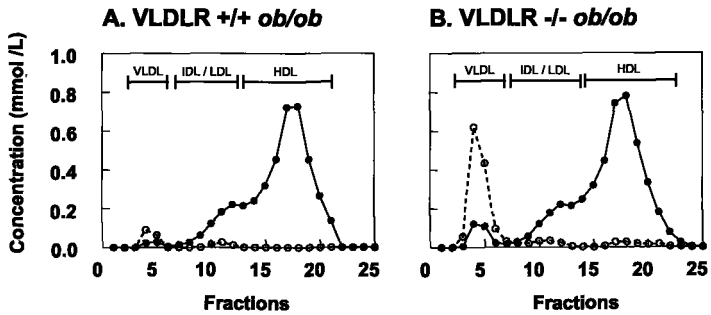

Figure 8: Lipoprotein profiles of *VLDLR*+/+ (8*A*) and *VLDLR*-/- (8*B*) mice on an *ob/ob* background
Plasma of *VLDLR*-/- *ob/ob* and *VLDLR*+/+ *ob/ob* mice was pooled and separated on the base of size by FPLC. The total cholesterol (TC, closed symbols) and triglyceride (TG, open symbols) content of each individual fraction was measured enzymatically, as described in the methods section.

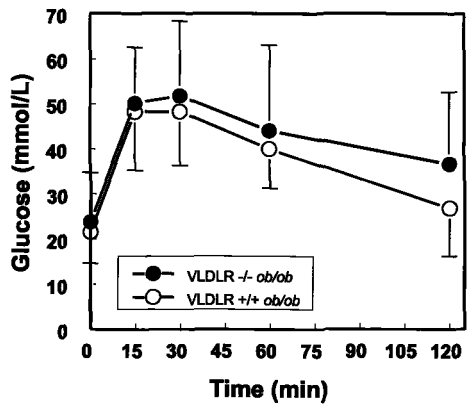

Figure 9: Glucose tolerance tests in *VLDLR*-/- and *VLDLR*+/+ mice on an *ob/ob* background
At 4 months of age, *VLDLR*+/+ *ob/ob* and *VLDLR*-/- *ob/ob* mice were intraperitoneal injected with a glucose bolus (2g/kg) after 4 hours of fasting and changes in plasma glucose concentration were monitored. Values represent the mean ± SD of 7 mice per group.

Discussion

Earlier studies have reported that *VLDLR*-/- mice at the age of 5 months have slightly lower body weights on both a chow and a high-sucrose diet, due to a 50% decrease in adipose tissue mass (13). However, it still remained to be determined whether this was due either to early developmental differences or to altered adipocyte FFA metabolism. In the present study, *VLDLR*-/- mice at the age of 6 months also display a slightly reduced body weight as compared to *VLDLR*+/+ controls when fed a chow diet. Strikingly, upon switching to the high fat, high caloric (HFC) diet, containing predominantly linoleic acid (corn oil), *VLDLR*-/- mice fail to gain any further weight. Furthermore, the average adipocyte size of *VLDLR*-/- mice was significantly

reduced as compared to their wild-type littermates, implying a reduction in cellular TG storage. The reduction in weight gain, adipose tissue mass and adipocyte size in the absence of the VLDLR also holds true for more severe forms of obesity, as in the genetically obese *ob/ob* model (Fig. 7). Altogether, these data indicate that in the absence of the VLDLR, mice are protected from development of obesity.

We investigated four possible explanations for the protection from obesity in the absence of the VLDLR: (1) increased energy expenditure; (2) fat malabsorption; (3) decreased food intake, and (4) decreased flux of fatty acids into peripheral tissues. In *ob/ob* mice it has been shown that obesity results partly from decreased energy metabolism reflected by a strong reduction in core body temperature (20). Body temperature in *VLDLR-/-* mice was similar to that in *VLDLR+/+* mice, both on the HFC diet as well as on the *ob/ob* background (37.0 ± 0.2 vs 37.1 ± 0.2 °C and 36.4 ± 0.6 vs 35.9 ± 0.4 °C, respectively), suggesting that the protection from obesity in the absence of the VLDLR is not likely to involve increased energy expenditure. Protection from obesity in the absence of the VLDLR is not likely due to fat malabsorption (Table 2). Furthermore, no significant differences were found with respect to food intake (Table 2).

Since the VLDLR is predominantly expressed in tissues active in FA metabolism such as the heart, muscle and adipose tissue, it is hypothesized that the VLDLR plays a role in the delivery of FA to peripheral tissues. Thus, in order to study the possibility of an altered flux of fatty acids into peripheral tissues with VLDLR deficiency, we performed FFA turnover experiments with continuous infusion of ^{3}H-palmitate. These experiments reveal a significant reduction in whole body FFA uptake in *VLDLR-/-* mice, which was reflected by a significant decrease in the peripheral FFA oxidation (Fig. 5*A*). Although this method does not allow exact identification of the affected peripheral tissues, these data suggest that absence of the VLDLR may impair FA transport from the circulation into adipose tissue and, as such, may contribute to the protection from obesity.

Given the observation that *VLDLR-/-* mice exhibit a significant reduction in whole body FFA uptake upon palmitate infusion, the question remains to be answered as to where these fatty acids are going. Although plasma FFA levels are elevated in *VLDLR-/-* mice on the *ob/ob* background, no significant differences in plasma FFA levels were found between *VLDLR-/-* and *VLDLR+/+* mice after 17 weeks of HFC feeding (Table 1). However, the appearance of tracer-FFA in plasma VLDL-TG is increased in *VLDLR-/-* mice on the HFC diet (Fig. 5*B*). These findings are in line with the observed hypertriglyceridemia in these mice and with recently generated data in our laboratory showing that *VLDLR-/-* mice crossbred onto the LDLR-deficient background exhibit elevated levels of TG as well (21). The mechanisms underlying the development of hypertriglyceridemia in *VLDLR-/-* mice may involve either an enhanced VLDL-TG production and/or a decrease in VLDL-TG lipolysis in peripheral tissues. Arguments in favor of an enhanced VLDL-TG production is that decreased peripheral uptake of FFA due to VLDLR deficiency will lead to an increased flux of FFA to the liver, which subsequently may result in an increased VLDL-TG production (22). However, recent studies showed that VLDLR deficiency does not affect VLDL-TG production in LDLR -/- mice (21). Argumentative for an impaired TG lipolysis is that, in coordination with LPL, the VLDLR is proposed to act as a

docking protein for an efficient VLDL-TG lipolysis. LPL is the main enzyme involved in the hydrolysis of TG and has been shown to bind to the VLDLR (3, 4). Furthermore LPL stimulates the binding of lipoprotein particles to the VLDLR through a bridging function between extracellular heparan sulphate proteoglycans and lipoprotein particles (3, 4). Based on these observations, absence of the VLDLR may hamper an efficient hydrolysis of VLDL-TG so that less FFA are generated and taken up by peripheral tissues. Such a hypothesis would be in line with studies by Weinstock et al. (23), showing that decreased FFA generation due to deficiency for adipose tissue LPL impairs growth rate and adipose tissue accretion of *ob/ob* mice very similar to what we currently find for VLDLR deficiency. Taken together, the results of the present study suggest that the VLDLR plays a role in adipose tissue TG storage, possibly through regulation of the entry of VLDL-derived fatty acids into adipose tissue.

In addition to protection from diet-induced obesity, *VLDLR-/-* mice are also protected from diet-induced insulin resistance. This was reflected by lower plasma glucose and insulin levels as compared to *VLDLR+/+* mice. Moreover, *VLDLR-/-* mice effectively cleared a glucose bolus that was administrated intraperitoneally, while *VLDLR+/+* mice stayed hyperglycemic. Most likely, protection from insulin resistance in VLDLR -/- mice is secondary to protection from obesity, since no significant differences were found in the glucose tolerance tests between chow-fed non-obese wild-type and *VLDLR-/-* mice that were matched for body weight (Fig. 3*B*). Furthermore, no obvious improvements in insulin sensitivity or glucose handling were observed in *VLDLR-/-* mice on the *ob/ob* background (Fig. 9). These latter findings can possibly be explained by the fact that although *VLDLR-/- ob/ob* mice show reduced obesity as compared to *VLDLR+/+ ob/ob* mice, body weights are still increased compared to normal wild-type mice on a chow diet (results not shown).

The results of the current study suggest that selective inhibition of the VLDLR may prevent obesity. Previous studies have shown that RAP inhibits ligand binding to the VLDLR *in vitro* (5). Overexpression of RAP in LDLR-deficient mice causes severe hypertriglyceridemia, which was suggested to be due to an impaired clearance of TG by the LDLR-related protein (LRP) (24). However, recently generated mice that are deficient in both the LDLR and LRP exhibit significantly lower plasma TG levels compared to the LDLR single deficient mice with RAP overexpression (25), indicating the existence of an additional pathway in TG removal next to the LDLR and LRP (26). Our current results indicate that the VLDLR might represent this additional pathway. Additional evidence is presented in studies showing that, in addition to RAP, apolipoprotein C1 (ApoC1) also inhibits lipoprotein binding to the VLDLR both *in vitro* and *in vivo* (27). Similar to LDLR single deficient mice transfected with RAP, overexpression of APOC1 on the LDLR-deficient background causes a more severe hypertriglyceridemia compared to the LDLR/LRP double-deficient mice (28). Furthermore, in line with the protection from obesity in *VLDLR-/-* mice, overexpression of ApoC1 is also associated with diminished adipose tissue mass (29).

In summary, VLDLR deficiency protects mice from diet-induced as well as genetically-determined obesity possibly through interference with the entry of fatty acids into adipose tissue. As such these data represent the first demonstration that the VLDLR participates in the

regulation of adipose tissue formation and suggests that selective inhibition of the VLDLR may offer a therapeutic target for treatment of obesity.

Acknowledgments

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CHAPTER 5

VLDL receptor deficiency enhances intimal thickening after vascular injury but does not affect atherosclerotic lesion area

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(Submitted)

Abstract

The very low density lipoprotein receptor (VLDLR) has been shown to modulate cell migration and foam cell formation *in vitro*. This suggests a role for the VLDLR in vascular pathology associated with intimal thickening and atherogenesis. In the present paper both intimal thickening and atherosclerosis were studied using VLDLR knockout and transgenic mouse models. The role of the VLDLR in intimal thickening was established in an *in vivo* model for vascular injury. A non-restrictive cuff was placed around the femoral artery of VLDLR deficient (*VLDLR-/-*), heterozygous deficient (*VLDLR+/-*) and wild type (WT) mice. Intimal thickening was assessed after 3 weeks by determining the intima to media (I/M) volume ratio. Both *VLDLR-/-* (I/M ratio 42%) and *VLDLR+/-* (I/M ratio 40%) mice showed a significant increase as compared to WT littermates (I/M ratio 25%). The effect of VLDLR deficiency on atherosclerosis was examined in *VLDLR-/-* mice on an LDLR deficient (*LDLR-/-*) background. In addition, we assessed whether increased endothelial VLDLR expression levels affect atherosclerotic lesion formation. Therefore, atheroslcerosis was studied in LDLR deficient mice that overexpress the VLDLR in endothelial cells (PVL;*LDLR-/-*). Both VLDLR deficiency and endothelial VLDLR overexpression did not affect atherosclerotic lesion size. Interestingly, *VLDLR-/-;LDLR-/-* mice showed a high incidence of necrosis in both fatty streaks and atherosclerotic plaques as compared to *LDLR-/-* mice (75% vs. 0% and 76% vs. 45%, respectively). In conclusion, deficiency for the VLDLR profoundly increased intimal thickening after vascular injury.

Introduction

The very low density lipoprotein (VLDL) receptor (VLDLR) is a multi ligand receptor that belongs to the low density lipoprotein receptor (LDLR) family. The VLDLR has been described to bind apolipoprotein E (apoE) rich VLDL (1, 2) and lipoprotein lipase (3), while VLDLR deficient (*VLDLR-/-*) mice reveal a moderate decrease in adipose tissue mass. This suggests a role for the VLDLR in lipid metabolism (4). In addition, the VLDLR binds a number of ligands which are not directly linked to lipoprotein metabolism, including receptor associated protein (RAP) (5), reelin (6), urokinase plasminogen activator (uPA)/plasminogen activator inhibitor (PAI)-1 complex (7) and several other proteinase/serpin complexes (8). Binding of reelin to the VLDLR has been hypothesized to be important for correct regulation of neuronal migration in the developing brain. *VLDLR-/-* mice that are also deficient for the apolipoprotein E receptor 2 display marked malformation of both cerebral cortex and cerebellum (9).

The VLDLR is most abundantly expressed in heart, skeletal muscle and adipose tissue (10-14). VLDLR expression is localized in endothelial and smooth muscle cells of arteries and veins, and has also been detected in neointimal smooth muscle cells and macrophage derived foam cells (15, 16). *In vitro* studies have suggested a role for the VLDLR in smooth muscle cell migration (17-19) and foam cell formation (20). Thus, the VLDLR could play a role in vascular

pathology associated with intimal thickening leading to and restenosis, and lipid accumulation leading to atherosclerosis.

Intimal thickening is a characteristic arterial response to injury, caused by migration of smooth muscle cells from the media into the intima. Intimal thickening plays a role in restenosis and the development of atherosclerotic lesions (21). In the present paper, intimal thickening has been studied in *VLDLR-/-*, VLDLR heterozygous knockout (*VLDLR+/-*) and wild type mice using a model for vascular injury. This model consists of placement of a nonocclusive cuff around the femoral artery, which has been reported to lead to reproducible intimal growth (22).

In addition to investigating its role in intimal thickening, we have examined the role of the VLDLR in atherosclerosis. The VLDLR could contribute to atherosclerotic lesion formation in several ways, such as mediating neointimal thickening by smooth muscle cells, facilitating lipid loading of macrophages and smooth muscle cells and mediating binding, uptake and/or transcytosis of VLDL by endothelial cells. Interestingly, induction of atherosclerotic lesion formation by feeding of a cholesterol-rich diet has been reported to lead to a marked induction of rabbit aortic VLDLR expression levels (16). In the present paper, the effect of VLDLR deficiency on lesion formation was tested in a mouse model for atherosclerosis, the LDLR deficient (*LDLR-/-*) mouse (23, 24). In addition, we have tested the hypothesis by Wyne et al, suggesting that the endothelial VLDLR mediates atherosclerotic lesion formation by transporting lipoproteins into the vessel wall (25). Therefore, atherosclerotic lesion formation was studied on an LDLR deficient background in transgenic mice overexpressing the VLDLR in the endothelium (PVL) (26). PVL mice carry the human VLDLR gene under control of the preproendothelin-1 promoter, which drives expression in endothelial cells (27).

The results of the present study demonstrate that VLDLR deficiency leads to a profound increase in cuff induced intimal thickening by smooth muscle cells. VLDLR deficiency and endothelial VLDLR overexpression did not affect lesion size or smooth muscle cell composition of the plaque. However, *VLDLR-/-;LDLR-/-* mice showed a relatively high number of necrotic lesions when compared to *LDLR-/-* mice.

Methods

Animals

VLDLR-/- mice were purchased from Jackson (Bar Harbor, Maine, USA). *VLDLR-/-* mice were bred to C57Bl6/J to produce *VLDLR+/-* mice. *VLDLR+/-* mice were bred to produce *VLDLR-/-*, *VLDLR+/-* and wild type mice for the neointima experiments. Experiments were performed using 10-12 weeks old male mice.

VLDLR-/-;LDLR-/- and LDLR-/- male littermates and PVL;*LDLR-/-* and *LDLR-/-* female littermates were generated as was described before (26). At ten weeks of age, mice were put on a high fat diet, containing 0.25% cholesterol (Hope Farms, Woerden, The Netherlands), for 8 weeks. Subsequently the mice were sacrificed by cervical dislocation.

Femoral artery cuff placement

12 *VLDLR-/-*, 9 *VLDLR+/-* and 14 wild type mice were anesthetized with Hypnorm (Bayer, Leverkusen, Germany, 25 mg/kg) and Dormicum (Roche, Basel, Switzerland, 25 mg/kg) i.p. The right femoral artery was dissected from its surroundings and a non-restrictive polyethylene cuff (0.28 mm inner diameter, 0.61 outer diameter and 1.5 mm length, Portex, Hythe, Great Britain), was placed loosely around it (22). After 3 weeks, mice were anesthetized with Hypnorm/Dormicum and the thorax was opened. Mild pressure perfusion with Zinc Formal-Fixx™ (dilution 1:4, Shandon Inc., Pittsburgh, PA, USA) was performed by cardiac puncture for 10 minutes. Subsequently, the femoral artery was harvested, fixated overnight in 3.7% formaldehyde in phosphate buffered saline and paraffin-embedded. Serial cross sections (5 μm) were cut throughout the entire length of the cuff.

Morphometry

Six cross sections in the middle of the cuff, each at a 50 μm interval, were used for analysis. Sections were stained with Weigert's elastin staining to visualize the elastic laminae. Image analysis software (Leica, Solms, Germany) was used to measure total cross sectional medial area between the external and internal elastic lamina. Total cross sectional intimal area was measured between the endothelial cell monolayer and the internal elastic lamina.

Atherosclerosis experiments

After receiving the high fat diet for 8 weeks, mice were fasted for 4 hours. Subsequently they were anesthetized and blood samples were collected by eye bleeding. Total serum cholesterol and triglyceride concentrations (without free glycerol) were measured enzymatically using commercial test kits (kit no. 236691 from Boehringer Mannheim GmbH, Mannheim, Germany, and kit no. 337-B from Sigma, St. Louis, MO, USA respectively). Subsequently mice were sacrificed and the heart was removed, cutting the aorta just above the atria. The heart was bisected perpendicular to the heart axis below the atrial tips, fixated in 4% formaldehyde and paraffin-embedded. Serial cross sections of 5 μm were cut throughout the entire length of the atrioventricular valve area. Three cross sections from this area were used for measuring lesion area by computer-aided morphometry (Kontron-Videoplan, Zeiss, Oberkochen, Germany). Lesions were examined microscopically for the degree of monocytic infiltration, fibrosis, necrosis and granulocyte infiltration. Furthermore, the number of fatty streaks and plaques displaying necrosis were determined.

Immunohistochemistry

Smooth muscle cells were visualized by α-smooth muscle actin staining (dilution 1:1500, Sigma, St. Louis, MO, USA). The primary antibody was detected with a peroxide-labeled rabbit anti-mouse antibody (dilution 1:300, Dako, Denmark). Macrophages were visualized using the rabbit AIA31240 antibody (dilution 1:5000, Accurate Chemical, New York, NY, USA). The primary antibody was detected with a biotinylated goat anti-rabbit antibody (Janssen Pharmaceuticals, Tilburg, The Netherlands). 3,3'-diaminobenzidine (DAB, Sigma, St. Louis, MO, USA) was used as chromagen for both macrophage and smooth muscle cell staining. Sections were counterstained either with hematoxylin or hematoxilin-phloxine-saffron (HPS).

Statistics

Data were reported as mean ± S.D. The Student's t-test was used to compare groups to their controls. P-values less than 0.05 were regarded significant.

Results

Intimal thickening

To determine the effect of the VLDLR on intimal thickening, a cuff was placed around the femoral artery of *VLDLR*-/-, *VLDLR*+/- and wild type mice. Three weeks later, the cuffs were harvested and analyzed. Figure 1 shows examples of Weigert's elastin stained sections of cuffed femoral arteries from *VLDLR*-/- (A) and wild type (B) mice, as were used for morphometric analysis. Thick cell layers were present in the subendothelial intimal region of femoral arteries of *VLDLR*-/- and *VLDLR*+/- mice, whereas these cell layers were relatively thin in wild type mice. Immunohistochemical analysis revealed that the subendothelial cell layers of the intima consisted exclusively of smooth muscle cells (Figure 1C). Morphometric analysis of the cuffs showed a significant increase in intimal thickening in *VLDLR*-/- (I/M ratio 42%) and *VLDLR*+/- (I/M ratio 40%) mice as compared to wild type (I/M ratio 25%) littermates (figure 2).

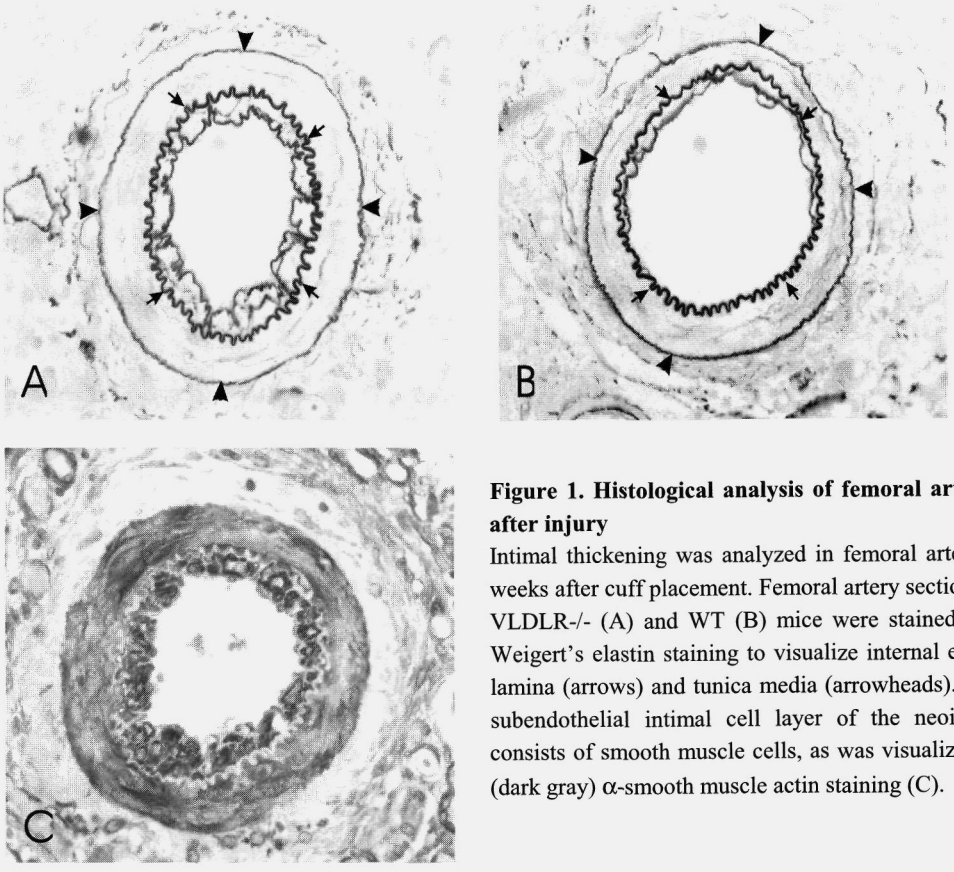


Figure 1. Histological analysis of femoral arteries after injury

Intimal thickening was analyzed in femoral artery, 3 weeks after cuff placement. Femoral artery sections of VLDLR-/- (A) and WT (B) mice were stained with Weigert's elastin staining to visualize internal elastic lamina (arrows) and tunica media (arrowheads). The subendothelial intimal cell layer of the neointima consists of smooth muscle cells, as was visualized by (dark gray) α-smooth muscle actin staining (C).

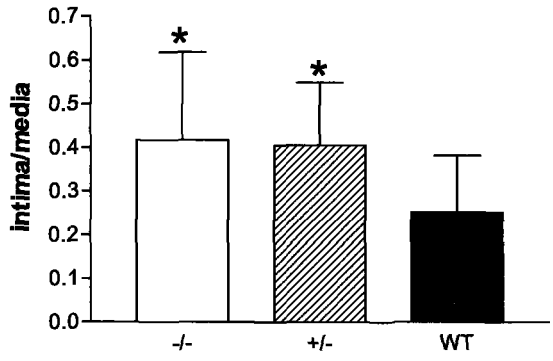


Figure 2. Intima/media ratio after vascular injury
Intima to media ratio was determined three weeks after cuff placement in 12 *VLDLR-/-* (-/-), 9 *VLDLR*+/- (+/-) and 14 wild type (WT) mice, as described in methods.
* = significantly different from WT littermates (P<0.05), using the Student's t-test.

Serum lipid levels

To assess the role of the VLDLR in atheroslcerosis, *VLDLR-/-* and PVL mice were crossbred with *LDLR-/-* mice to generate *VLDLR-/-;LDLR-/-* and PVL;*LDLR-/-* mice. After feeding of an atherogenic diet all animals were hypercholesterolemic (table I). No significant differences in serum cholesterol levels were detected between VLDLR deficient or overexpressing mice and their control groups. Serum triglyceride levels in PVL;*LDLR-/-* mice were significantly lower than in *LDLR-/-* control animals, as was described before (paper submitted). In contrast, no difference in serum triglyceride levels was found between *VLDLR-/-;LDLR-/-* mice and *LDLR-/-* controls (table I).

Table I: Serum cholesterol and triglyceride levels of the different mouse models after receiving an atherogenic diet

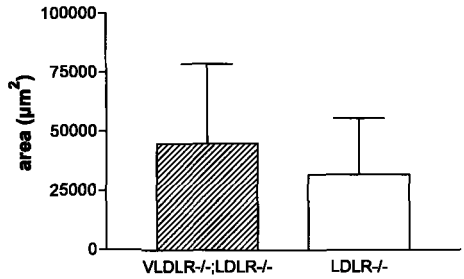
	N	Sex	Cholesterol (mM)	Triglycerides (mM)
VLDLR-/-;LDLR-/-	8	M	25.7 ± 5.6	7.5 ± 2.4
LDLR-/-	11	M	25.5 ± 5.9	6.4 ± 3.2
PVL;*LDLR-/-*	16	F	47.2 ± 10.3	1.7 ± 0.5*
LDLR-/-	20	F	49.9 ± 10.1	3.5 ± 1.7

Serum cholesterol and triglyceride concentrations were measured in the different mouse strains. Lipid levels were measured in mice that were fasted for 4 hours. Mice were kept on a high fat diet for 8 weeks. Values shown are means ± S.D., * = significantly different from LDLR-/- controls (P<0.05), using the Student's t-test.

Atherosclerosis

After 8 weeks of feeding an atherogenic diet, atherosclerosis was quantified in the aortic root area. Both VLDLR deficiency and endothelial VLDLR overexpression had no significant effect on atherosclerotic lesion area (figure 3). However, *VLDLR-/-;LDLR-/-* mice displayed a

high incidence of plaques with necrosis when compared to *LDLR-/-* littermates (Table II, figure 4A and 4B). Moreover, necrosis was detected in the majority of fatty streaks of *VLDLR-/-;LDLR-/-* mice and was absent in fatty streaks of *LDLR-/-* controls (Table II, figure 4C). Necrosis in lesions of *VLDLR-/-;LDLR-/-*mice was often accompanied by granulocyte infiltration (figure 4C). Immunohistochemical analysis of the lesions revealed that necrosis occurred in the macrophage rich areas (figure 4D). In the cuff experiments, *VLDLR-/-* mice showed enhanced intimal thickening as compared to wild type littermates, implying an increase in smooth muscle cell infiltration of the intima. To detect possible changes in smooth muscle cell infiltration of atherosclerotic plaques as a result of VLDLR deficiency, *VLDLR-/-;LDLR-/-* and *LDLR-/-* sections were stained for actin (figure 4A and 4B). No differences were found in smooth muscle cell number and location between atherosclerotic plaques of both groups.



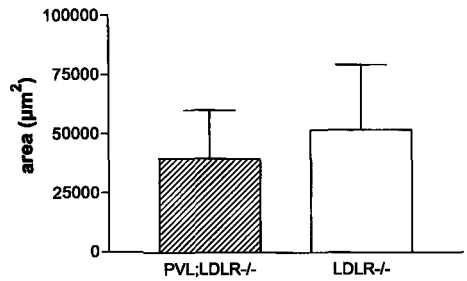


Figure 3. Atherosclerotic lesion area
Top panel shows the mean atherosclerotic lesion area (± S.D.) as determined in 8 *VLDLR-/-;LDLR-/-* and 11 *LDLR-/-* littermates that were fed a high fat diet. Lower panel shows the mean atherosclerotic lesion area (± S.D.) as determined in 16 PVL;*LDLR-/-* and 20 *LDLR-/-* littermates that were fed a high fat diet.

Table II: Necrosis in atherosclerotic lesions

	Fatty Streak	Plaque
VLDLR-/-;LDLR-/-	9/12** (75%)	19/25* (76%)
LDLR-/-	0/15 (0%)	13/31 (45%)

Values shown are number of necrotic lesions from total, as determined in 8 *VLDLR-/-;LDLR-/-* mice and 11 *LDLR-/-* littermates. Values between brackets represent percentage of necrotic lesions from total. *= significantly different from *LDLR-/-* littermates using Fisher's exact test ($P<0.05$).

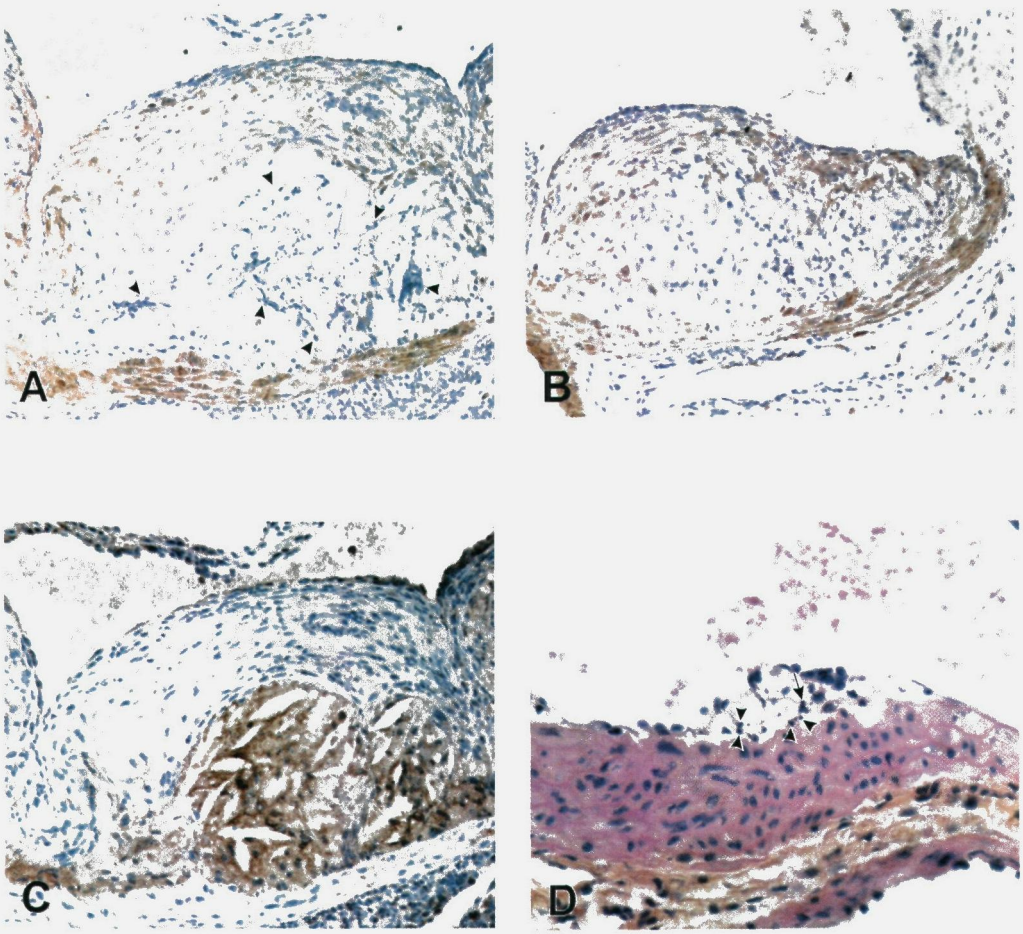


Figure 4. Histological analysis of atherosclerotic plaques

α-Smooth muscle actin staining of heart sections of *VLDLR-/-;LDLR-/-* mice (A) and *LDLR-/-* littermates (B). Smooth muscle cell distribution does not differ in both plaques. *VLDLR-/-;LDLR-/-* plaque is severely necrotic (arrowheads). Panel (C) shows a AIA31240 macrophage (brown) staining of a *VLDLR-/-;LDLR-/-* plaque (same plaque as depicted in panel A). Panel (D) shows necrosis (arrowheads) and granulocyte infiltration (arrow) characteristic for fatty streaks of *VLDLR-/-;LDLR-/-* mice.

Discussion

In the present study, the role of the VLDLR in intimal thickening and atherosclerosis was studied in mice. A non-occlusive cuff model to induce vascular injury in mice was used to examine the effect of the VLDLR on intimal thickening. *VLDLR+/-* and *VLDLR-/-* mice both showed a profound increase in intimal thickening when compared to wild type controls. VLDLR deficiency did not affect atherosclerotic lesion size in *LDLR-/-* mice that were fed an atherogenic diet. However, *VLDLR-/-;LDLR-/-* mice did show a high incidence of lesions with necrosis when compared to *LDLR-/-* controls. Endothelial overexpression of the VLDLR in *LDLR-/-* mice did not influence the atherosclerotic process.

After cuff treatment in the intimal thickening studies, smooth muscle cells were present in the subendothelial intimal region of the femoral arteries. Therefore, smooth muscle cells must have migrated across the internal elastic lamina. Furthermore, immunohistological analysis revealed proliferating smooth muscle cells in the intima (data not shown). Taken together, these results suggest the VLDLR inhibits smooth muscle cell migration and/or proliferation. Lipoprotein receptors, including VLDLR and LDLR related protein (LRP), have been reported before to modulate cell migration *in vitro* (17, 28, 29). Similar to what we found *in vivo*, these studies showed an increase in cell migration when either the VLDLR or LRP are blocked or absent. In contrast, *in vitro* studies using RAP to neutralize both VLDLR and LRP function in smooth muscle cells revealed an inhibitory (18, 19) or no effect (30) on cell migration. The mechanisms underlying these apparent contrasting effects of lipoprotein receptor function on cell migration remain to be elucidated.

In addition to intimal thickening, we examined the role of the VLDLR in atherosclerotic lesion formation, using *LDLR-/-* mice as a model for atherosclerosis. Endothelial VLDLR overexpression decreased serum triglyceride levels of *LDLR-/-* females fed a high fat diet, as has been reported previously (26). In contrast to the situation in female mice (26), VLDLR deficiency did not lead to an increase in serum triglyceride levels of male *VLDLR-/-;LDLR-/-* mice fed a high fat diet, suggesting a sex difference in the response of serum triglycerides to VLDL deficiency.

Lipoprotein receptors, including the VLDLR, have been hypothesized to play a role in atherosclerosis by mediating lipid loading of smooth muscle cells and macrophages (for review see (31)). The VLDLR is not downregulated by intracellular cholesterol (2, 20), and induces lipid loading and foam cell formation *in vitro* (20). Remarkably, we found no effect of VLDLR deficiency on the mean atherosclerotic lesion area in *LDLR-/-* mice, suggesting the VLDLR does not play an essential role in atherosclerotic foam cell formation.

Smooth muscle cell mediated intimal thickening has been proposed to play a role in atherosclerotic neointima formation (21). In our studies, histological analysis revealed no differences in smooth muscle cell presence in atherosclerotic lesions between *VLDLR-/-;LDLR-/-* mice and *LDLR-/-* controls. Thus the role of the VLDLR in smooth muscle cell mediated intimal thickening found in response to vascular injury could not be established for atherosclerotic neointima formation.

Remarkably, VLDLR deficiency led to a high incidence of plaques displaying necrosis in macrophage rich areas, which was often accompanied by infiltration of granulocytes. Furthermore, necrosis was found in the majority of fatty streaks of *VLDLR;LDLR-/-* mice, while it was not found in fatty streaks of *LDLR-/-* mice. These results indicate that certain cells, probably macrophages, become necrotic more rapidly in the absence of the VLDLR. The nature of the mechanism underlying this increase in necrosis remains to be elucidated.

Similar to VLDLR deficiency, endothelial VLDLR overexpression did not affect atherosclerotic lesion size in *LDLR-/-* mice. These results do not support the hypothesis that the VLDLR mediates development of atherosclerosis by contributing to lipoprotein transport into the vessel wall. Furthermore, no differences were detected between the composition of atherosclerotic lesions of PVL;*LDLR-/-* mice and *LDLR-/-* controls. Thus no evidence was found to support a role for the endothelial VLDLR in atheroslcerosis.

In summary, we demonstrated a profound stimulatory effect of VLDLR deficiency on intimal thickening after vascular injury. Expression modulation of the VLDLR in *LDLR-/-* mice did not affect atherosclerotic lesion size. However, VLDLR deficiency markedly enhanced necrosis of cells, both in early and advanced lesions.

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CHAPTER 6

VLDL binding to the apolipoprotein E receptor 2 is enhanced by lipoprotein lipase and does not require apolipoprotein E

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Abstract

The apolipoprotein (apo) E receptor 2 (apoER2) is a recently cloned member of the low density lipoprotein (LDL) receptor (LDLR) family, showing a high homology with the LDLR and the very low density lipoprotein (VLDL) receptor (VLDLR). In the present study the binding characteristics of the apoER2 with respect to apoE and lipoprotein lipase (LPL) were investigated. Therefore, VLDL was isolated from apoE deficient mice, and from mice expressing the human APOE2 (Arg158→Cys) and APOE3-Leiden isoforms on an *Apoe-/-* and *Ldlr-/-* double knockout background. ApoE rich rabbit β-VLDL was used as a positive control for binding. Binding experiments using Chinese hamster ovary (CHO) cells expressing the human apoER2 showed, that the receptor was able to bind VLDL containing either of both apoE isoforms, as well as the apoE deficient VLDL. Hence, in contrast to the VLDL receptor, the apoER2 is not strictly dependent on apoE for VLDL binding. Since LPL has been described to enhance the binding of lipoproteins to several members of the LDLR family, including the LDL receptor related protein (LRP), VLDL receptor, gp330 and the LDLR itself, VLDL binding experiments were performed in the presence of LPL. Addition of LPL resulted in a significant increase in apoER2 binding for all VLDL fractions used in this study. In conclusion, lipoprotein binding of VLDL to the apoER2 is enhanced in the presence of LPL, and is not restricted to apoE containing lipoproteins.

Introduction

The apolipoprotein (apo) E receptor 2 (apoER2) was initially cloned from a human placenta cDNA library on the basis of homology to the coated pit signaling domain of the low density lipoprotein receptor (LDLR) family members (1). It shows high homology to the LDLR and very low density lipoprotein (VLDL) receptor (VLDLR), even up to the level of the positioning of the exon/intron boundaries of the genes (2). *In vitro*, the human apoER2 binds and internalizes apoE rich rabbit β-VLDL with high affinity. The affinity for human VLDL and LDL is much lower. In humans, apoER2 expression has been detected in brain and placenta, while in rabbits expression has been found in brain, testis and ovary (1). LR8b, the chicken homologue of the apoER2, is specifically expressed in brain, and binds and internalizes the activated form of α_2 macroglobulin (3, 4). Recent findings suggest a role for the apoER2 in transmitting extra-cellular signals to intra-cellular signaling processes in a molecular pathway that regulates neuronal migration during fetal brain development (5). ApoE has been hypothesized to play a role in modulating this signaling pathway. Since apoER2 expression levels remain high in the adult brain, it seems likely that the apoER2 is also involved in other physiologically relevant processes.

ApoE plays a key role in the lipid metabolism as a ligand for receptor mediated uptake of apoE containing lipoproteins. It is involved in the clearance of chylomicrons, VLDL and intermediate density lipoprotein (IDL) by the liver. In addition, apoE plays a role in reverse cholesterol transport (for reviews see (6, 7)), VLDL production (8) and VLDL triacylglycerol

lipolysis (9). Despite extensive knowledge of the functions of apoE in lipoprotein metabolism, the role of apoE in the lipid metabolism of the brain is poorly understood. ApoE is synthesized and secreted in high levels by astrocytes, and is found incorporated in phospholipid discoid particles and apoE enriched HDL residing in the cerebrospinal fluid (10). These lipoprotein particles are thought to be taken up by brain cells via receptor mediated endocytosis, a process in which apoE receptors of the LDL receptor family such as the apoER2 could play a role.

LPL is the principal enzyme responsible for hydrolysis of triacylglycerols of circulating lipoproteins. Like apoE, LPL is present in the brain, and is expressed in a number of cell types including neurons in the hippocampus, Purkinje cells of the cerebellum, and cells deep within the cortex (11). In addition to its lipolytic activity, LPL can enhance VLDL catabolism by performing a bridging function between extra-cellular heparan sulfate proteoglycans and the lipoprotein particle, leading to rapid internalization via receptor mediated pathways (12-14). LPL can also bind directly to lipoprotein receptors of the LDLR family, and perform a bridging function between the lipoprotein particle and the receptor (15-23). Whether LPL performs similar functions in the brain remains to be elucidated.

To study the ligand binding characteristics of the apoER2, we analyzed the effect of different apoE variants on the binding of lipoproteins to the apoER2. VLDL was isolated from APOE2 mice on an *Apoe-/-;Ldlr-/-* double knockout background. VLDL was also isolated from APOE3Leiden mice on an *Apoe-/-;Ldlr-/-* double knockout background. The APOE3Leiden variant contains a tandem duplication of codons 120-126, and is associated with a dominant inheritance of Familial Dysbetalipoproteinemia (24, 25). Both VLDL fractions bound to the apoER2 with high affinity. VLDL from *Apoe-/-* mice also bound to the apoER2, showing that apoER2 binding is not restricted to apoE containing lipoproteins. In addition, the effect of LPL on binding of the VLDL fractions to the apoER2 was analyzed. *In vitro* binding studies revealed that LPL enhanced binding of all VLDL fractions to the apoER2 significantly.

Methods

Mice

Ldlr-/- mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). Transgenic mice expressing human APOE2 and APOE3-Leiden in the absence of endogenous mouse apoE have been described previously (26). *Ldlr-/-*, *Apoe-/-*, APOE2 and APOE3-Leiden genotype was confirmed by PCR analysis on tailtip DNA, as described earlier (27, 28). Mice were fed ad lib a regular chow diet (SRM-A: Hope Farms, Woerden, The Netherlands).

Immunoblotting

Membrane protein fractions of both apoER2 transfected and control CHO cell lines were isolated. Cells were lysed on ice by incubation in 150 mM NaCl, 10 mM EDTA, 100 mM Tris (pH 8.0), 1% (v/v) NP-40 and a protease inhibitor cocktail tablet Complete™, Mini (Boehringer Mannheim, Mannheim, Germany) in water. Cell extracts were subjected to SDS-PAGE on a 6% (w/v) gel under non-reducing conditions, and transferred to nitrocellulose membranes by

electrophoresis. Filters were blocked for 1 hour in 3% (w/v) dried non-fat milk, 2% (v/v) normal goat serum and 0.05% Tween 20 (v/v) in phosphate buffered saline (blocking buffer). The membranes were then incubated in blocking buffer containing a 3000-fold dilution of the antiserum for 1 hour. Subsequently the membranes were washed 3 times in blocking buffer and incubated with a 1000-fold diluted goat anti-rabbit IgG horseradish peroxidase conjugate (Promega, Madison, USA) in blocking buffer for 30 minutes. The membranes were then washed three times in blocking buffer and 2 times in phosphate buffered saline. Antibody binding was visualized by using the ECL detection kit (Amersham, Amersham, UK).

Antiserum

The antiserum is a rabbit polyclonal antiserum generated against a synthetic peptide (EKDQFQCRN) corresponding to a segment of the first repeat of the binding domain of the apoER2, as described by Sun and Soutar (29).

Lipoprotein and LPL isolation

After a 5 hour fasting period, blood was collected from *Apoe-/-*, APOE2;*Apoe-/-;Ldlr-/-* and APOE3-Leiden;*Apoe-/-;Ldlr-/-* mice. For the isolation of rabbit β-VLDL, a rabbit was fed on a high cholesterol diet for at least two weeks, and blood was collected after a 5 hour fasting period. The sera were ultracentrifuged to obtain the VLDL fractions ($d < 1.006$ g/ml). VLDL protein was determined according to Lowry et al. (30). A portion of the VLDL was labeled with ^{125}I by the iodine monochloride method (31). After iodination, VLDL was dialyzed extensively at 4 °C against phosphate buffered saline (PBS) for 24 hours. The apoB content of the VLDL fractions was determined by isopropanol precipitation as described by Egusa et al. (32). VLDL particle size was determined by photon correlation spectroscopy using a Malvern 4700 C system (Malvern Instruments, Malvern, UK). Measurements were performed at 25°C and a 90° angle between laser and detector.

LPL was isolated from fresh bovine milk as described previously (33). The isolated fraction was suspended in 20 mmol/l NaH_2PO_4, 50% glycerol (v/v) and stored in aliquots at –80 °C. Prior to the experiments, LPL was heat-inactivated at 56 °C for 30 minutes.

Binding studies

To study the binding of VLDL derived from transgenic mice to the apoER2, binding studies were performed using an LDL receptor deficient CHO cell line (ldlA7) that was stably transfected with either the human apoER2 cDNA (apoER2 CHO) or pSV2-Neo (control CHO) (1). Binding experiments were performed essentially as described previously (34). The cells were cultured in 24-well plates in HAM's F10 medium, supplemented with 10% (v/v) fetal calf serum, streptomycin (200 μg/ml) and penicillin (200 U/ml) at 37°C and at a 5% CO_2 concentration in air. Cells were washed with HAM's F10 3 times and incubated for 3 h at 4°C with increasing amounts of ^{125}I-labeled VLDL in HAM's F10, either in the presence or absence of a 20-fold excess of unlabeled VLDL. Specific binding was calculated as binding in the

absence minus binding in the presence of excess unlabeled VLDL. Dissociation constants were calculated by nonlinear regression using GraphPad Prism (GraphPad Software Inc, San Diego). The binding of the different VLDL fractions in the presence or absence of LPL (5 μg/ml) was determined essentially as described previously (33). Binding was studied at a concentration of 10 μg total protein/ml of labeled lipoprotein both to the apoER2 and control CHO cells. For the LPL binding experiments, binding of VLDL to the apoER2 was defined as the amount of VLDL protein bound to the apoER2 CHO cells minus the amount of VLDL protein bound to control CHO cells.

Results

Binding in vitro of apoE-/-, apoE2 and apoE3-Leiden VLDL to the apoER2

To determine whether the human apoER2 is expressed in the CHO cells, protein extracts of both apoER2 and control CHO cells were immunoblotted using rabbit polyclonal antibodies to the apoER2 (29). A protein with an apparent relative molecular mass of ~130 kDa was detected in the apoER2 CHO cells (figure 1). This protein was not detected in control CHO cells, indicating that the apoER2 CHO cells do express the human apoER2.

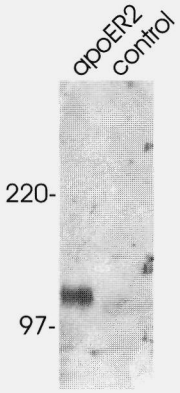

Figure 1. Western blot analysis of CHO cell extracts using a polyclonal antibody raised against ApoER2

Membrane protein fractions of both apoER2 CHO (apoER2) and control CHO (control) cell lines were isolated and subjected to SDS-PAGE on a 6% gel under non-reducing conditions, and subsequently transferred to a nitrocellulose membrane. The membrane was incubated with a polyclonal antiserum against the human apoER2. The position of molecular mass size markers (in kDa) run on the same gel is indicated on the left.

The effect of apoE and different isoforms of apoE on lipoprotein binding to the apoER2 was studied using VLDL that was isolated from several mouse models. VLDL binding in the absence of apoE was studied using VLDL derived from *Apoe-/-* mice. ApoE rich rabbit β-VLDL was used as a positive control for binding. Binding of different apoE variants to the apoER2 receptor in the absence of endogenous apoE was studied using VLDL derived from APOE3-

Leiden;*Apoe-/-;Ldlr-/-* and APOE2;*Apoe-/-;Ldlr-/-* mice. These mice have relatively high levels of VLDL in their plasma, which is rich in apoE (35). As shown in figure 2, all ligands that were used in this study bind to the apoER2.

The amount of apoB present in the different VLDL fractions was determined by specifically precipitating apoB in these fractions. Subsequently the protein mass of the apoB precipitate was determined (table 1). ApoB precipitation proved to be very efficient, as the percentage of radioactive label that precipitated with apoB was identical to the percentage of label present in apoB as determined by VLDL analysis using SDS-PAGE and Phosphor-Imaging quantification (results not shown). K_d values, which were calculated on the basis of the amount of apoB bound to the cells, are provided in table 1. The data show that the human apoER2 binds all VLDL fractions used in this study. The affinity of ApoER2 for rabbit β-VLDL was significantly higher than that for the other VLDL fractions. No significant differences in apoER2 affinity for APOE3-Leiden, APOE2 and Apoe-/- VLDL were found.

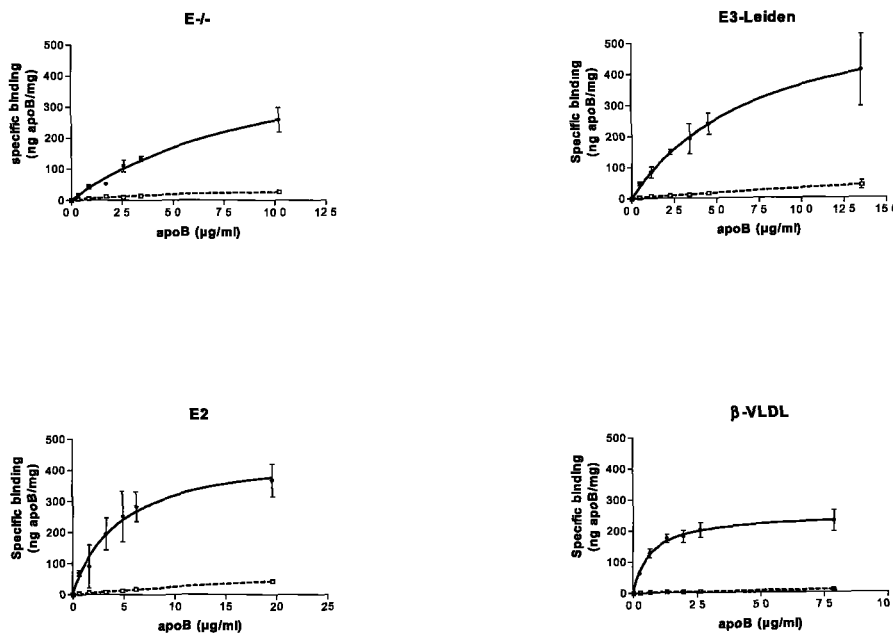

Figure 2. Specific binding of VLDL to apoER2 expressing and control CHO cells
Control (o) and apoER2 (•) CHO cells were incubated for three hours at 4 °C with increasing concentrations of ^{125}I-labeled VLDL from *Apoe-/-* mice (E-/-), APOE3-Leiden;*Apoe-/-;Ldlr-/-* mice (E3Leiden), APOE2;*Apoe-/-;Ldlr-/-* (E2) mice and rabbit β-VLDL (β-VLDL), in either the presence or absence of a 20-fold excess of unlabeled VLDL, as described in Methods. Specific binding was calculated as the amount of apoB bound per mg cell protein, which reflects particle number. Each value represents the mean ± S.D. of three experiments.

Table 1. VLDL particle composition and its apoER2 binding characteristics

	Particle size (nm)	ApoB (% of total protein)	K_d (µg apoB/ml)
β-VLDL	82 ± 18	26.3	0.67 ± 0.06*
E-/-	49 ± 11	34.0	10.9 ± 2.6**
E3-Leiden E-/- Ldlr-/-	96 ± 26	45.1	7.6 ± 0.5**
E2 E-/- Ldlr-/-	71 ± 12	65.5	4.6 ± 0.8**

The specific binding of ^{125}I-labeled VLDL to the apoER2 was determined as described in the Methods section. Dissociation constants were calculated by non linear regression methods based on the amount of apoB protein, which reflects particle number. Particle size was determined by photon correlation spectroscopy. The values represent the mean ± S.E.M. for three measurements. * = K_d value differs significantly from E-/-, E3-Leiden and E2 VLDL, ** = K_d value differs significantly from rabbit β-VLDL, $P < 0.05$, Student's t-test.

VLDL binding in vitro in the presence of LPL

The effect of LPL on the binding of VLDL by the apoER2 was studied with control and apoER2 expressing CHO cells. None of the VLDL fractions bound to the control cells in the absence of LPL, and also in the presence of LPL the binding capability of the control cells was low (data not shown). As shown in figure 3, addition of LPL to apoER2 CHO cells leads to a vast increase in specific binding of all of the VLDL fractions used in the present study.

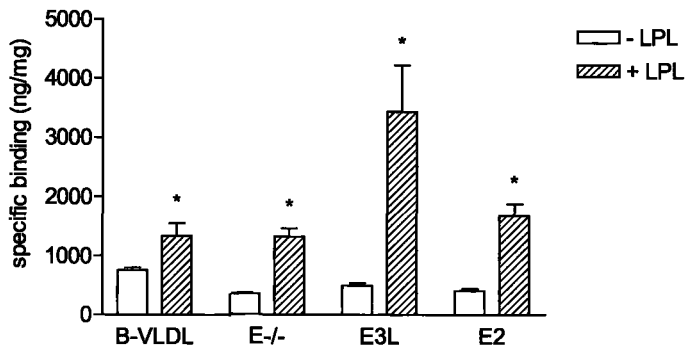

Figure 3. Effect of LPL on binding of VLDL to the apoER2

Control and apoER2 CHO cells were incubated for three hours at 4 °C with 10 µg total protein/ml ^{125}I-labeled VLDL from *Apoe-/-* mice (E-/-), APOE3-Leiden;*Apoe-/-;Ldlr-/-* mice (E3L), APOE2;*Apoe-/-; Ldlr-/-* (E2) mice and rabbit β-VLDL (β-VLDL). The incubations were performed either in the presence or absence of a 20-fold excess of unlabeled VLDL, with and without addition of 5 µg/ml heat inactivated LPL. The effect of LPL on specific binding of VLDL to the apoER2 was determined by subtracting specific VLDL binding to control CHO cells from specific VLDL binding to apoER2 CHO cells. Each value represents the mean ± S.D. of three experiments. * = significantly different from binding in the absence of LPL, $P < 0.05$, Student's t-test.

Discussion

In the present study we demonstrate that the apoER2 is, despite its name, a receptor capable of binding apoE deficient VLDL. In addition, LPL was found to enhance VLDL binding to the apoER2. Hence, the apoER2 differs from the highly homologous VLDLR, which requires apoE for lipoprotein binding. The apoER2 bound APOE3-Leiden and APOE2 VLDL with high affinity, as was also found for the VLDLR (36, 37). High affinity binding of these apoE variants distinguishes both the apoER2 and the VLDLR from the LDLR, which shows a severe binding defect of apoE2 and a moderate binding defect of apoE3-Leiden (26, 38). However, both the apoER2 and the LDLR are not dependent on apoE for lipoprotein binding.

Our experiments did not exclude a role for apoE in modulating the binding of VLDL to the apoER2. The apoER2 showed the lowest affinity for Apoe-/- VLDL, which could indicate that apoE enhances binding. Whether differences in VLDL particle size and lipid content, as has been described previously (35) for the particles used in the present study, play an additional role in modulating binding to the apoER2 remains to be determined. LPL increased lipoprotein binding to the apoER2, as was also found for many other LDLR family members (15-19). The LPL mediated increase in binding was found for all VLDL fractions used in the present study.

Recently, compelling evidence has been found to support a role for both the apoER2 and the closely related VLDLR in neuronal migration during fetal brain development (5). Interestingly, the VLDLR also plays a role in lipid metabolism. The VLDLR has been found to be involved in the storage of lipids in fat tissue (39). In addition, the VLDLR has been found to lower serum cholesterol levels when ectopically expressed in the liver of several hypercholesterolemic mouse models, indicating that the VLDLR is capable of lipid transport from the circulation into the cells (36, 40). In the present paper, we show that the apoER2 displays distinct, but overlapping lipoprotein binding characteristics as compared with the VLDLR, suggesting that the apoER2 has a similar dual function in neuronal migration and lipid metabolism.

Both the apoER2 and LPL have been shown to be expressed in the cerebral cortex, cerebellum and hippocampus (1, 11, 41). The chicken homologue of the apoER2 is expressed in neurons all over the brain, including the Purkinje cells of the cerebellum, and in cells that constitute brain barriers such as endothelial cells of blood vessels, cells of the arachnoid membrane, and cells of the choroid plexus (4). LPL in the brain is also expressed by neurons, including the Purkinje cells, and LPL protein is found in many vascular structures throughout the central nervous system (11). The similarities in expression pattern between LPL and the apoER2, together with the data presented in this paper on enhanced lipoprotein binding to the apoER2 by LPL, support a role for the apoER2 in the lipid metabolism of the brain. Based on its localization, it could be hypothesized that the apoER2 is involved in transport of lipids into neurons and across brain barriers, a process that could be facilitated by LPL.

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CHAPTER 7

Living up to a name: the role of the very low density lipoprotein receptor in lipid metabolism

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Abstract

The very low density lipoprotein receptor (VLDLR) is a member of the low density receptor (LDLR) family. The VLDLR was hypothesized to mediate fatty acid entry into peripheral tissues based on its expression in tissues active in fatty acid metabolism and its capacity to bind apolipoprotein (apo) E rich VLDL *in vitro*. This hypothesis initially proved difficult to establish, since VLDLR knockout mice were reported to display normal plasma lipid levels. Moreover, VLDLR knockout mice that were also deficient for a second LDLR family member, the apoE receptor 2, revealed a role for the VLDLR in neuronal migration during brain development. However, in line with what its name suggests, recent studies using VLDLR deficient and transgenic mice have provided compelling evidence that the VLDLR does play a role in VLDL-triglyceride metabolism, and is important for triglyceride storage in the adipocyte.

Introduction

The very low density lipoprotein (VLDL) receptor (VLDLR) was originally cloned on the basis of its homology to the low density lipoprotein (LDL) receptor (LDLR). The human VLDLR gene is located on chromosome 9 (1-3). Its genomic organization is strikingly similar to that of the low density lipoprotein receptor (LDLR) and the apolipoprotein (apo) E receptor 2 (apoER2) (4). The VLDLR displays approximately 95% amino acid conservation between human (1-3, 5), mouse (6, 7), rabbit (8) and rat (9), and 84% amino acid conservation between human and chicken (10). Thus compared with the LDLR, which displays 76% amino acid conservation between human and rabbit, the VLDLR is very well conserved throughout evolution.

The VLDLR is most abundantly expressed in heart, skeletal muscle and adipose tissue, but not in liver (2, 5-7, 11). VLDLR expression is localized in endothelial and smooth muscle cells of arteries and veins (12). Since heart and skeletal muscle use free fatty acids (FFA) as an energy source, and adipose tissue uses FFA for energy storage, the VLDLR was hypothesized to play a role in the delivery of fatty acids to these peripheral tissues.

The VLDLR binds numerous ligands, including apoE rich VLDL, intermediate density lipoproteins (IDL) and chylomicrons (1, 8, 13), lipoprotein lipase (LPL) (13), receptor associated protein (RAP) (14), thrombospondin-1 (15), urokinase plasminogen activator (uPA)/plasminogen activator inhibitor (PAI)-1 complex (16) and several other proteinase/serpin complexes (17). The biological significance of many of these VLDLR-ligand interactions is still poorly understood.

VLDLR deficient (*VLDLR-/-*) mice could be a valuable tool to study VLDLR function *in vivo*. Surprisingly, the initial studies on *VLDLR-/-* mice revealed no alterations in plasma lipid and lipoprotein levels, although adipose tissue mass of *VLDLR-/-* mice was reduced (18). Thus it remained unclear whether the VLDLR is involved in lipid metabolism. Further doubts were raised when it was reported that both VLDLR and the closely related apoER2 are involved in neuronal migration in the developing brain (19). Trommsdorff and co-workers showed that mice

deficient for both the VLDLR and the apoER2 display severe malformations of the cerebral cortex and cerebellum. The VLDLR and the apoER2 appear to be involved in a signal transduction pathway, in which binding of the large extracellular matrix protein reelin to the receptor induces tyrosine phosphorylation of the intracellular adaptor protein mDAB1 (20).

In addition to neuronal migration via reelin, *in vitro* studies revealed that the VLDLR modulates cell migration via a pathway depending on uPA (21), as was described before for the LDLR related protein (LRP) (22). Both LRP (23) and VLDLR (21) mediate catabolism of the uPA receptor (uPAR), which plays an important role in regulation of cell migration (24). It has been hypothesized that uPA, complexed with its inhibitor PAI-1, binds simultaneously to the uPAR and to either the LRP or the VLDLR, leading to the internalization of the whole complex (23, 25).

The hypothesis that the VLDLR plays a role in lipid metabolism dates from almost a decade ago (8), this has only recently been proven. This review will focus on recent studies establishing the role of the VLDLR in lipid metabolism, and will present a possible mechanism of action.

Regulation of VLDLR expression

VLDLR gene expression regulation has been subject of several studies, since expression regulation does reveal basal information about gene function. Several elements present in the promoter region of the VLDLR have previously been recognized to play a role in the regulation of genes implicated in lipid metabolism and maintenance of energy balance. The 5'-flanking region of the VLDLR contains two copies of a potential sterol regulatory element (SRE)-1, which was reported previously to mediate down-regulation of the LDLR by sterols (1, 26). However, VLDLR mRNA expression levels are unaffected when human monocyte leukemia THP-1 cells as well as rabbit resident alveolar macrophages are cultured in the presence of sterols, indicating that regulation via the SRE may not be straightforward (1, 27). Two transcription factors, CCAAT/enhancer-binding protein β (C/EBPβ) and nuclear factor-Y (NF-Y), have been reported to bind to regulatory elements in the VLDLR promoter region (28). C/EBPs play a role in the regulation of energy hemostasis and in adipocyte differentiation (29-31). NF-Y plays a major role in the regulation of lipoprotein lipase (LPL), the principal enzyme responsible for the hydrolysis of triglycerides of circulating lipoproteins (32). Finally, the VLDLR promoter region contains a half site for the estrogen receptor (1). Administration of estradiol has been reported to dramatically increase VLDLR mRNA levels in rabbit cardiac ventricles (33).

Other factors that affect VLDLR expression include granulocyte-macrophage colony stimulating factor (34), thyroid hormone (9) and 1α-25-dihydroxyvitamin D_3 (35). In several studies the role of diet in VLDLR expression regulation has been examined. A study in rats by Jokinen et al. (9) has revealed no effect of fasting and refeeding on VLDLR mRNA and protein levels. In contrast, a study in mice by Kwok et al. (36) has revealed an increase in heart and a decrease in epididymal fat VLDLR protein levels after fasting. Tiebel et al. (11) have

investigated the effect of prolonged feeding of an atherogenic diet on VLDLR mRNA expression levels in wild type, LDLR deficient (*LDLR-/-*), apoE deficient (*apoE-/-*) and *LDLR-/-;apoE-/-* mice. Remarkably, downregulation of the VLDLR in heart and skeletal muscle upon feeding the atherogenic diet has been found only in *LDLR-/-* mice. In contrast, VLDLR mRNA expression is upregulated upon feeding of the atherogenic diet in adipose tissue of all mouse models studied, except *LDLR-/-;apoE-/-* mice. Thus the effect of the diet on VLDLR expression seems to depend upon several factors, including the species studied and the presence or absence of other lipoprotein receptors and/or apoE. The mechanisms by which these factors influence the effect of the diet on VLDLR expression are still under investigation.

The VLDLR and plasma VLDL-triglyceride levels

The initial studies on VLDLR deficient (*VLDLR-/-*) mice revealed no alterations in lipoprotein profile and total plasma cholesterol, triglyceride, and FFA levels as compared with wild type mice (18). This indicates that either the VLDLR does not play a major role in lipoprotein metabolism, or backup mechanisms can compensate for VLDLR deficiency. To stress the possible backup mechanisms, we have studied VLDLR transgenic and knockout mice under conditions of LDLR deficiency combined with a high fat diet and/or prolonged fasting. Under these conditions, endothelial VLDLR overexpression is associated with a decrease, while VLDLR deficiency is associated with an increase in plasma triglyceride levels (37). This effect on plasma triglyceride levels is not due to differences in VLDL synthesis. In the absence of the VLDLR, the lipid composition of nascent VLDL is unaffected, while circulating VLDL displays high triglyceride content, suggesting the VLDLR modulates peripheral hydrolysis of VLDL triglycerides (37).

These findings are in line with previous studies on the effect of RAP on plasma triglyceride levels. The VLDLR is one of the LDLR family members blocked by RAP (14). Similar to VLDLR deficiency in *LDLR-/-* mice, adenovirus mediated overexpression of RAP into the circulation of mice that lack both the LDLR and the LRP in their livers results in an increase of plasma triglycerides (38). No RAP-binding proteins have been detected in the livers of these mice. Therefore, the increase in plasma triglyceride levels must have been mediated by an extra-hepatic RAP-sensitive mechanism, possibly the VLDLR.

The VLDLR and adipose tissue mass

In line with the hypothesis that the VLDLR is involved in peripheral free fatty acid delivery, VLDLR deficient (VLDLR-/-) mice show a 15-20% reduction in body weight due to a reduction in adipose tissue mass (18). Interestingly, transgenic mice overexpressing apolipoprotein C1 also exhibit reduced adipose tissue mass (39). ApoC1 strongly inhibits lipoprotein binding to the VLDLR (40), while it does not affect LPL mediated hydrolysis of

triglycerides *in vitro* (41). It can be postulated that apoC1 mediates its effect on adipose tissue by blocking lipoprotein binding to receptors such as the VLDLR.

Recently, we have studied the effect of VLDLR deficiency on profound weight gain in mice (42). Obesity has been induced either by feeding a high fat, high caloric (HFC) diet or by crossbreeding mice onto the genetically obese *ob/ob* background. After 17 weeks of feeding the HFC diet, *VLDLR-/-* mice remain lean, while wild type littermates become obese. Similarly, weight gain is less profound in *ob/ob* mice that are deficient for the VLDLR. VLDLR deficiency leads to marked hypertriglyceridemia in both the genetically and the diet induced obesity model. In addition, *VLDLR-/-* mice on the HFC diet display decreased plasma glucose and insulin levels, and, in contrast to wild type controls, do not develop insulin resistance. *VLDLR-/-* mice exhibit reduced whole body free fatty acid uptake as compared to control mice, with no differences in food intake and fat absorption. Histological analysis of adipose tissue of both obesity models reveals decreased adipocyte size in VLDLR deficient mice as compared to controls, suggesting adipocyte triglyceride storage is reduced when the VLDLR is absent. These results indicate that the VLDLR indeed plays a role in the entry of fatty acids into peripheral tissues, such as the adipose tissue.

VLDLR and LPL metabolism

The mechanism responsible for the effects of VLDLR expression modulation on lipoprotein metabolism remains to be elucidated. Studies using adenoviral vectors have shown that ectopic expression of the VLDLR in mouse liver results in enhanced internalization of lipoproteins (43, 44). Thus, when expressed in liver, the VLDLR seems to act as a clearance receptor for lipoproteins similar to the LDLR and LRP. However, the studies using VLDLR transgenic and knockout mice reveal that the VLDLR in the periphery primarily affects VLDL triglyceride content (37). This suggests that at its natural site of expression, the VLDLR facilitates hydrolysis of triglycerides instead of clearing entire lipoprotein particles.

The principal enzyme responsible for the hydrolysis of triglycerides of circulating lipoproteins is LPL. FFA liberated by triglyceride hydrolysis can enter adjacent tissues, either to be stored (45) or oxidized (46). Similar to the VLDLR, the major sites of LPL production are the adipose tissue, skeletal muscle and heart. In these tissues, LPL is produced by the adipocytes and the skeletal and heart muscle cells. Subsequently, the LPL is secreted and transported to the luminal side of the vascular endothelium. Interestingly, recent studies by Ira Goldberg and co-workers have shown that the VLDLR mediates transport of LPL across a layer of cultured endothelial cells (personal communication). Therefore, the VLDLR might regulate LPL levels on the vascular wall by mediating transport of LPL derived from adipocytes and muscle cells from the basal to the luminal side of endothelial cells.

In the vascular lumen, LPL is bound to heparan sulphate proteoglycans (HSPG) (47). In addition to binding to HSPG, LPL contains a carboxy-terminal binding site for lipoprotein receptors of the LDLR family. LPL is reported to bind to LRP (48-51), LDLR (52, 53), megalin (54), and the VLDLR (13), thereby enhancing binding and internalization of lipoproteins. The

mechanism behind LPL facilitated lipoprotein uptake by the lipoprotein receptors remains to be determined. It is proposed that LPL mediates lipoprotein binding to HSPG, which in turn transfers the LPL-lipoprotein complex to the lipoprotein receptor (55, 56). On the other hand, Beisiegel *et al* proposed that LPL promotes binding directly by bridging the lipoprotein particle to the receptor (48). On an LPL heterozygous knockout background, muscle specific expression of catalytically inactive LPL lowers both plasma triglyceride and cholesterol levels. Furthermore, muscle uptake of radiolabeled VLDL triglycerides and apolipoproteins is increased, implying LPL bridging of lipoproteins to lipoprotein receptors and/or HSPG occurs *in vivo* (57).

VLDLR and LPL are present on the vascular wall in the same tissues, can bind to each other and both seem to be involved in hydrolysis of triglycerides. Furthermore, deficiency for adipose tissue LPL in *ob/ob* mice results in a similar reduction in weight gain and adipose tissue mass as we have detected in VLDLR deficient *ob/ob* mice (58). Therefore, we hypothesize that the VLDLR and LPL act together in delivering FFA to peripheral tissues. We propose a model in which the VLDLR binds triglyceride-rich VLDL, bringing it into close contact with LPL bound to either the VLDLR itself or to HSPG, thereby enhancing the efficiency of triglyceride hydrolysis.

Conclusions

The VLDLR forms an intriguing example of a receptor with multiple and diverse functions. The VLDLR seems to regulate biological processes by binding and/or internalization of ligands, but also by transducing extracellular signals across the cell membrane. In addition to its role in neuronal cell migration during brain development, evidence from animal studies now shows that the VLDLR modulates VLDL triglyceride metabolism, and that it plays an important role in the building of adipocyte triglyceride stores. This indicates that the VLDLR is indeed involved in delivery of FFA to peripheral tissues, and exposes the VLDLR as a potential target for prevention of diet induced obesity and insulin resistance.

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Chapter 8

General discussion & future perspectives

General discussion

Synthesis, processing and catabolism of lipoproteins are tightly regulated to retain a correct distribution of lipids in the body. The LDLR plays an important role in hepatic clearance of lipoproteins, and genetic defects in the LDLR have been described to result in increased plasma LDL levels and premature atherosclerosis.

A search for homologous receptors that might play a role in lipoprotein metabolism has resulted in the discovery of numerous receptors, together forming the LDLR family. However, *in vitro* studies have shown that LDLR family members not only bind lipoproteins, but also numerous other ligands, including plasma proteases, protease inhibitors and lipases. Therefore it has been questioned whether the LDLR family members function in lipid metabolism, act as receptors for numerous proteins or both. The LRP was the first receptor with homology to the LDLR to be cloned (1). Similar to the LDLR, the LRP is expressed in the liver and mediates clearance of chylomicron remnants (2). For the remaining mammalian LDLR family members a role in lipoprotein metabolism has yet to be established.

Of the LDLR family members, the apoER2 and the VLDLR display the highest homology to the LDLR. Recent findings have implicated the apoER2 and the VLDLR in cell signaling processes that control neuronal migration in the developing brain (3, 4). The apoER2 is not likely to play a major role in systemic lipoprotein metabolism, since it is exclusively expressed in brain, placenta and testis (5). The VLDLR is most abundantly expressed in heart, skeletal muscle and adipose tissue, but not in liver (6-10). The VLDLR is present in the vessel wall, where expression is localized in endothelial and smooth muscle cells of arteries and veins (11). Since heart and skeletal muscle use fatty acids as an energy source, and adipose tissue uses fatty acids for energy storage, the VLDLR has been hypothesized to play a role in the delivery of fatty acids to peripheral tissues. Furthermore, the VLDLR is expressed in smooth muscle cells and in macrophages of the atherosclerotic plaque (12). Therefore, the VLDLR might contribute to atherosclerotic plaque formation by modulating lipid loading of both smooth muscle cells and macrophages, thereby enhancing foam cell formation. The aim of the present thesis was to study the physiological functions of the VLDLR, and its role in lipid metabolism and atherosclerosis in particular, using VLDLR knockout and transgenic mouse models.

Mouse models to study the VLDLR

The first mouse model that was generated to study the biological functions of the VLDLR is the *VLDLR-/-* mouse (13). *VLDLR-/-* mice display 15-20% reduction in body weight as compared to wild type mice due to a reduction in adipose tissue mass. However, lipoprotein profile and total plasma cholesterol, triglyceride and FFA levels of *VLDLR-/-* mice are indistinguishable from those of wild type controls (13). Thus, a role for the VLDLR in lipid metabolism could not be established, although the reduction in adipose tissue mass in *VLDLR-/-* mice might suggest a role for the VLDLR in FFA delivery to adipocytes. It could be

hypothesized that the relatively mild phenotype of *VLDLR-/-* mice is due to backup mechanisms that compensate for VLDLR deficiency. Therefore, we chose to study the effect of VLDLR overexpression in mice.

In Chapter 2, we describe the generation of VLDLR transgenic mice, using two large DNA fragments consisting of overlapping cosmids. Together the two DNA fragments constitute the entire human VLDLR gene, including 15 kb 5' and 23 kb 3' sequences. The expression pattern of the human transgene partly overlaps with the endogenous VLDLR expression pattern of the mouse, and is characterized by high-level testis expression in germ cells. The human VLDLR transgene is not expressed in heart and aorta, possibly because regulatory sequences necessary to drive expression in these tissues are localized outside the region spanned by the transgene DNA construct. The early generations of VLDLR-Tg mice show atrophy of the testis, and reduced paternal transmission of the transgene in two independent mouse strains. This suggests that VLDLR overexpression disturbs the development of male germ cells. Interestingly, ApoER2 deficient mice have also been reported to display male fertility problems (3). Taken together, these findings suggest that lipoprotein receptors might be involved in spermatogenesis.

To study the effect of VLDLR overexpression in endothelial cells lining the vessel wall, we generated transgenic mice using the human VLDLR cDNA in combination with the preproendothelin-1 promoter, as described in Chapter 3. Histological analysis of these PVL mice reveals no abnormalities. Moreover, lipoprotein profile and total plasma cholesterol and triglyceride levels of PVL mice are similar to those of wild type controls.

VLDLR in lipoprotein metabolism

Both PVL and *VLDLR-/-* mice display normal plasma lipid levels when fed a chow diet, indicating that either the VLDLR is not important in lipid metabolism, or backup mechanisms can compensate VLDLR function. To increase the strain on possible backup mechanisms, we have studied the VLDLR under conditions of decreased hepatic lipoprotein clearance in combination with dietary stress. Therefore, PVL and *VLDLR-/-* mice were studied on an LDLR deficient background, combined with a high fat diet and/or prolonged fasting. As discussed in Chapter 3, the results of these studies show that the VLDLR indeed plays a role in lipoprotein metabolism. Endothelial VLDLR overexpression is associated with decreased, while VLDLR deficiency is associated with increased plasma triglyceride levels. The increase in plasma triglyceride levels in the absence of the VLDLR is not due to alterations in VLDL triglyceride synthesis. In the absence of the VLDLR, the lipid composition of nascent VLDL is unaffected, while circulating VLDL displays high triglyceride content. Thus, the VLDLR enhances clearance of triglycerides from circulating VLDL particles, suggesting it modulates peripheral hydrolysis of VLDL triglycerides.

Decreased triglyceride clearance from circulating VLDL particles suggests that the reduction in adipose tissue mass of *VLDLR-/-* mice is due to reduced FFA delivery to adipocytes. To further investigate the role of the VLDLR in the adipose tissue, the effect of VLDLR deficiency on profound weight gain has been studied in *VLDLR-/-* mice, as discussed in

Chapter 4. Obesity was induced in *VLDLR-/-* mice either by feeding a high fat, high caloric (HFC) diet or by crossbreeding *VLDLR-/-* mice onto an ob/ob background. After 17 weeks of feeding the HFC diet, *VLDLR-/-* mice remain lean, while wild type littermates become obese. Similarly, weight gain of ob/ob mice is less profound in the absence of the VLDLR. In both the genetically and diet-induced obesity model, VLDLR deficiency reduces adipose tissue mass and induces hypertriglyceridemia. Histological analysis of adipose tissue reveals decreased adipocyte size in *VLDLR-/-* mice as compared to wild type controls, suggesting adipocyte triglyceride storage is reduced. In addition, *VLDLR-/-* mice exhibit reduced whole body free fatty acid uptake as compared to wild type controls. Taken together, these findings strongly support a role for the VLDLR in FFA delivery to peripheral tissues that are active in FFA metabolism, such as the adipose tissue. Furthermore, they expose the VDLR as a potential target in the prevention of diet induced obesity.

VLDLR in atherosclerosis

Thus far, the LDLR is the only member of the LDLR family for which a role in atherosclerosis has been established (14-17). In addition to the LDLR, the VLDLR has also been hypothesized to play a role in atherosclerosis. *In vitro* studies have shown that the VLDLR is not downregulated by intracellular cholesterol levels and mediates accumulation of cellular lipids (18, 19). This suggests the VLDLR could function as an atherogenic recepor by contributing to the transformation of macrophages and smooth muscle cells into foam cells. Furthermore, the VLDLR has been hypothesized to affect plaque formation by mediating binding, uptake and/or transcytosis of VLDL by endothelial cells (20). As discussed in Chapter 5, we have detected no differences between the mean atherosclerotic lesion area of *VLDLR-/-;LDLR-/-* mice and *LDLR-/-* controls, demonstrating the VLDLR is not essential for foam cell formation. In addition, we have detected no differences between the mean atherosclerotic lesion area between PVL;*LDLR-/-* mice and *LDLR-/-* controls. Together with the data from the *VLDLR-/-;LDLR-/-* mice, this does not support the hypothesis that the endothelial VLDLR affects atherosclerotic plaque formation. Remarkably, we detected a relatively high incidence of necrotic lesions in *VLDLR-/-;LDLR-/-* as compared to *LDLR-/-* mice. The mechanism behind this increase in necrosis in the absence of the VLDLR remains to be determined.

Recent *in vitro* experiments have revealed an inhibitory effect of the VLDLR on cellular migration (21, 22). Studies using RAP to block lipoprotein receptor function have shown that the VLDLR might also be involved in migration of smooth muscle cells. However, these studies suggest a stimulatory effect of lipoprotein receptors on migration (23, 24). As discussed in Chapter 5, we have used intimal thickening as an *in vivo* model to study the effect of the VLDR on smooth muscle cell migration. Intimal thickening is a characteristic arterial response to injury, caused by migration of smooth muscle cells from the media into the intima. In addition, migration of smooth muscle cells from the media to the intima takes place in atherosclerotic plaque formation and during restenosis (25). Our studies in *VLDLR-/-* mice demonstrate that cuff induced intimal thickening is enhanced in both *VLDLR+/-* and *VLDLR-/-* mice as compared

with wild type controls. This suggests the VLDLR has an inhibitory effect on smooth muscle cell migration *in vivo*. In contrast to the situation in the intimal thickening experiments, we have not detected differences in smooth muscle cell infiltration of atherosclerotic lesions between *VLDLR-/-;LDLR-/-* mice and *LDLR-/-* controls in the atherosclerosis experiments. Thus we could not establish a role for the smooth muscle cell VLDLR in atherosclerotic neointimal thickening.

Lipoprotein binding characteristics of apoER2

In addition to examining the physiological functions of the VLDLR, we have compared its lipoprotein binding capabilities to that of the closely related apoER2. In contrast to the VLDLR, the lipoprotein binding characteristics of the apoER2 have not been extensively studied. The name apoER2 has been assigned to the receptor on the basis of its capacity to bind and internalize apoE rich rabbit β-VLDL (5) and its high homology to the VLDLR, which is known to bind apoE enriched lipoproteins (26, 27). As discussed in Chapter 6, our studies reveal that the apoER2 is capable of binding VLDL containing the APOE3-Leiden and APOE2 variants with high affinity, as has been described before for the VLDLR (28, 29). Our studies also reveal that, in contrast to the VLDLR, the apoER2 binds apoE deficient VLDL. Thus, similar to the LDLR, the apoER2 does not depend on apoE for lipoprotein binding.

LPL has been reported to enhance lipoprotein binding and internalization by the VLDLR (27), LRP (30-33), LDLR (34, 35), and megalin (36). LPL binds directly to these receptors, and performs a bridging function between the lipoprotein particle and the receptor. We now show that LPL also enhances binding of lipoproteins to the apoER2 (Chapter 6).

Taken together, these *in vitro* studies show that the apoER2 might play a role in lipoprotein metabolism. The apoER2 is expressed in tissues in which lipids are transported across barrier systems, including testis, placenta and brain (5). Moreover, in the brain the apoER2 has been detected in cells that constitute brain barriers such as endothelial cells of penetrating blood vessels, cells of the arachnoid membrane, and cells of the choroid plexus (37). Therefore, it could be hypothesized that the apoER2 is involved in the transport of lipids and/or other ligands, across these barriers.

Future perspectives

From *in vivo* and *in vitro* studies it has become clear that the VLDLR is a multifunctional receptor, involved in multiple physiological processes. The studies presented in this thesis show that the VLDLR interacts with lipoprotein metabolism. However, the exact nature of the interaction remains to be elucidated. Studies by Kobayashi et al indicate that the VLDLR affects total plasma cholesterol and triglyceride levels by internalizing lipoproteins *in vivo*, when ectopically expressed in the liver (38). In contrast, our studies using VLDLR transgenic and knockout mice reveal that the VLDLR mainly affects VLDL triglyceride content, suggesting the

VLDLR affects triglyceride hydrolysis instead of clearing entire lipoprotein particles. As discussed in Chapter 7, LPL might be the link between the VLDLR and VLDL-triglyceride metabolism.

In order to clarify the pathways in which the VLDLR is involved, it is imperative that more knowledge becomes available on the exact expression pattern of the VLDLR. Although the tissue expression pattern of the VLDLR has been extensively studied in several species (6-8, 19, 26, 39, 40), controversy still exists regarding which cell types express the VLDLR. In a study by Wyne et al it has been shown that in skeletal muscle, heart, ovary, and brain the VLDLR is exclusively expressed in endothelial cells (20). However, several other studies have reported VLDLR expression in vascular smooth muscle cells (11, 12), macrophages in the atherosclerotic plaque (12, 41), and neurons in the brain (3). In addition, *in vitro* studies have revealed that VLDLR mRNA is expressed in differentiated 3T3-L1 adipocytes, but not in the 3T3-L1 preadipocytes (7, 42).

The expression of the VLDLR by adipocytes is puzzling, since lipoprotein particles are too large to passively diffuse across the endothelium in adipose tissue capillaries. Our studies in PVL mice demonstrate that endothelial VLDLR expression suffices to lower plasma triglyceride levels in *LDLR-/-* mice. To establish whether endothelial VLDLR expression alone can mediate diet induced obesity, the obesity studies discussed in Chapter 5 should be repeated with PVL mice on a VLDLR deficient background.

The VLDLR has the potential to affect many cellular processes by mediating cell signaling and by binding and/or internalizing multiple ligands. Future research should focus on VLDLR ligands that can trigger signaling events and the intracellular pathways involved in VLDLR signaling. On the other hand, the mechanisms underlying VLDLR involvement in cellular migration, lipoprotein trafficking and diet induced obesity require further investigation. Interestingly, two different splice variants of the VLDLR have been described, which are differentially expressed in a cell- and tissue-type specific manner (9, 19, 43, 44). This differential expression pattern may reflect functional differences between the two VLDLR variants.

Studies on LDLR family members in knockout mice have demonstrated that the absence of one receptor can be compensated by another LDLR relative. Mice deficient for two LDLR family members, such as the *apoER2-/-;VLDLR-/-* (3) and *LRP-/-;LDLR-/-* (2) double knockouts, have revealed receptor functions that would be difficult to detect in single knockouts. Thus, crossbreeding of mice deficient for LDLR family members that are believed to act in identical pathways, or that are expressed in the same tissues or cell types, can be an effective strategy in elucidating receptor function. To overcome the problem of embryonic and neonatal lethality, as seen in LRP and megalin deficient and apoER2-/-;VLDLR-/- mice, inducible knockouts will have to be generated to study receptor function. In addition, the rapidly increasing number of transgenic and knockout mouse models involving genes constituting ligands and intracellular adapter proteins for LDLR family members will become important tools in receptor research. The knowledge gained by studying these mouse models will provide better insight in the physiological roles of LDLR family members, and may become useful in the development of strategies to prevent diseases, including atherosclerosis, obesity and diabetes.

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Summary

Summary

Lipoprotein metabolism needs to be tightly regulated to ensure correct distribution of lipids throughout the body. Receptors of the LDLR family, including the LRP and the LDLR itself, have been shown to play important roles in hepatic lipoprotein clearance. The VLDLR is a member of the LDLR family that has been described to bind and internalize apoE rich lipoproteins *in vitro*. These lipoproteins include VLDL, IDL and chylomicrons. The VLDLR is predominantly expressed in heart, skeletal muscle and adipose tissue, but not in liver. The VLDLR is localized in smooth muscle cells and endothelial cells of arteries and veins. Based on its binding characteristics and its expression pattern, the VLDLR has been hypothesized to play a role in the delivery of FFA to peripheral tissues.

The initial studies on *VLDLR-/-* mice reported that they display normal plasma lipid and lipoprotein levels. The only indication that the VLDLR is involved in lipid metabolism is the observation that *VLDLR-/-* mice show a decrease in adipose tissue mass. Thus, either the VLDLR does not play an important role in lipoprotein trafficking, or backup mechanisms can compensate for VLDLR deficiency. An important biological function of the VLDLR was revealed when *VLDLR-/-* mice were crossbred with *apoER2-/-* mice. The absence of both VLDLR and apoER2 resulted in malformations of cerebral cortex, cerebellum and hippocampus, and showed that both VLDLR and apoER2 play a role in signaling pathways controlling neuronal migration during brain development.

To examine the VLDLR *in vivo*, we generated two transgenic mouse models that overexpress the VLDLR. Chapter 2 discusses the generation of VLDLR transgenic mice by coinjection of two overlapping genomic human VLDLR fragments into murine zygotes (VLDLR-Tg mice). Human VLDLR expression in these mice is controlled by endogenous promoter sequences present in the construct. Three transgenic founders were born, of which two generated transgenic offspring. Using fiber-FISH, Southern and Northern blot analysis, we have shown that the use of two overlapping DNA fragments resulted in large integration sites, containing a high percentage of reconstituted, transcriptionally active transgenes. In both mouse strains, the expression pattern of the human VLDLR transgene only partly overlapped with the endogenous mouse VLDLR expression pattern, and was characterized by high expression levels in the testis. In contrast to the endogenous VLDLR, the human VLDLR was not expressed in heart and aorta. Human VLDLR expression in testis was detected in germ cells up to the meiotic stage by *in situ* mRNA analysis. Remarkably, in the F1 generation of both VLDLR-Tg mouse strains the testis was atrophic and giant cells were detected in the semineferous tubuli. Furthermore, male VLDLR-Tg mice transmitted the transgene to their progeny with low frequencies. This could imply that VLDLR overexpression in the germ cells disturbed spermatogenesis. Interestingly, male *apoER2-/-* mice were reported previously to display fertility problems, indicating that members of the LDLR family play a role in sperm cell development.

Chapter 3 describes the generation of a transgenic mouse model (PVL), using the human VLDLR cDNA in combination with the preproendothelin promoter to overexpress the VLDLR

in the endothelium. Subsequently, both PVL and *VLDLR-/-* mice were studied under conditions of decreased hepatic lipoprotein clearance in combination with dietary stress. Therefore, PVL and *VLDLR-/-* mice were studied on an LDLR deficient background, combined with a high fat diet and/or prolonged fasting. The absence of the VLDLR was associated with an increase, while overexpression of the VLDLR was associated with a decrease in serum triglyceride levels. No differences were detected in VLDL production or nascent VLDL composition between *VLDLR-/-;LDLR-/-* and *LDLR-/-* littermates. However, the triglyceride content of circulating VLDL in *VLDLR-/-;LDLR-/-* mice was relatively high as compared with *LDLR-/-* mice. These observations showed that the VLDLR is involved in VLDL-triglyceride metabolism, and suggested that the VLDLR affects peripheral uptake of VLDL-triglycerides.

To address the question whether decreased adipose tissue FFA uptake is responsible for the reduction in adipose tissue mass seen in *VLDLR-/-* mice, the effect of VLDLR deficiency on profound weight gain was studied, as discussed in Chapter 4. Obesity was induced either by feeding of a high fat, high caloric (HFC) diet or by crossbreeding mice onto an ob/ob background. After 17 weeks of HFC feeding, *VLDLR-/-* mice remained lean, whereas wild type mice became obese. Similarly, absence of the VLDLR resulted in less profound weight gain of ob/ob mice. VLDLR deficiency reduced adipose tissue mass and induced hypertriglyceridemia in both the genetically and diet-induced obesity model. In addition, *VLDLR-/-* mice fed the HFC diet exhibited significantly decreased levels of plasma glucose and plasma insulin, and cleared a glucose bolus more rapidly than wild type littermates. These results show that *VLDLR-/-* mice are protected from diet-induced obesity and insulin resistance. The reduction in adipose tissue mass in the absence of the VDLR was accompanied by a decrease in average adipocyte size in both obesity models, implying adipocyte triglyceride storage was reduced. Moreover, *VLDLR-/-* mice showed a significant reduction in whole body FFA uptake as compared with wild type littermates. Thus, it seems likely that the reduced adipose tissue triglyceride stores in *VLDLR-/-* mice result from a decrease in FFA uptake from the circulation.

In addition to its expression in healthy blood vessels, the VLDLR is expressed in atherosclerotic plaques. VLDLR expression in plaques is localized in endothelial cells and in macrophage and smooth muscle cell derived foam cells. The VLDLR is not downregulated by intracellular cholesterol levels, and mediates accumulation of cellular lipids *in vitro*. Taken together, these observations led to the hypothesis that the VLDLR is involved in atherosclerosis by mediating foam cell formation and/or transport of lipids from the circulation into the plaque. As discussed in Chapter 5, the role of the VLDLR in atherosclerosis was studied in PVL and *VLDLR-/-* mice on an LDLR deficient background. After feeding of a high fat diet for 8 weeks, no differences were detected between the mean atherosclerotic lesion area of *VLDLR-/-;LDLR-/-* mice and *LDLR-/-* controls, demonstrating the VLDLR is not essential for foam cell formation. In addition, we have detected no differences between the mean atherosclerotic lesion area between PVL;*LDLR-/-* mice and *LDLR-/-* controls. Together with the data from the *VLDLR-/-;LDLR-/-* mice, this indicates that lipid or lipoprotein transport mediated by the endothelial VLDLR is not essential for atherosclerotic plaque formation. Remarkably, *VLDLR-/-;LDLR-/-* displayed a high incidence of necrotic lesions as compared with *LDLR-/-* mice. The mechanism behind this increase in necrosis remains to be determined.

Recent *in vitro* studies revealed that the VLDLR, similar to the LRP, inhibits cellular migration via a uPA dependent pathway. In contrast, studies using RAP to block receptor function revealed a stimulatory effect of LDLR family members on migration of smooth muscle cells. As discussed in Chapter 5, we used cuff induced intimal thickening as an *in vivo* model to study the effect of the VLDR on smooth muscle cell migration. Intimal thickening is a characteristic arterial response to injury, caused by migration of smooth muscle cells from the media into the intima. Intimal thickening is associated with vascular pathological processes, including restenosis and atherogenesis. Three weeks after cuff placement, *VLDLR-/-* and *VLDLR+/-* mice displayed a profound increase in intimal thickening as compared with wild type littermates, suggesting that the presence of the VLDLR reduces smooth muscle cell migration *in vivo*. In contrast, immunohistological analysis revealed no differences in smooth muscle cell infiltration of atherosclerotic lesions between *VLDLR-/-;LDLR-/-* mice and *LDLR-/-* controls in the atherosclerosis experiments. Thus, we could not establish a role for the smooth muscle cell VLDLR in atherosclerotic neointimal thickening.

The name apoER2 was assigned to the receptor only on the basis of its high homology to the VLDLR, which is known to bind apoE enriched lipoproteins, and its capacity to bind and internalize apoE rich rabbit β-VLDL. In Chapter 6, the lipoprotein binding characteristics of the apoER2 with respect to apoE and LPL were investigated using CHO cells expressing the apoER2. To study the effects of apoE deficiency and different apoE isoforms on the binding of VLDL to the apoER2, VLDL was isolated from *apoE-/-*, APOE2;*apoE-/-;LDLR-/-* and APOE3Leiden;*apoE-/-;LDLR-/-* mice. Rabbit ß-VLDL was used as a positive control for binding. All VLDL fractions used were shown to bind to the apoER2, revealing that apoER2 binding is not restricted to apoE containing lipoproteins. Several members of the LDLR family were described to display enhanced binding and internalization of lipoproteins in the presence of LPL. Therefore, we repeated the VLDL binding experiments with LPL added to the culture medium. LPL was shown to markedly enhance lipoprotein binding to the apoER2 with all VLDL fractions tested.

Samenvatting in het Nederlands

Het lipoproteine metabolisme behoort strikt gereguleerd te worden om een correcte distributie van lipiden te waarborgen. Receptoren van de LDL receptor (LDLR) familie, inclusief de LRP en de LDLR zelf, spelen een belangrijke rol in de klaring van lipoproteinen door de lever. De VLDL receptor (VLDLR) is een lid van de LDLR familie die *in vitro* apolipoproteine (apo) E rijke lipoproteinen, zoals VLDL, IDL en chylomicronen, bindt en internaliseert. De VLDLR komt tot expressie in hart, skelet spier en vetweefsel, maar niet in de lever. De VLDLR is gelocaliseerd in gladde spiercellen van zowel slagaders als aders. Gebaseerd op de bindingskarakteristieken en het expressiepatroon werd de VLDLR verondersteld een rol te spelen in het transport van vrije vetzuren naar de perifere weefsels.

De initiële studies aan VLDLR deficiënte (*VLDLR-/-*) muizen laten geen afwijkingen zien in plasma lipide- en lipoproteineniveaus. De enige indicatie dat de VLDLR een rol speelt in het lipid metabolisme, is de observatie dat *VLDLR-/-* muizen een afname in vetweefselmassa vertonen. Dit betekent ofwel dat de VLDLR geen belangrijke rol in het lipoproteinemetabolisme speelt, of dat backup mechanismen de afwezigheid van de VLDLR in deze muizen compenseren. Een belangrijke biologische functie van de VLDLR kwam aan het licht toen *VLDLR-/-* muizen werden gekruist met apoE receptor 2 (apoER2) knockout muizen. De afwezigheid van beide receptoren resulteerde in afwijkingen in de morfologie van de cerebrale cortex, het cerebellum en de hippocampus, en toonde dat de VLDLR en de apoER2 een rol spelen in signaal transductie pathways die de neuronale migratie gedurende de onwikkeling van de hersenen controleren.

Om de VLDLR *in vivo* te bestuderen, hebben we 2 transgene muismodellen gegenereerd die de VLDLR tot overexpressie brengen. Hoofdstuk 2 bespreekt het genereren van VLDLR transgene muizen door injectie van twee overlappende genomische humane VLDLR fragmenten in zygoten (VLDLR-Tg muizen). De expressie van de humane VLDLR in deze muizen wordt gereguleerd door de endogene promotor sequenties die aanwezig zijn in het construct. Van de drie transgene founders genereerden er two transgene nakomelingen. Fiber-FISH, Southern en Nortern blot analyse lieten zien dat het gebruik van de twee overlappende DNA fragmenten resulteerde in grote integratie sites, die een hoog percentage herstelde, transcriptioneel actieve transgenen bevatten. In beide muislijnen overlapte het expressiepatroon van het humane VLDLR transgen maar gedeeltelijk met het endogene VLDLR expressiepatroon van de muis, en werd het gekarakteriseerd door hoge expressie niveaus in de testis. De humane VLDLR kwam, in tegenstelling tot de endogene VLDLR, niet tot expressie in hart en aorta. *In situ* mRNA analyse toonde dat de humane VLDLR in de testis tot expressie kwam in spermatocyten tot aan de meiose. De F1 generatie van beide muislijnen vertoonde atrofie van de testis, en "giant cells" in de seminifere tubili. Tevens was de efficiëntie waarmee de mannelijke VLDLR-Tg muizen het transgen overbrachten op de nakomelingen erg laag. Dit impliceert dat overexpressie van de VLDLR in spermatocyten de spermatogenese verstoort. Mannelijke apoER2 deficiënte muizen vertonen eveneens vruchtbaarheidsproblemen, wat aangeeft dat de leden van de LDLR familie misschien een rol spelen in de spermatogenese.

Hoofdstuk drie beschrijft het genereren van een transgeen muismodel (PVL) met behulp van het humane VLDLR cDNA in combinatie met de preproendotheline promotor om de VLDLR tot overexpressie te brengen in het endotheel. Vervolgens zijn de PVL en de *VLDLR-/-* muizen bestudeert onder condities van verminderde klaring van lipoproteinen door de lever in combinatie met dieet-geïnduceerde stress. Daarvoor zijn de PVL en *VLDLR-/-* muizen op een LDLR deficiënte achtergrond bestudeerd, gecombineerd met een vetrijk dieet en/of langdurig vasten. Afwezigheid van de VLDLR was geassocieerd met verhoogde serum triglycerideniveaus, terwijl overexpressie van de VLDLR was geassocieerd met verlaagde serum triglycerideniveaus. Tussen de *VLDLR-/-;LDLR-/-* muizen en *LDLR-/-* controles zijn geen verschillen geconstateerd in zowel VLDL productie als de samenstelling van nieuw geproduceerd VLDL. Echter, het triglyceride gehalte van circulerend VLDL in *VLDLR-/-;LDLR-/-* muizen was relatief hoog vergeleken met *LDLR-/-* muizen. Deze bevindingen tonen aan dat de VLDLR betrokken is bij het metabolisme van VLDL-triglyceriden, en waarschijnlijk een rol speelt bij de perifere opname van deze VLDL-triglyceriden.

Om te bestuderen of een verminderde vrije vetzuur opname door het vetweefsel verantwoordelijk is voor de verminderde vetweefselmassa van *VLDLR-/-* muizen, is het effect van VLDLR deficiëntie op een sterke gewichtstoename bestudeerd (hoofdstuk 4). Zwaarlijvigheid werd geïnduceerd door middel van een calorie- en vetrijkrijk diëet, of door de muizen op een ob/ob achtergrond te kruisen. Na 17 weken het calorie- en vetrijk dieet gegeten te hebben, waren de *VLDLR-/-* slank en de controle muizen zwaarlijvig. Op een ob/ob achtergrond resulteerde VLDLR deficiëntie eveneens in een gereduceerde gewichtstoename. VLDLR deficiëntie reduceerde de vetweefsel massa en induceerde hypertriglyceridemia, in zowel het genetisch als het diëet geïnduceerde model voor zwaarlijvigheid. Tevens vertoonden de *VLDLR-/-* muizen op het calorie- en vetrijk diëet lagere plasma glucose en insuline niveaus, en een snellere klaring van een glucose bolus dan controle muizen. Deze resultaten tonen aan dat VLDLR muizen beschermd zijn tegen diëet geïnduceerde zwaarlijvigheid en insuline resistentie. In beide modellen voor zwaarlijvigheid ging de reductie in vetweefsel massa in afwezigheid van de VLDLR gepaard met een afname in de gemiddelde adipocyt grootte, wat impliceert dat de opslag van triglyceriden in de adipocyt was gereduceerd. *VLDLR-/-* muizen vertoonden eveneens een significante reductie in totale vrije vetzuur opname. Daardoor is het aannemelijk dat de afname in opslag van triglyceriden in vetweefsel van *VLDLR-/-* muizen veroorzaakt wordt door een verminderde opname van vrije vetzuren uit de circulatie.

Behalve in gezonde bloedvaten, komt de VLDLR ook voor in atherosclerotische plaques. VLDLR expressie in plaques is gelocaliseerd in endotheel cellen, en in schuimcellen afkomstig van gladde spiercellen en macrofagen. De VLDLR wordt niet verminderd tot expressie gebracht bij hoge intracellulaire cholesterol niveaus, en kan *in vitro* accumulatie van cellulaire lipiden mediëren. Deze observaties leidden tot de hypothese dat de VLDLR betrokken is bij schuimcelvorming en/of transport van lipiden vanuit de circulatie naar de plaque. Zoals besproken in hoofdstuk 5, is de rol van de VLDLR in het atherosclerotisch proces bestudeerd in PVL en *VLDLR-/-* muizen op een *LDLR-/-* achtergrond. Na een periode van 8 weken op een vetrijk diëet vertoonden *VLDLR-/-;LDLR-/-* muizen en *LDLR-/-* controles geen verschillen in gemiddeld atherosclerotisch lesie oppervlak. Dit toont aan dat de VLDLR niet noodzakelijk is

voor schuimcelvorming. PVL;*LDLR-/-* muizen en *LDLR-/-* controles vertoonden eveneens geen verschillen in gemiddeld atherosclerotisch lesie oppervlak. Tesamen met de data van de *VLDLR-/-;LDLR-/-* muizen tonen deze studies aan dat de VLDLR op het endotheel niet noodzakelijk is voor transport van lipiden vanuit de circulatie naar de plaque. Opmerkelijk was de observatie dat necrotische lesies vaker voorkwamen bij *VLDLR-/-;LDLR-/-* muizen dan bij *LDLR-/-* muizen. Het mechanisme achter deze toename in necrose is nog niet opgehelderd.

Recente *in vitro* studies tonen aan dat de VLDLR, evenals de LRP, migratie van cellen inhibeert via een uPA afhankelijke pathway. Studies waarbij RAP wordt gebruikt om de receptor functie te blokkeren tonen echter een stimulerend effect van LDLR familieleden op de migratie van gladde speircellen. Wij gebruikten cuff-geïnduceerde intima verdikking als een *in vivo* model om het effect van de VLDLR op migratie van gladde spiercellen te bestuderen (hoofdstuk 5). Verdikking van de intima is een karakteristieke arteriële respons op vaatwandschade, die wordt veroorzaakt door migratie van gladde spiercellen vanuit de media naar de intima. Intima verdikking is geassocieerd met pathologische vasculaire processen, inclusief restenose en atherosclerose. Drie weken na het plaatsen van de cuff vertoonden zowel VLDLR-/- muizen als VLDLR+/- muizen een grotere verdikking van de intima dan wild type muizen, wat suggereert dat aanwezigheid van de VLDLR de migratie van gladde spiercellen remt. Echter, in de atherosclerose experimenten kon geen verschil worden aangetoond in gladde spiercelinfiltratie van plaques tussen *VLDLR-/-;LDLR-/-* muizen en *LDLR-/-* controles. Daardoor kan niet geconcludeerd worden dat de VLDLR een rol speelt bij atherosclerotische neointima vorming door gladde spiercellen.

De apoER2 kreeg zijn naam op basis van zijn hoge mate van homologie met de VLDLR, die apoE rijke lipoproteinen bindt, en zijn capaciteit om apoE rijk ß-VLDL te binden en te internalizeren. In hoofdstuk 6 zijn de bindingdkarakteristieken van de apoER2 met betrekking tot apoE en LPL onderzocht met behulp van CHO cellen die de apoER2 tot expressie brengen. Om de effecten van apoE deficiëntie en verschillende apoE isovormen op de binding van VLDL aan de apoER2 te bestuderen is VLDL geïsoleerd uit *apoE-/-*, APOE2;*apoE-/-;LDLR-/-* en APOE3Leiden;*apoE-/-;LDLR-/-* muizen. Konijnen ß-VLDL is gebruikt als positieve controle voor binding. Alle VLDL fracties bonden aan de apoER2, wat aantoont dat binding aan de apoER2 niet beperkt is tot lipoproteinen die apoE bevatten. Verscheidene leden van de LDLR familie vertonen verhoogde binding en internalisatie van lipoproteinen in aanwezigheid van lipoproteïne lipase (LPL). Daarom hebben we de apoER2 bindingsexperimenten herhaald in aanwezigheid van LPL in het celkweek medium. LPL verhoogde de binding van alle geteste VLDL fracties aan de apoER2.

List of publications

Full papers

1) Modulating effects of thyroid state on the induction of biotransformation enzymes by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Schuur AG, Tacken PJ, Visser TJ, Brouwer A. *Envirion. Toxicol. Phar.* 5: (1) 7-16 Jan. 1998.
2) Very low density lipoprotein binding to the apolipoprotein receptor 2 is enhanced by lipoprotein lipase, and does not require apolipoprotein E. Tacken PJ, de Beer F, van Vark LC, Havekes LM, Hofker MH, Willems van Dijk K. *Biochem. J.* 347: 357-362 Apr. 2000.
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Curriculum Vitae

Paulus Johannes Tacken werd geboren op 24 mei 1971 in Sint Oedenrode. In 1989 behaalde hij zijn VWO diploma aan het Mgr. Zwijsencollege te Veghel. In datzelfde jaar begon hij aan de studie Biologie aan de Landbouwuniversiteit Wageningen. Het propaedeutisch examen Biologie werd behaald in 1990. Het doctoraal examen Biologie, oriëntatie Cel, werd afgelegd in 1995. In het kader van het doctoraal examen werden twee afstudeervakken en een stage uitgevoerd. Het eerste afstudeervak, met als titel "De invloed van het schildklierhormoon op de activiteit van P-450 en glucuronyltransferase enzymen na inductie door TCDD", werd uitgevoerd bij de vakgroep Toxicologie, Landbouwuniversiteit Wageningen (ir. G. Schuur en dr. A Brouwer). Het tweede afstudeervak, met als titel "Search for cytokines in Carp", werd uitgevoerd bij de vakgroep Experimentele Diermorfologie en Celbiologie, Landbouwuniversiteit Wageningen (dr. Lidy Verburg). De stage, met als titel "Iron metabolism and the immune system: Cytokine expression in hemochromatotic patients", werd uitgevoerd aan de sectie Immunologie van het Instituut Biomedische Wetenschappen aan de Universiteit van Porto, Portugal (prof. dr. M. de Sousa).

Van maart 1996 tot maart 2000 was hij werkzaam als assistent in opleiding (AIO) op een door de Nederlandse Hartstichting gesubsidieerd samenwerkingsproject tussen de vakgroep Humane en Klinische Genetica, Leids Universitair Medisch Centrum, Leiden (dr. M.H. Hofker en dr. ir. K. Willems van Dijk) en het Gaubius Laboratorium, TNO-Preventie en Gezondheid (prof. dr. ir. L.M. Havekes), beide te Leiden. De resultaten van het onderzoek staan beschreven in dit proefschrift.

Vanaf december 2000 is hij werkzaam als post-doc op een door de Europese Unie gefinancierd project, met als titel "Control of lung infection, allergy and inflammation: assessment of the therapeutic potential of recombinant forms of the lung surfactant proteins SP-A and SP-D", aan de faculteit Diergeneeskunde, afdeling Biochemie, Universiteit Utrecht.