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New Aspects in Stabilization and Activation Mechanisms of Lipoprotein Lipase

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

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Uudsed aspektid lipoproteiinlipaasi stabiilsuse ja aktiivsuse regulatsioonis

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CONTENTS

LIST OF PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals.

I **M. Reimund**, M. Larsson, O. Kovrov, S. Kasvandik, G. Olivecrona, A. Lõokene, Evidence for two distinct binding sites for lipoprotein lipase on glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1 (GPIHBP1)., *J. Biol. Chem.* 290 (2015) 13919–13934.

II **M. Reimund***, O. Kovrov*, G. Olivecrona, A. Lõokene, Lipoprotein lipase activity and interactions studied in human plasma by isothermal titration calorimetry., *J. Lipid. Res.* 58 (2017) 279–288.

III **M. Reimund***, A. Wolska*, R. Risti, S. Wilson, D. Sviridov, A. T. Remaley, A. Lõokene, Apolipoprotein C-II mimetic peptide is an efficient activator of lipoprotein lipase in human plasma as studied by a calorimetric approach., *Biochem. Biophys. Res. Commun.* 519 (2019) 67–72.

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AUTHOR'S CONTRIBUTION TO THE PUBLICATIONS

I The author participated in planning the experiments and in analyzing and interpreting the results, performed surface plasmon resonance, fluorescence anisotropy, enzyme stability and chemical cross-linking experiments (except for the mass spectrometric analysis, which was conducted by S. Kasvandik), and contributed to writing the manuscript.

II The author participated in planning the experiments and in analyzing and interpreting the results, performed isothermal titration calorimetry experiments together with O. Kovrov, and contributed to writing the manuscript.

III The author was responsible for planning the experiments and for analyzing and interpreting the results, performed part of the isothermal titration calorimetry experiments, and wrote the manuscript, with contributions from the co-authors.

OTHER PUBLICATIONS AND AN INVENTION BY THE AUTHOR

The following publications are not discussed in this thesis. The invention is related to the publication II of this thesis.

1. S. Kaabel, J. Adamson, F. Topić, A. Kiesilä, E. Kalenius, M. Öeren, **M. Reimund**, E. Prigorchenko, A. Lõokene, H. J. Reich, K. Rissanen, R. Aav, Chiral hemicucurbit[8]uril as an anion receptor: selectivity to size, shape and charge distribution., *Chem. Sci.* 8 (2017) 2184 – 2190.
2. L. Villo, R. Risti, **M. Reimund**, K. Kukk, N. Samel, A. Lõokene, Calorimetric approach for comparison of angiopoietin-like protein 4 with other pancreatic lipase inhibitors., *Biochim. Biophys. Acta. Mol. Cell. Biol. Lipids.* (2019) In press, DOI: 10.1016/j.bbalip.2019.158553
3. Invention: Method for calorimetric determination of the lipoprotein lipase activity in human plasma environment; Owner: Tallinn University of Technology; Authors: A. Lõokene, **M. Reimund**, O. Kovrov, G. Olivecrona; Priority number: US62/350,747; Priority date: 16.06.2016; International application number: PCT/EP2017/064850

INTRODUCTION

Lipoprotein lipase (LPL) is the central enzyme in the metabolism of blood plasma triglycerides. It catalyzes the hydrolysis of dietary derived or endogenously synthesized triglycerides within triglyceride-rich lipoproteins. The produced fatty acids are used in the heart and skeletal muscle for energy production or in adipose tissue for energy storage. A lack of LPL activity causes massive increase in plasma triglyceride levels (clinically called severe hypertriglyceridemia), which results in a life-threatening pancreatitis, whereas decreased LPL activity leads to mild-to-moderate hypertriglyceridemia, which significantly increases the risk of atherosclerotic cardiovascular disease. Mild-to-moderate hypertriglyceridemia is relatively common and affects about one in four people in modern Western societies. Since current therapeutic options to treat hypertriglyceridemia are not efficient enough to reduce the risk of atherosclerotic cardiovascular disease, pharmaceutical companies are actively developing novel therapeutic agents. One way to reduce plasma triglyceride levels and the risk of atherosclerotic cardiovascular disease is to increase the activity of LPL.

LPL is synthesized in adipocytes and myocytes, but is then secreted and transported across the capillary endothelium by glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1). Once on the luminal surface of the capillary endothelium, GPIHBP1-bound LPL acts on the triglyceride-rich lipoproteins circulating in the blood. The physiological regulation of LPL activity needs to be kept under dynamic and careful control. Numerous components influence LPL activity in plasma. Several apolipoproteins (apo) are components of substrate lipoproteins and act as activators or inhibitors of the enzyme. LPL requires apoC-II for its activity in plasma; homozygotes for apoC-II deficiency have severe hypertriglyceridemia. Additionally, apoA-V increases LPL activity. In contrast, apoC-I and apoC-III on lipoproteins and circulating angiopoietin-like proteins 3, 4 and 8 all inhibit LPL activity.

Although remarkable progress has been made through investigations of the structural and functional properties of LPL and its numerous ligands, many aspects of LPL regulation are still unclear, probably because several ligands have only been identified relatively recently. For example, the exact role of GPIHBP1's two domains in the lipoprotein metabolism has remained unclear. Moreover, the complexity of the LPL system and the lack of suitable techniques for monitoring lipolysis in a plasma environment have hampered our understanding of how LPL activity is regulated under physiological conditions. Such a method could also be used to evaluate the efficiency of novel therapies developed to increase LPL activity.

The present thesis is divided into three parts. In the first part, the role and contribution of GPIHBP1's two functionally important domains in the interaction with LPL were investigated using various biophysical methods, including surface plasmon resonance, fluorescence anisotropy and chemical cross-linking combined with mass spectrometry. In the second part, a novel isothermal titration calorimetry (ITC) based approach was developed, which made it possible to study LPL's activity and stability in real-time near physiological conditions, i. e. in undiluted human plasma. The ITC-assay enabled us to study the effects of different endogenous and exogenous LPL regulators on its activity. Finally, the ITC-based approach was applied to investigate the efficiency and mechanism of the action of a new triglyceride-lowering drug candidate that activates LPL – apoC-II mimetic peptide – in human plasma.

ABBREVIATIONS

ANGPTL	angiopoietin-like
Apo	apolipoprotein
ASCVD	atherosclerotic cardiovascular disease
CETP	cholesteryl ester transfer protein
CHO	Chinese hamster ovary
DGGR	1,2-*O*-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin) ester
FRET	Förster resonance energy transfer
GPIHBP1	glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1
HDL	high-density lipoprotein
HDX-MS	hydrogen deuterium exchange mass spectrometry
HSPG	heparan sulfate proteoglycan
ITC	isothermal titration calorimetry
IDL	intermediate-density lipoprotein
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LMF1	lipase maturation factor 1
LPL	lipoprotein lipase
LRP	LDL receptor-related protein
MTP	microsomal triglyceride transport protein
NEFA	non-esterified fatty acid
SEC	size-exclusion chromatography
SPR	surface plasmon resonance
TRL	triglyceride-rich lipoprotein
VLDL	very-low density lipoprotein

1 LITERATURE REVIEW

1.1 Lipoproteins

Lipids, such as triglycerides, cholesterol and phospholipids, are an important source of energy or cell membrane components. Because various tissues require lipids, efficient transport systems are needed for the transport of water-insoluble lipids in the aqueous environment of the bloodstream. Therefore, lipids are assembled with specific amphipathic proteins called apolipoproteins (apo) to form soluble globular complexes known as lipoproteins, which are responsible for the controlled inter-organ transport of lipids.

Lipoproteins contain a hydrophobic core, consisting of triglycerides, cholesteryl esters and a small amount of fat-soluble vitamins. The core is shielded by a more hydrophilic surface, containing a phospholipid bilayer, non-esterified cholesterol and apolipoproteins. Lipoproteins are classified into four major lipoprotein classes based on their density, which depends on the ratio of lipids to protein: chylomicron, very-low density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) (Table 1, listed in the order of less dense to more dense particles) [1]. Additionally, in many classifications, intermediate-density lipoprotein (IDL), particles with a density in a range between VLDL and LDL, are considered a separate lipoprotein class [2]. More dense particles, such as HDLs, contain about 50% lipids and 50% protein (% of dry weight), whereas less dense particles, such as chylomicrons, contain 98-99% lipids and only 1-2% protein. The density of a lipoprotein is inversely proportional to its diameter. The largest lipoproteins, such as chylomicrons, have diameters as large as 1200 nm and smaller lipoproteins, such as HDLs, have diameters of 5 nm. It is important to note, however, that each discrete lipoprotein class contains a heterogeneous population of particles because lipoproteins undergo continuous changes in their density, size and composition due to the action of different plasma enzymes and proteins. Therefore, lipoproteins in plasma can be further divided into subclasses and can even be considered to form a continuous spectrum of particles [1,2].

Table 1. *Properties and compositions of the major lipoprotein classes [1,2].*

	Chylomicron	**VLDL**	**LDL**	**HDL**
Density (g/ml)	<0.94	0.94-1.006	1.006-1.063	1.063-1.210
Diameter (nm)	80-1200	30-80	18-25	5-12
Major apolipoproteins	B-48, A, C, E	B-100, C, E	B-100	A, C, E
% of total lipoprotein weight				
Protein	1-2	8-10	20-25	52-60
Lipids	98-99	90-92	75-80	40-48
% of total lipid weight				
Triglycerides	81-89	50-58	7-11	6-7
Cholesterol	1-3	4-9	10-12	6-8
Cholesteryl esters	2-4	15-23	47-51	24-45
Phospholipids	7-9	19-21	28-30	42-51

Nascent lipoproteins are mainly synthesized in the liver (VLDL, HDL) or in the small intestine (chylomicron, HDL), and are matured in plasma after incorporating additional apolipoproteins or lipids. Also, lipoproteins are formed from transformations of

precursor lipoproteins in plasma. For example, an LDL is formed in circulation after the progressive enzymatic degradation of triglycerides in a VLDL [1,2].

Lipoproteins in different classes have distinct functions. Chylomicron and VLDL are triglyceride-rich lipoproteins (TRLs), whose main role is to transport dietary derived (chylomicron) or endogenously synthesized (VLDL) triglycerides to the heart, skeletal muscle and adipose tissue, to be used or stored as energy. In contrast, LDL and HDL lipid cargoes mainly contain cholesteryl esters/cholesterol and phospholipids. While LDL is responsible for cholesterol transport to extrahepatic tissues, HDL's function is to acquire free cholesterol from extrahepatic tissues and transport it back to the liver for excretion as bile acids or neutral sterols, a process called "reverse-cholesterol transport" [1,2]. Because LDL has long been known to be causally related to the development of atherosclerotic cardiovascular disease (ASCVD), LDL has also been called "bad cholesterol". The reduction of LDL cholesterol using statins has been, and is, the first-choice of therapy for patients at risk of ASCVD [3]. At the same time, it was previously shown that high HDL levels reduce the risk of ASCVD [4] and thus these particles have been referred to as "good cholesterol". However, clinical trials with the aim of increasing HDL levels via cholesteryl ester transfer protein (CETP) inhibition have failed to show correlations between increased HDL levels and a reduced risk of ASCVD [5]. Importantly, in the last decade, it has been established that chylomicrons, VLDLs and their remnants play an independent and causal role in the development of ASCVD (discussed more thoroughly in the chapter 1.6 "Hypertriglyceridemia: risk of cardiovascular disease and treatment") [6].

Apolipoproteins play a crucial role in the functioning and regulation of the lipoprotein metabolism. In addition to ensuring the solubility of hydrophobic lipids and stabilizing the structure of lipoproteins, they bind to cell surface lipoprotein receptors and are responsible for receptor-mediated endocytosis of lipoproteins, or enable the transfer of lipids from lipoproteins to cells or vice versa. Moreover, they function as activators or inhibitors of the enzymes involved in the lipoprotein metabolism and thereby are responsible for the controlled modulation of lipoproteins in plasma [1]. Plasma apolipoproteins can be divided into two groups: apolipoprotein Bs and exchangeable apolipoproteins. ApoB-48 and apoB-100 are non-exchangeable signature proteins for chylomicrons or VLDLs/LDLs, respectively, which stay with the corresponding particle until it is cleared from circulation via endocytosis. Exchangeable apolipoproteins, including apoAs, apoCs and apoE, transfer between different lipoproteins in plasma, depending on the properties of lipoprotein surfaces [7]. The modifications in lipid composition (e. g. during triglyceride hydrolysis) changes the surface pressure of lipoproteins, which affects the ability of an apolipoprotein to be bound to a certain lipoprotein. The exchangeable apolipoproteins form amphipathic α-helices, in which one side is hydrophobic and interacts with lipids in lipoproteins, and the other side is hydrophilic and interacts with aqueous environment and with different ligands [7].

Interestingly, while apolipoproteins account for the majority of protein mass in lipoproteins, recent proteomic studies using mass spectrometry have revealed that lipoproteins also carry a lot of other proteins, which may have various functional roles. For example, it has been demonstrated that an HDL contains more than 90 distinct proteins [8], whereas an LDL contains more than 20 distinct proteins [9].

1.2 Metabolism of triglyceride-rich lipoproteins

Dietary fat mainly contains triglycerides, but also sterols (e. g. cholesterol), phospholipids and other lipids (e. g. fat-soluble vitamins) [10]. During digestion in the small intestine, lipids are emulsified by bile salts, which act as detergents [10]. Emulsification leads to effective hydrolysis of lipids by digestive enzymes. Triglyceride hydrolysis into monoglycerides and fatty acids is catalyzed by a pancreatic lipase, while phospholipases are responsible for phospholipid hydrolysis [10,11]. A small amount of dietary cholesterol is esterified (10-15%), which also needs to be hydrolyzed by cholesteryl esterase [12]. Lipolysis products form bile acid-stabilized micelles, which are absorbed by enterocytes: epithelial cells that form the surface of the lumen in the small intestine. Additionally, free fatty acids can enter the enterocytes via specific proteins [12].

In the enterocytes, fatty acids and monoglycerides are re-esterified into triglycerides, which together with cholesteryl esters, phospholipids and fat-soluble vitamins are assembled with a large apolipoprotein – apoB-48 – to form nascent chylomicrons [12]. This process takes place in the endoplasmic reticulum and is mediated by a microsomal triglyceride transport protein (MTP). Another apolipoprotein – apoA-IV – is added to chylomicrons in the endoplasmic reticulum [12]. After further processing in the Golgi, where nascent chylomicrons also acquire apoA-I, these particles are secreted through the lymphatics into the bloodstream, where other functionally important apolipoproteins (e. g. apoC-I, apoC-II, apoC-III, apoE and apoA-V) transfer from HDLs to nascent chylomicrons [12].

In blood circulation, mature chylomicrons interact with LPL, which is bound to glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1) at the vascular endothelium of adipose tissue, skeletal muscle and the heart [13,14]. This starts the rapid hydrolysis of triglycerides mediated by many LPL molecules simultaneously, each requiring LPL's cofactor apoC-II for effective triglyceride degradation [15]. Released fatty acids and monoglycerides are taken up by the parenchymal cells for energy storage (adipocytes) or for energy production (myocytes in the heart and skeletal muscle). Additionally, some of the triglycerides are removed from chylomicrons in plasma by CETP, which exchanges triglycerides and cholesteryl esters between chylomicrons and HDL or LDL [16]. Chylomicrons lose most of their triglycerides during LPL-mediated lipolysis. As a result, particle size is reduced, apoCs and apoAs shuttle back to HDLs and chylomicron remnants are formed, whose remaining triglycerides may be further hydrolyzed by hepatic lipase.

Finally, chylomicron remnants, enriched with apoE, are mainly cleared from circulation in the liver via receptor-mediated endocytosis. As endothelial cells in the liver are fenestrated, chylomicron remnants in circulation can bind to hepatocyte surface heparan sulfate proteoglycans (HSPGs), LDL-receptor (LDLR) or LDLR-related protein (LRP) via apoE [17]. Additionally, some of the LPL and hepatic lipase may remain associated with chylomicron remnants and facilitate remnant binding to LRP or HSPGs [17,18]. Chylomicrons are normally cleared from circulation after 4-6 h of eating a fat-containing meal [19]. Figure 1 provides an illustration of the chylomicron metabolism.

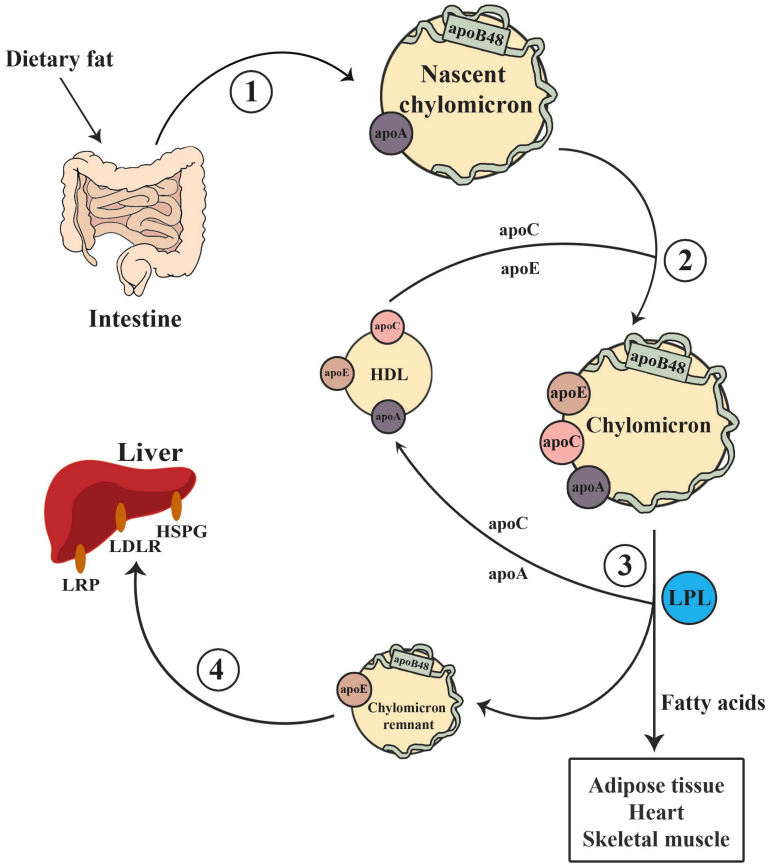

Figure 1. *Metabolism of chylomicrons. (1) Nascent chylomicrons are secreted through the lymphatics into the bloodstream. (2) Mature chylomicrons are formed after acquiring apoC-I, apoC-II, apoC-III and apoE from HDLs. (3) Triglycerides within chylomicrons are degraded by LPLs, which are bound via GPIHBP1 to the vascular endothelium of adipose tissue, heart and skeletal muscle. Released fatty acids and monoglycerides are taken up by the same tissues. During this process, apoCs and apoAs transfer back to HDLs. (4) Formed chylomicron remnants are taken up by the liver via apoE interactions with different receptors. This illustration was created by Robert Risti and it was inspired by Figure 1 from [20].*

Regardless of being in the fed or fasted state, VLDL particles are constantly produced in the liver from endogenously synthesized lipids to provide a continuous supply of triglycerides for the heart and skeletal muscle as a fuel. Importantly, however, VLDL production is accelerated after an increase in chylomicron concentration in circulation (after a fat-containing meal). Chylomicron remnants are taken up by the liver, where they are completely degraded in the lysosomes to fatty acids, glycerol, cholesterol and amino acids. Increased fatty acid concentration in the liver stimulates triglyceride synthesis, which enhances VLDL production [21].

VLDLs are assembled in the endoplasmic reticulum, where MTP mediates apoB-100 lipidation by triglycerides, cholesteryl esters, cholesterol and phospholipids [21,22]. ApoB-100 is one of the largest proteins, containing 4536 amino acid residues, and is a full-length version of apolipoprotein B, whereas apoB-48, a signature molecule for chylomicrons, is a truncated form containing 48% of apoB-100's N-terminus [21,22].

Like chylomicrons, VLDLs contain a single apoB molecule. After a nascent VLDL is secreted from hepatocytes into circulation, several apolipoproteins (apoCs, apoE and apoA-V) transfer from HDLs to VLDLs. Matured VLDL particles undergo a similar LPL-mediated degradation of their triglycerides as chylomicrons at the vascular endothelium of the heart, skeletal muscle and adipose tissue. Also, CETP can transfer some triglycerides from VLDLs to HDLs and LDLs in exchange for cholesteryl esters [16]. During these processes, apoCs return to HDL and smaller VLDLs or intermediate-density lipoproteins (IDLs) are generated. Some IDLs are cleared from circulation by receptor-mediated endocytosis in the liver via apoB-100 or apoE interaction with LDLR [17]. Other IDLs are further lipolyzed to LDLs by hepatic lipase [23]. LDLs are taken up by extrahepatic tissues or hepatocytes via apoB-100 interaction with LDLR [17]. Figure 2 provides an illustration of the VLDL metabolism.

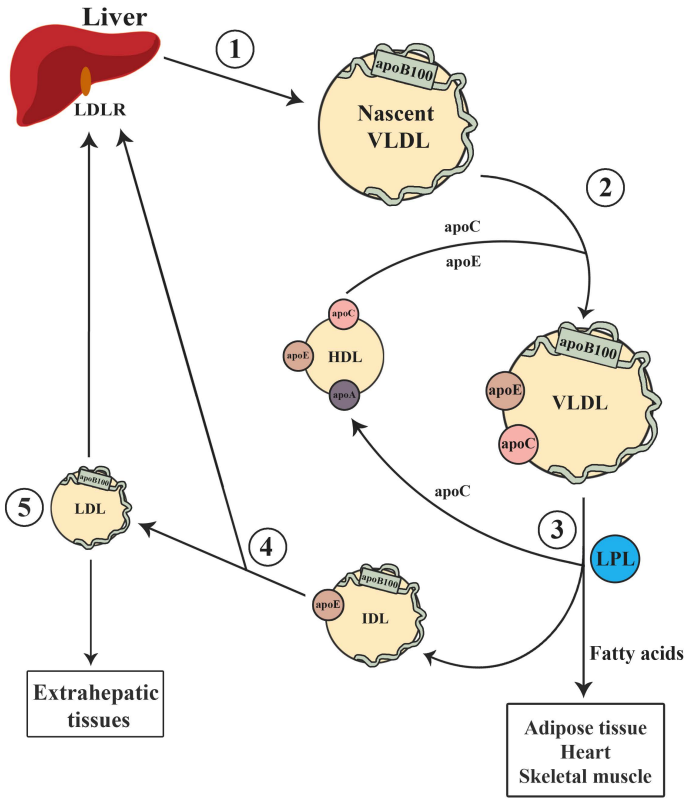

Figure 2. *Metabolism of VLDLs. (1) Nascent VLDLs are produced in the liver and secreted into the bloodstream. (2) Mature VLDLs are formed after acquiring apoC-I, apoC-II, apoC-III and apoE from HDLs. (3) Triglycerides within VLDLs are degraded by LPLs, which are bound via GPIHBP1 to the vascular endothelium of adipose tissue, the heart and skeletal muscle. Released fatty acids are taken up by the same tissues. During this process, apoCs transfer back to HDLs. (4) Intermediate-density lipoproteins (IDLs) (also called VLDL remnants) are formed, which can be taken up by the liver via interactions with LDLR or can undergo further lipolysis by hepatic lipase to form LDLs. (5) LDLs are taken up by extrahepatic tissues or by the liver via LDLR. This illustration was created by Robert Risti and it was inspired by Figure 2 from [20].*

1.3 Lipoprotein lipase

LPL is the main enzyme in the plasma triglyceride metabolism. Its activity is crucial in determination of plasma triglyceride concentration [13]. LPL catalyzes the hydrolysis of core triglycerides in circulating TRLs, such as chylomicrons and VLDLs, at the vascular endothelium of adipose tissue, skeletal muscle and the heart. A lack of LPL activity causes severe hypertriglyceridemia due to an extremely high concentration of chylomicrons, which finally leads to pancreatitis [24,25], whereas decreased LPL activity significantly increases the risk of ASCVD [26–28]. The latter is the main reason why many pharmaceutical companies are currently developing therapies for increasing LPL activity [29].

LPL activity needs to be regulated carefully in a tissue-specific manner depending on the physiological state of the body (e. g. fasting/eating and exercise) [30]. After eating a fat-containing meal, LPL activity is increased at the vascular endothelium of adipose tissue, where triglycerides in chylomicrons must be rapidly degraded by LPL to store energy-rich fatty acids in the adipocytes. While fasting, LPL activity is decreased in adipose tissue and increased at the vascular endothelium of the heart and skeletal muscle to hydrolyze VLDL-triglycerides to provide energy-rich fatty acids to myocytes to be used as a fuel. Similarly, during exercise, energy-rich fatty acids must be directed to active muscles and LPL activity is increased there [30]. Because LPL activity must be regulated rapidly, it occurs mainly at the post-translational level in plasma and in extracellular spaces by different regulator proteins and not at the transcriptional level [15,30].

There are numerous components that are responsible for the regulation of LPL activity (discussed more thoroughly in the chapter 1.4 “Extracellular regulators of lipoprotein lipase”). Firstly, HSPGs and GPIHBP1 are responsible for transporting freshly secreted LPL from parenchymal cells to the vascular endothelium and stabilizing its activity [31]. Secondly, ANGPTLs inactivate LPL in a tissue-specific manner depending on the physiological state of the body [32]. Thirdly, apolipoproteins are components of substrate lipoproteins and act as activators or inhibitors of LPL. The most important apolipoprotein is apoC-II, which is a cofactor for LPL, and without apoC-II circulating TRLs are not hydrolyzed [33].

LPL is mainly synthesized in parenchymal cells in adipose tissue, skeletal muscle and the heart. In the endoplasmic reticulum of adipocytes and myocytes, the proper folding of LPL monomers requires glycosylation of the enzyme and help from several protein disulfide isomerases to correctly form its disulfide bonds [34]. For the final maturation and secretion of active LPL that can form dimers from the endoplasmic reticulum, lipase maturation factor 1 (LMF1) is essential: without LMF1, LPL is misfolded and degraded [34,35]. In line with this, biallelic loss-of-function mutations in the LMF1 gene lead to severe hypertriglyceridemia and similar phenotypes, as in the case of mutations in the LPL gene [25,35]. Additionally, it has been proposed that the presence of Ca^{2+} ions is needed for the formation of active LPL [36].

After LPL is secreted from adipocytes or myocytes into subendothelial space, the enzyme first binds with the cell surface or extracellular matrix HSPGs, then moves across the subendothelial space until it binds with GPIHBP1 on the abluminal surface of endothelial cells and is finally transported by GPIHBP1 across the endothelial cells to the capillary lumen, where LPL acts on TRLs [31,37]. How LPL travels across the subendothelial space from HSPGs to GPIHBP1 is unclear. However, it was recently shown that even in the absence of GPIHBP1, LPL moved through the subendothelial space from HSPGs near the myocytes to HSPGs near the endothelial cells [38]. Because

subendothelial space is filled with HSPGs, each having a different number of sulfo groups [39], and because LPL's affinity for HSPGs depends on how much negative charge they carry [15,40,41], it has been proposed that "directed diffusion" due to an electrostatic gradient of HSPGs is the mechanism that moves LPL from the parenchymal cell surface to the abluminal side of endothelial cells [38].

Once LPL is transported across the endothelial cells to the capillary lumen, many GPIHBP1-LPL units simultaneously interact with TRLs to rapidly hydrolyze their triglycerides [13]. Figure 3 provides an illustration of the LPL-mediated endothelial lipolysis.

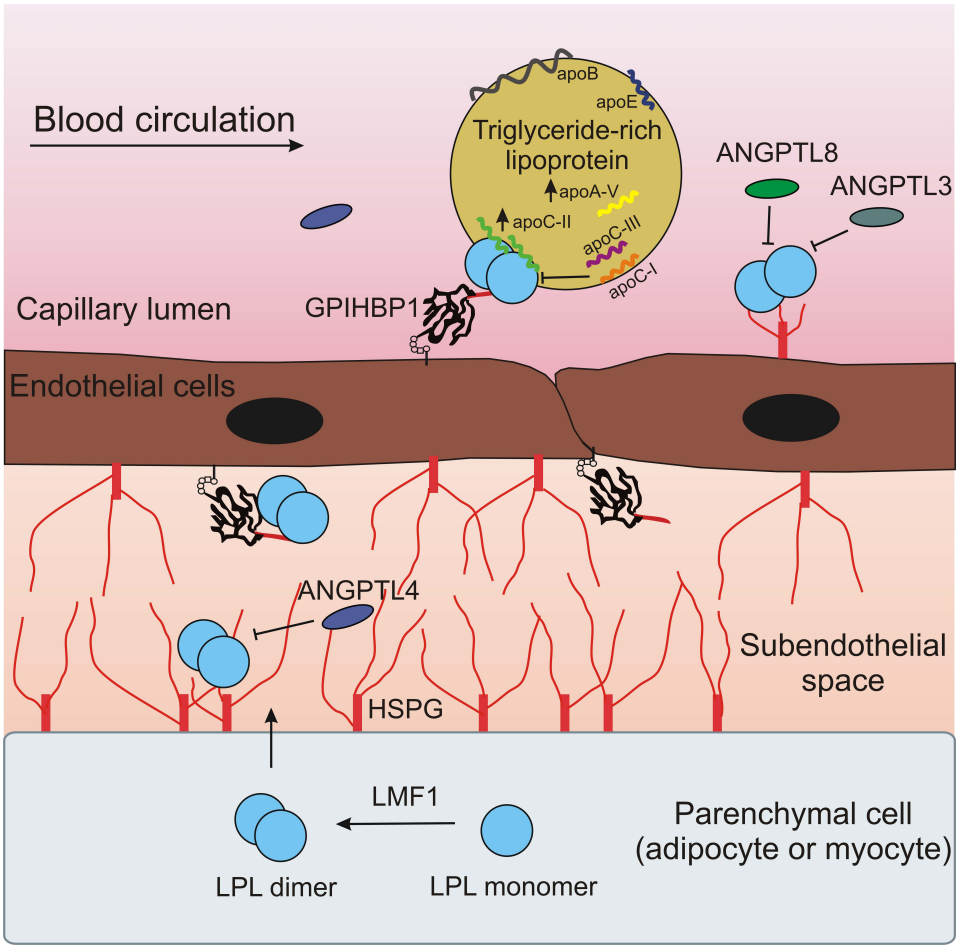

Figure 3. *LPL-mediated endothelial lipolysis. LPL is synthesized in parenchymal cells. LMF1 is needed in the endoplasmic reticulum for the formation of active LPL, which can form dimers. The enzyme is secreted into subendothelial space, where it binds to the cell surface or extracellular matrix HSPGs. Endothelial membrane protein GPIHBP1 attracts LPL from the HSPGs and transports the enzyme across the endothelial cells to the luminal side of the endothelium, where GPIHBP1-bound LPL acts on triglyceride-rich lipoproteins. Substrate lipoproteins contain numerous apolipoproteins, which activate (apoC-II and apoA-V) or inhibit (apoC-I and apoC-III) LPL. It has been proposed that while all ANGPTLs inhibit LPL in plasma, ANGPTL4 can inactivate LPL already in the subendothelial space or intracellularly. Additionally, it has been suggested that GPIHBP1-bound LPL is protected from the inactivating effects of ANGPTLs. This figure is created partially based on a drawing in [42].*

It has been suggested that most of the active LPL molecules are anchored to the endothelial cells via GPIHBP1 during the lipolysis of TRLs [14]. In line with this, plasma contains only inactive LPL monomers [43,44]. However, after an oral fat load (e. g. after eating a fat-containing meal), LPL activity in plasma is slightly increased [45], suggesting that not all LPL molecules must be bound to GPIHBP1 during lipolysis.

LPL catalyzes the hydrolysis of triglyceride ester bonds in positions sn-1 and sn-3 [46,47]. The formed products are fatty acids and monoglycerides, which are taken up by parenchymal cells. Lipolysis is terminated when the corresponding tissue becomes saturated with fatty acids, most likely because the accumulation of fatty acids on the interface of the LPL-lipoprotein complex results in the dissociation of this complex [48,49]. Importantly, while LPL prefers triglycerides as substrates, it can also catalyze the hydrolysis of ester bonds in the sn-1 position of phospholipids. The formed products are lysophospholipids [50]. However, the rate of hydrolysis of phosphatidylcholine within chylomicrons was shown to be about 5% of that of triglyceride [50].

Human LPL monomer consists of 448 amino acid residues and has a molecular weight of 55 kDa. LPL belongs to the same protein family as pancreatic lipase, hepatic lipase and endothelial lipase [15]. The crystal structures of LPL-GPIHBP1 complexes were very recently published by two independent groups (Figure 4) [51,52]. LPL consists of two domains: the N-terminal domain, which has an α/β-hydrolase fold containing both α-helices and β-strands, and the C-terminal domain, which contains only antiparallel β-strands forming a β-sandwich structure. The domains are connected via the hinge region. The N-terminal domain (residues 1-313) contains the active site (catalytic triad: Ser132, Asp156, His241), which is covered by a 22-residue lid. The lid opens and exposes its hydrophobic inner side for lipoprotein binding during LPL catalyzed triglyceride hydrolysis (Figure 4). The C-terminal domain (314-448) contains the binding site for the Ly6 domain of GPIHBP1 and for lipoproteins. While a loop containing several tryptophan residues in the C-terminal domain has been shown to be important for lipoprotein binding, other regions in this domain probably also participate in the interaction with lipoproteins, as was shown in a study where tryptophan residues were mutated [53]. One side of LPL contains many positively charged lysine and arginine residues spanning the N-terminal domain, the hinge region and the C-terminal domain (Figure 4). These residues form a large positively charged binding site for heparan sulfate chains in HSPGs and probably for the N-terminal domain of GPIHBP1.

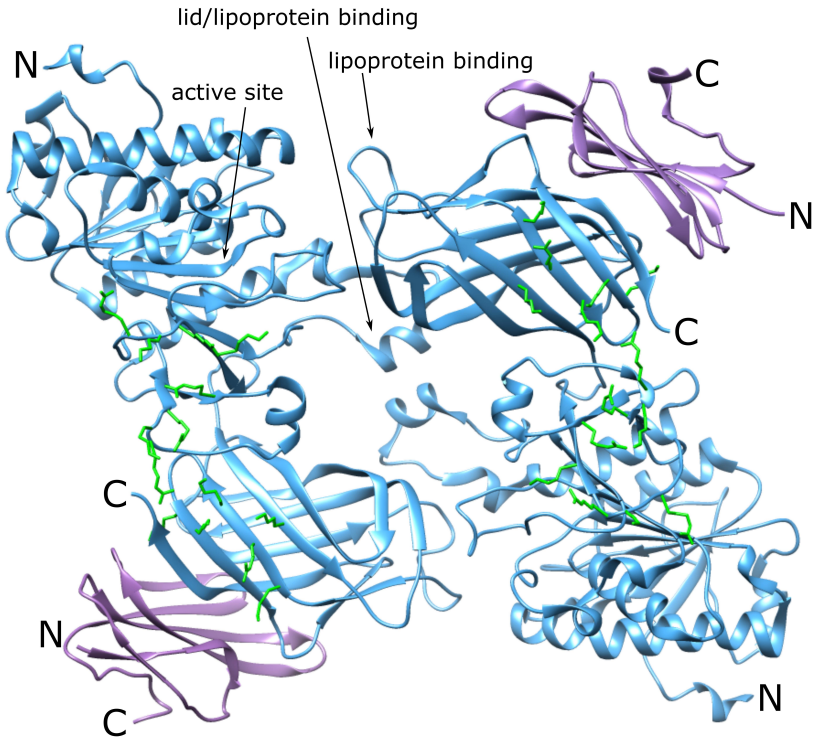

Figure 4. *Crystal structure of the complex between LPL and GPIHBP1 (PDB ID 6OB0) [51]. Head-to-tail arrangement of two LPL molecules (blue) in complex with GPIHBP1 (purple) in the crystallographic asymmetric unit are shown. Only the Ly6 domain of GPIHBP1 was identified in the structure. The N-terminal domain of GPIHBP1 was not identified. The active site and the lid in the N-terminal domain of LPL are indicated by an arrow in one LPL molecule. The lid is in an open conformation. The loop in the C-terminal domain of LPL, which is important for lipoprotein binding, is indicated by an arrow in the second partner LPL molecule. Side chains of positively charged residues that are important for heparin or HSPG binding are shown in green in both LPL molecules (residues 263, 279, 280, 282, 294, 296, 297, 300, 319, 403, 405, 407, 413, 414) [54–58].*

Several studies have demonstrated that purified active LPL forms homodimers. Firstly, ultracentrifugation in a sucrose gradient revealed that active LPL sedimented as a protein, whose molecular weight was close to 100-110 kDa [59]. The treatment of active LPL with 0.75 M guanidinium chloride resulted in a loss of activity and slower sedimentation in sucrose gradient ultracentrifugation. The estimated molecular weight of LPL corresponded to the LPL monomer. Secondly, the dimeric structure of LPL was confirmed by Förster resonance energy transfer (FRET) experiments, where differently labeled samples were used [60,61]. Thirdly, LPL has been shown to form homodimers by different molecular biology-based approaches. In these studies it was proposed that the dimer was arranged in a head-to-tail fashion, where the N-terminal domain of one monomer interacted with the C-terminal domain of the second monomer [62–64]. A similar orientation of LPL monomers in a dimer was also identified in the crystallographic asymmetric unit of LPL/GPIHBP1 complexes (Figure 4) [51,52] and in solution using small-angle x-ray scattering [52]. In the crystal structures, where the lid was in the closed conformation, the loop containing tryptophan residues in the C-terminal domain of one monomer interacted with the catalytic pocket in the

N-terminal domain of the second monomer. In such a conformation, however, acyl chains of lipoprotein-triglycerides cannot access the active site and it was proposed that this is the stable conformation of the LPL dimer, which does not hydrolyze triglycerides [31]. In contrast, a recent study used distance constraints for LPL-LPL intermolecular interactions derived from single-molecule FRET experiments to model a LPL dimer [61]. It was proposed that LPL monomers can interact in a way where the dimer interface does not interfere with the movement of the lid. According to this model, the active site is more accessible to triglycerides. It is important to note, however, that while there is a great amount of experimental data demonstrating that active LPL can form dimers, also at very low enzyme concentrations [60,61], there is no experimental proof of the oligomeric state of LPL while hydrolyzing triglycerides in lipoproteins. Therefore, based on current knowledge, it would be reasonable to conclude that the active form of LPL can form dimers, whereas the inactive form is a monomer, which cannot dimerize. Importantly, active LPL dimers are not stable complexes. Using FRET measurements, it has been demonstrated that LPL dimers are very dynamic, so that monomers rapidly exchange partners to form new dimers [60].

In addition to LPL's catalytic function in plasma triglyceride degradation, the enzyme has another important role: LPL enhances chylomicron remnant uptake by cells [18,65,66]. Lipoprotein-bound active or inactive LPL can interact with a variety of receptors, including LRP, LDLR and HSPGs, and promote chylomicron remnant uptake in this way.

More than 100 distinct mutations in the LPL gene have been identified [67]. Most of them cause a loss of enzymatic activity in the enzyme. Homozygotes or compound heterozygotes against LPL loss-of-function mutations result in severe hypertriglyceridemia and pancreatitis [24,25]. It is important to note, however, that biallelic loss-of-function mutations in the LPL gene are rare: one or two per million subjects [68]. Interestingly, one gain-of-function mutation, LPL S447X, has also been identified [69]. This variant of LPL lacks the last two residues, serine 447 and glycine 448. Carriers of LPL S447X have an anti-atherogenic lipid profile, with a lower plasma triglyceride concentration and higher HDL cholesterol [70]. A recent study demonstrated that this variant of LPL catalyzed the hydrolysis of lipoprotein triglycerides as efficiently as the natural LPL containing all 448 residues, whereas LPL S447X was more effective in promoting lipoprotein uptake by cell surface receptors [71].

1.4 Extracellular regulators of lipoprotein lipase

1.4.1 GPIHBP1

GPIHBP1 is a 28 kDa glycoprotein attached to the surface of capillary endothelial cells via its GPI-anchor. Although it was first discovered in 2003 as an HDL-binding protein [72], later more thorough studies found no evidence of this interaction occurring [73]. GPIHBP1's role in the plasma triglyceride metabolism became apparent in 2007, when knockout mice were generated and characterized [74]. GPIHBP1 deficient mice had severe chylomicronemia with triglyceride levels as high as 5000 mg/dl, very similar to plasma triglyceride levels in mice lacking LPL. This concentration of triglycerides was 50-100-fold higher than in the wild type mice. The same study also demonstrated the ability of GPIHBP1 to bind LPL. Together with the finding that GPIHBP1 is expressed in the endothelial cells of the heart, skeletal muscle and adipose tissue, the authors suggested that GPIHBP1 may serve as a platform for LPL-mediated TRL degradation on the luminal side of capillary endothelium. Indeed, a subsequent study suggested that

GPIHBP1-bound LPL is mainly responsible for the binding of TRLs from the bloodstream (this is called “margination”) [14]. This finding changed the view of LPL-mediated TRL hydrolysis. Since LPL was discovered in the 1950s, it was thought that LPL was bound to the vascular endothelium mainly via electrostatic interactions with HSPGs. However, because HSPGs are components of the glycocalyx of virtually all cells, the interaction with HSPGs is not specific enough to ensure the concentration of LPL, and later the binding of TRLs, only to the vascular endothelium of the heart, skeletal muscle and adipose tissue [15]. Importantly, GPIHBP1 is highly expressed in endothelial cells of these tissues and provides the specificity.

Since it was found that GPIHBP1 is involved in plasma triglyceride metabolism, its functions in the regulation of the TRL metabolism have been extensively studied. It is now clear that GPIHBP1 does more than act as a platform for the LPL-mediated lipolysis of TRLs. Soon after the first study in 2007, it was demonstrated that GPIHBP1 is responsible for the transport of LPL from subendothelial space to the vascular endothelium across the endothelial cells [37]. Without GPIHBP1, LPL accumulates in the subendothelial space. The movement of GPIHBP1 was shown to be bidirectional and GPIHBP1-bound LPL was transported across the endothelial cells in transcytotic vesicles [75]. How LPL finds its way from parenchymal cells of the heart, skeletal muscle and adipose tissue to the vascular endothelium had been a mystery before the discovery of GPIHBP1. Interestingly, the gene for GPIHBP1 has not been identified in lower vertebrates (e. g. birds, fish and reptiles), although they have the gene for LPL [76]. Therefore, these species must have another mechanism to transport LPL to the vascular endothelium.

A mature GPIHBP1 on the endothelial cell surface contains two domains, the N-terminal and the Ly6 domain, and a GPI-anchor, which is added to the protein in the endoplasmic reticulum [31,74]. GPIHBP1 belongs to the group of mammalian GPI-anchored Ly6 proteins [77]. The characteristic Ly6 domain of this protein family is usually 60-80 amino acid residues long and contains 3-5 disulfide bonds, which are arranged to adapt to a three-fingered structural motif. Based on the recently reported crystal structures of GPIHBP1-LPL complexes, the Ly6 domain of GPIHBP1 contains four antiparallel β-strands and ten cysteines, forming five disulfide bonds which stabilize its classic three-finger fold (Figure 4) [51,52]. The Ly6 domain of GPIHBP1 is glycosylated via Asn78 and Asn82, which are crucial for the secretion of GPIHBP1 on the surfaces of endothelial cells. GPIHBP1 is distinguished from other mammalian Ly6 protein family members by its N-terminal domain: a highly negatively charged region. In the human GPIHBP1 sequence, 21 residues out of 26 are aspartates or glutamates. Moreover, it was recently demonstrated that the tyrosine within the N-terminal domain is sulfated [38]. While the sequence of the Ly6 domain is relatively conserved among species, the sequence of the N-terminal domain is variable [31,76]. For example, the mouse N-terminal domain of GPIHBP1 contains 17 negatively charged residues, whereas the N-terminal domain in opossum GPIHBP1 contains 32 negatively charged residues [31]. Based on small-angle x-ray scattering and HDX-MS measurements, the N-terminal domain was reported to be intrinsically disordered and adopted a flexible and extended conformation which occupies a large mushroom-shaped space with a diameter of 112 Å [38,78].

Both domains of GPIHBP1, the N-terminal and the Ly6 domain, are important for interaction with LPL. The importance of the N-terminal domain was predictable, as LPL has a large positively charged area, which binds to such negatively charged biopolymers

as heparin, HSPGs and even polynucleotides, with a high affinity (Figure 4) [40,79]. In agreement with that, early studies showed that polyaspartate, polyglutamate peptides and heparin prevented LPL from binding to GPIHBP1 [80]. Furthermore, an antiserum against the N-terminal domain of GPIHBP1 blocked the interaction with LPL, and a variant of GPIHBP1 which lacked the N-terminal domain, or part of it, did not bind to LPL [80]. Similarly, the importance of the Ly6 domain was demonstrated by replacing this domain in GPIHBP1 with the Ly6 domain of CD59, a member of the same protein family [80]. This variant did not bind to LPL. Also, when any of the conserved cysteines in the Ly6 domain were mutated, GPIHBP1 lost its ability to interact with LPL, demonstrating that the proper fold of the Ly6 domain is crucial [81]. In another study, each residue in the Ly6 domain of GPIHBP1 was mutated to alanine and the ability of these variants to bind LPL were examined [82]. Twelve additional residues besides cysteines were shown to be crucial in the interaction with LPL and mutating any of these abolished the ability of GPIHBP1 to bind LPL. These residues were located mostly in the same region (finger 2, residues 89-110), suggesting the direct role of these residues in the interaction with LPL. Taken together, all of these studies demonstrated that by mutating, deleting or replacing the N-terminal domain or the Ly6 domain of GPIHBP1 it loses its ability to bind LPL. It is important to note, however, that these studies used qualitative, mostly cell culture-based, binding assays with "yes and no" answers, without measuring the affinity of the interactions to investigate the contribution of GPIHBP1's two domains. Our study published in 2015, discussed in the results and discussion section of this thesis (Publication I), was the first to investigate the role of the two domains of GPIHBP1 in interaction with LPL in more detail using quantitative biophysical binding assays. Also, subsequent studies have thoroughly examined these interactions by using different biophysical methods [38,78].

The N-terminal domain of GPIHBP1 binds to LPL via electrostatic interactions. The exact binding site on LPL remains to be determined, but it is probably close to regions on LPL responsible for heparin/HSPG binding (Figure 4). The exact residues or regions in the N-terminal domain of GPIHBP1 which interact with LPL are unknown. The Ly6 domain binds to the C-terminal domain of LPL mainly through hydrophobic contacts, as was demonstrated by site-directed mutagenesis studies and as was seen in the crystal structures [51,52,82]. While all three fingers of the Ly6 domain participate in the interaction, most contacts are made by fingers 2 (residues 86-111) and 3 (112-130), based on the crystal structures (Figure 4).

The N-terminal domain of GPIHBP1 has been shown to stabilize the catalytic activity of LPL. Using HDX-MS and LPL activity assays, the presence of the N-terminal domain in GPIHBP1 was demonstrated to be crucial to prevent the spontaneous unfolding of the catalytic domain of LPL [78]. Moreover, it has been shown that GPIHBP1-bound LPL is not inactivated by ANGPTLs and that both domains of GPIHBP1 are needed for this protective effect [83,84].

More than 20 different mutations have been identified in the GPIHBP1 gene in patients with familial chylomicronemia syndrome and severe hypertriglyceridemia [85]. Most of the mutations are in the Ly6 domain and disrupt the interaction between GPIHBP1 and LPL. These patients have very low post-heparin plasma LPL activity because LPL is not transported to the vascular endothelium. No mutations causing familial chylomicronemia syndrome have been reported in the N-terminal domain of GPIHBP1 [31]. Patients carrying mutations in the GPIHBP1 gene have plasma triglyceride concentrations as high as 2000 mg/dl [31], which is more than 10-fold higher than in

normolipidemic individuals. Only homozygotes for mutated GPIHBP1 have familial chylomicronemia syndrome; heterozygotes have normal plasma triglyceride levels. The majority of the identified mutations involve one of the conserved cysteines in the Ly6 domain or adjacent residue [85]. For example, the Q115P mutation, involving a residue next to a cysteine, was the first one discovered [86]. Most of the conserved cysteines or adjacent residues, such as Q115, do not participate directly in the interaction with LPL, suggesting that they disrupt the overall three-finger fold of the Ly6 domain. *In vitro* cell culture studies suggested that these mutations cause the formation of disulfide-linked dimers and multimers on the Chinese hamster ovary (CHO) cell surface which were unable to bind LPL [87]. Multimerization was not seen *in vivo*, however, when GPIHBP1 C63Y knockin mice were generated and studied. Most of the GPIHBP1 in these mice was found to be monomeric [88]. Interestingly, the C63Y mutation did not alter the N-terminal domain of GPIHBP1 but these mice still had severe chylomicronemia because LPL was accumulated in the subendothelial space and was not transported to the vascular endothelium. Moreover, it was recently shown that, in certain cases, autoantibodies against GPIHBP1 can cause chylomicronemia [89]. These antibodies bind to the Ly6 domain of GPIHBP1 and disrupt the interaction between LPL and GPIHBP1 [38], so that LPL is not transported to the vascular endothelium. Taken together, these results clearly demonstrate the importance of the Ly6 domain for transporting LPL across the endothelial cells.

1.4.2 Heparan sulfate proteoglycans and heparin

Heparan sulfate and heparin are negatively charged linear polysaccharides which belong to the glycosaminoglycan family. Both molecules are composed of disaccharide units of uronic acid and D-glucosamine, which are partially sulfated and acetylated through hydroxy groups and D-glucosamine nitrogen [90]. Heparan sulfate proteoglycans are formed in the Golgi when one or more heparan sulfate chains are covalently attached to a core protein. HSPGs are synthesized by almost all animal cells and cover almost all cell surfaces. Because several HSPGs are secreted into the extracellular matrix, they are also a major component of extracellular matrices [90].

HSPGs play many important roles in the plasma triglyceride metabolism [15,39]. They participate in lipoprotein remnant clearance from circulation in the liver and are important components of subendothelial space in LPL regulation [39]. HSPGs retain freshly secreted LPL in the subendothelial space and don't let it diffuse into the lymph. Additionally, as discussed in the previous LPL chapter, HSPGs participate in the transport of LPL across the subendothelial space from the parenchymal cell surface to the basolateral side of endothelial cells. LPL binds to heparan sulfate and heparin via electrostatic interactions and this interaction is characterized by very fast association and dissociation rate constants as it was shown by using surface plasmon resonance (SPR) [40]. Because subendothelial space is filled with heparan sulfate chains, it was suggested that LPL dynamically moves between different HSPG molecules. However, the abundance of heparan sulfate chains makes the overall dissociation of LPL from HSPGs very low.

In contrast to HSPGs, heparin is synthesized by only connective tissue-type mast cells and is not attached to cell membranes [91]. Heparin is smaller and more negatively charged than HSPGs and it is best known as a biopharmaceutical that is used as an anticoagulant [91]. In addition, heparin is used in patients with severe hypertriglyceridemia for the possible diagnosis of LPL deficiency. As discussed above, active LPL is bound to the vascular endothelium mainly via GPIHBP1, and plasma contains only an inactive form

of LPL. Intravenous heparin injection releases active LPL into circulation and can be used to estimate the amount of functional endothelium-bound LPL [44].

1.4.3 Apolipoprotein C-II

ApoC-II plays a crucial role in the plasma triglyceride metabolism, as it is an essential activator of LPL [92]. When lipoproteins do not contain apoC-II, triglycerides are not hydrolyzed by LPL [93,94]. Therefore, a genetic deficiency of apoC-II results in a similar phenotype as in the case of LPL deficiency, where plasma triglyceride levels are extremely high [95]. The prevalence of homozygous apoC-II deficiency, however, is even lower than LPL deficiency [19].

ApoC-II is produced mainly in the liver but a small amount of apoC-II is also synthesized in the intestine [33]. After secretion to plasma, apoC-II binds to HDL, but once nascent chylomicron or VLDL enters circulation, apoC-II transfers to these TRLs. During the end of LPL-mediated lipolysis of a chylomicron and a VLDL, apoC-II transfers back to an HDL. Therefore, an HDL is like a reservoir that provides apoC-II for TRLs when needed and otherwise keeps apoC-II in circulation for a long time.

ApoC-II has a molecular weight of 8916 Da. Mature human apoC-II consists of 79 amino acid residues and lipid-bound apoC-II contains three amphipathic α-helices spanning residues 14-38, 50-54 and 63-76, as was seen in the nuclear magnetic resonance structures of apoC-II/SDS micelle complexes [96,97]. The N-terminal helices are needed for binding lipoproteins, while the C-terminal helix is responsible for binding and activating LPL. Although only the C-terminal helix is required to activate LPL when an emulsion of triolein is used as a substrate [98], all three helices are necessary for LPL activation with a natural lipid substrate (chylomicrons) [93]. The sequence of the N-terminal part of apoC-II varies among species, while the C-terminal part is more conserved. Specifically, seven amino acid residues were identified as being conserved among the species. Of these, four residues (human sequence Tyr63, Ile66, Asp69 and Gln70) were shown to reside on the same side of the helix and to be crucial for LPL activation [99].

LPL binds to lipoproteins even in the absence of apoC-II [93,100]. This shows that apoC-II is not needed for the initial binding of LPL to a lipid/water interface. It has been proposed that apoC-II may help to adapt the correct orientation of LPL on lipoproteins to allow the enzyme to hydrolyze triglycerides [100]. In addition, it was recently suggested that apoC-II helps to preserve LPL activity when triglycerides in lipoproteins are degraded, the particle shrinks and surface pressure is increased [101]. Without apoC-II, LPL can be bound to these partially degraded lipoproteins, but it is not able to catalyze the hydrolysis of triglycerides.

The exact binding site of apoC-II on LPL is unknown. Using chemical cross-linking, it was shown, however, that the C-terminal fragment of apoC-II binds to the N-terminal domain of LPL (residues 65-86) in the absence of lipoproteins or lipids [102]. Because apoC-II adopts a helical conformation when attached to lipids [103] and because the complex formed between apoC-II and LPL without lipids was shown to be non-productive [104], these cross-linking results need to be confirmed in the presence of lipids.

The apoC-II concentration in normolipidemic individuals is approximately 4.5 μM (4 mg/dl) and this appears to be sufficient for full activation of LPL as heterozygotes for apoC-II deficiency typically have normal triglyceride levels in plasma [105]. Moreover, it has been shown that plasma apoC-II concentrations correlate positively with triglyceride levels in patients with hypertriglyceridemia [106,107] and, in one case, high plasma levels of apoC-II have been reported to be the cause of hypertriglyceridemia [108].

Also, transgenic mice overexpressing human apoC-II have hypertriglyceridemia [109]. The reason for triglyceride accumulation in these mice has remained unclear. Because triglyceride-rich VLDLs accumulated in these mice, it was suggested that it is more likely that the high levels of apoC-II inhibited LPL-mediated TRL hydrolysis rather than TRL remnant clearance via receptors. On the other hand, it has been shown that apoC-I and apoC-II can displace apoE from VLDLs, which results in the lowered ability of VLDL to bind hepatic receptors [110]. This suggests that high apoC-II levels may inhibit the receptor-mediated endocytosis of lipoprotein remnants.

1.4.4 Apolipoproteins C-I and C-III

ApoC-I and apoC-III are negative regulators of plasma triglyceride metabolism, whose overexpression in mice results in severe hypertriglyceridemia [111,112]. ApoC-III has been extensively studied and has become an important therapeutic target for lowering triglycerides in patients with severe hypertriglyceridemia. This is because large human genetic studies have clearly demonstrated that loss-of-function mutations in apoC-III result in decreased triglyceride concentrations and a decreased risk of ASCVD [113,114]. In contrast, apoC-I has been less extensively studied. This is probably related to the fact that so far no apoC-I mutations have been found in humans that affect plasma triglyceride concentration and the risk of ASCVD [30]. ApoC-III and apoC-I have been shown to influence the plasma triglyceride metabolism in several negative ways. Firstly, both proteins inhibit LPL activity [115]. It has been proposed that apoC-III and apoC-I prevent the interaction between LPL and a lipoprotein [116]. Secondly, apoC-III and apoC-I prevent the binding of TRL remnants via apoE or apoB to liver receptors (LDLR, LRP) and thereby inhibit lipoprotein remnant clearance from circulation [115,117]. Thirdly, it has been suggested that apoC-III promotes VLDL production and secretion [118]. Although apoC-III plays a crucial role in the determination of plasma triglyceride concentration, its physiological role is unclear [30]. It has been speculated that apoC-III may be needed during fasting to prevent too rapid clearance of TRL remnants from circulation [117]. Because TRL remnants still contain triglycerides, the energy-rich fatty acids may be needed in the heart and skeletal muscle.

ApoC-III and apoC-I are mainly synthesized in and secreted from the liver and they are attached to TRLs and HDL in plasma. Mature apoC-III and apoC-I have molecular weights of 8.8 kDa and 6.6 kDa, respectively [115]. While the plasma concentrations of all apoCs are in a similar micromolar range, apoC-III's concentration is the highest, which in normolipidemic individuals is approximately 11-13 μM (10-12 mg/dl) [1,115,119]. The apoC-I concentration in plasma is approximately 4.5-9 μM (3-6 mg/dl) [1,115].

1.4.5 Apolipoprotein A-V

Although the apoA-V concentration in plasma is more than 1000-fold lower than the concentration of apoCs (0.6 – 10 nM, which corresponds to 2.4 – 40 μg/dl) [120], apoA-V has been shown to play an important role in the plasma triglyceride metabolism. The apoA-V gene is one of the five genes (the others are LPL, apoC-II, LMF1 and GPIHBP1) in which biallelic loss-of-function mutations cause monogenic chylomicronemia with extremely high triglyceride concentrations [121]. Human genetic studies have demonstrated that apoA-V deficiency causes hypertriglyceridemia and an increased risk of ASCVD [122,123]. This is in line with mouse studies. The deletion of apoA-V in mice causes an increase in plasma triglyceride concentration, whereas the overexpression of apoA-V reduces plasma triglyceride levels [124]. ApoA-V is synthesized in the liver like apoCs, but it is larger, with a molecular weight of 40 kDa. ApoA-V is bound to TRLs and

HDLs in plasma. The exact mechanism of how apoA-V lowers triglycerides is unclear. While *in vitro* experiments indicate that it does not directly increase LPL's catalytic activity [125], it has been proposed that *in vivo* apoA-V stimulates LPL-mediated lipolysis [126]. ApoA-V interacts with heparin, HSPGs [125] and GPIHBP1 [73]. It also interacts with different receptors, including LRP, which are needed for lipoprotein remnant clearance [127]. Therefore, its effect may be to enhance TRL binding to the endothelial cell surface to enable LPL to hydrolyze triglycerides more efficiently [128]. Additionally, apoA-V probably promotes lipoprotein remnant clearance [126].

1.4.6 Angiopoietin-like proteins 3, 4 and 8

The angiopoietin-like proteins 3, 4 and 8 are post-translational physiological regulators of LPL, and all three inhibit LPL activity in a tissue-specific manner depending on the metabolic needs of adipose tissue, skeletal muscle and the heart [30,32]. The importance of ANGPTLs in plasma triglyceride metabolism is evident from human genetics. Loss-of-function mutations in the ANGPTL3 and ANGPTL4 genes in humans lead to lowered plasma triglyceride levels and a decreased risk of ASCVD [129–132]. This is supported by mouse studies, which show that the deletion of the ANGPTL3, 4 or 8 gene results in increased LPL activity and reduced plasma triglyceride levels, whereas overexpression of these proteins causes hypertriglyceridemia due to reduced LPL activity [133–137].

While ANGPTL4 is expressed in many cells and tissues, its production is upregulated during fasting in adipocytes, where its role is to inactivate LPL to direct the energy-rich fatty acids derived from circulating lipoprotein-triglycerides to skeletal muscle and the heart [30]. In the (re)-fed state, the expression of ANGPTL4 is reduced in adipose tissue, which causes an increase in LPL activity and more fatty acids are taken up by adipocytes. In addition, during exercise ANGPTL4 expression is induced in resting muscles to inactivate LPL and to direct the triglyceride-derived fatty acids to active muscles to be used as fuel [138]. It has been proposed that ANGPTL4 acts on LPL in the subendothelial space [139] or intracellularly [140]. However, while LPL is not synthesized in the hepatocytes, liver-specific overexpression and the secretion of human ANGPTL4 into circulation increased plasma triglyceride levels in mice [141]. Moreover, the injection of recombinant ANGPTL4 into mice increased the concentration of plasma triglycerides [142]. This demonstrates that ANGPTL4 may also inhibit LPL in plasma at the vascular endothelium.

ANGPTL3 is mainly expressed in the liver and its expression levels remain unchanged during fasting or re-feeding. ANGPTL8 is expressed both in the liver and in adipose tissue, and the production of ANGPTL8 is induced in the fed state [32]. While ANGPTL3 is a known LPL inhibitor, ANGPTL8 alone has no effect on LPL activity. Recent studies have suggested that ANGPTL8 forms a complex with ANGPTL3 in the liver, which after secretion into circulation is a more potent inactivator of LPL than ANGPTL3 alone [143–145]. The proposed role for ANGPTL3 and ANGPLT8 is to inactivate LPL in the heart and skeletal muscle in the fed state to direct triglyceride-derived fatty acids for storage to adipose tissue and probably to protect the heart and skeletal muscle from lipotoxicity [32]. Interestingly, it was recently demonstrated that ANGPTL8 also forms a complex with ANGPTL4 [145]. In contrast to ANGPTL3/ANGPTL8 complex, this complex had reduced ability to inactivate LPL. It was proposed that during the fed state, ANGPTL8 reduces the inactivating effect of ANGPLT4 on LPL in adipose tissue. This allows adipocytes to take up more fatty acids after re-feeding.

ANGPTL3 and ANGPTL4 contain the N-terminal coiled-coil domain, which is responsible for inactivating LPL, and the C-terminal fibrinogen-like domain [32]. It has been shown that *in vivo* proprotein convertases cleave ANGPTL3 and ANGPTL4 between the two domains. Therefore, *in vivo*, ANGPTL3 and ANGPTL4 exist as individual N-terminal and C-terminal domains or full-length variants in plasma and in the subendothelial space[146]. In contrast, ANGPTL8 contains only the N-terminal coiled-coil domain [32,146].

Different mechanisms have been proposed for the effects of ANGPTL3 and ANGPTL4 on LPL. It has been shown that ANGPTL4 and ANGPTL3 promote the conversion of LPL dimers to an inactive monomeric form of LPL, which cannot form dimers anymore [147,148]. In contrast, it has been demonstrated that ANGPTL4 forms a complex with LPL, which has lower catalytic activity, and after the complex dissociates LPL regains its activity [149]. In another study using HDX-MS experiments, it was shown that ANGPTL4 promoted the spontaneous unfolding of the N-terminal catalytic domain of LPL [83]. This resulted in the conversion of LPL dimers to monomers. Furthermore, it was shown in the same study that the inactivating effects of ANGPTL3 and ANGPTL4 on LPL could be prevented with GPIHBP1 [83]. Also, others have demonstrated that when LPL is bound to GPIHBP1, it is resistant to the inactivating effect of ANGPTLs [84], including the ANGPTL3/ANGPTL8 complex [145].

1.5 Assays for determining lipoprotein lipase activity

LPL possesses broad substrate specificity [150]. In addition to triglycerides of lipoproteins, it catalyzes the hydrolysis of triglycerides with various fatty acid compositions that are incorporated into artificial emulsions, such as gum arabic-stabilized triglyceride emulsions or Intralipid (an emulsion of soybean triglycerides in egg yolk phosphatitylcholine used for parenteral nutrition) [151]. Furthermore, LPL is also active on phospholipids [50], simple water-soluble esters and artificial chromogenic or fluorogenic substrate analogs [152–155]. This diversity has made it possible to develop numerous assays for the determination of LPL activity.

The LPL assays can be divided into two groups: those that require apoC-II and albumin as a fatty acid acceptor to avoid product inhibition and those that do not need their presence. Only with natural lipoproteins, presented as isolated fractions with added albumin or in a plasma environment, the presence of apoC-II is essential. In the absence of apoC-II, LPL has no activity on chylomicrons [93]. At the same time, LPL has basal activity in emulsions of long-chain triglycerides (e. g. Intralipid) in the absence of apoC-II [100,151]. In this case, the addition of apoC-II increases the activity in a concentration-dependent manner, reaching maximum activity at a concentration several folds lower than the physiological concentration of apoC-II in human plasma. At higher concentrations, apoC-II starts to inhibit LPL activity [156]. The presence of apoC-II is not needed when LPL activity is measured against water soluble substrates or emulsions of short-chain triglycerides [50].

The LPL activity on lipoproteins or long-chain triglycerides is called lipolytic activity, while its activity measured in substrate systems that do not need the presence of apoC-II is called esterase activity. A comparison of various LPL activity assays is presented in Table 2. It is important to stress that the results must be interpreted carefully when measurements are performed using substrate systems which do not require apoC-II. Many previous studies have shown that LPL activity on non-physiological substrates (esterase activity) is less affected by its regulator proteins [93] and is more resistant to

thermal inactivation [60] or proteolytic cleavage [153] than when lipoproteins are used as substrates. Currently, the main approach for measuring LPL activity on natural lipoproteins – isolated or in a plasma environment – is based on the determination of released fatty acids at certain time points using enzymatic assays (NEFA kit) [157,158]. This approach has several limitations. The most important is that relatively diluted lipoprotein or plasma samples should be used for reliable results. Therefore, the obtained results can be different than in the case of undiluted plasma (this is more thoroughly discussed in the results and discussion section of publications II and III in this thesis). Additionally, the NEFA assay cannot be followed in real-time and thus important functional effects remain undiscovered.

Table 2. *Characteristics of different substrate systems used for measuring LPL activity.*

Type of substrate	Examples	Type of activity	Requirement for apoC-II	Detection	Sensitivity	Real-time detection
Soluble substrate	PNPB[153], DGGR[152]	Esterase	Not needed	Absorbance Fluorescence	Low High	Yes Yes
Emulsion of short-chain triglycerides	Tributyrin [153], tripropionin [159]	Esterase	Not needed	Radioactivity [160] pH-stat [153]	High Low	No Yes
Emulsion of long-chain triglycerides	Intralipid [151], triolein [151]	Lipolytic	Not needed, but addition of apoC-II increases activity	Radioactivity pH-stat NEFA kit	High Low High	No Yes No
Isolated lipoproteins	CM, VLDL [157]	Lipolytic	Needed	NEFA kit [157]	High	No
Blood plasma	Human plasma [158]	Lipolytic	Needed	NEFA kit	High	No

PNPB - p-nitrophenyl butyrate; DGGR – 1,2-O-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin) ester; CM – chylomicron; NEFA – non-esterified fatty acid.

1.6 Hypertriglyceridemia: risk of cardiovascular disease and treatment

While lifestyle modification (e. g. a healthy diet, an increase in physical activity and a reduction of alcohol intake) is the cornerstone in the treatment of subjects with a risk of ASCVD, pharmacological modulation is often needed for a sufficient reduction of the risk [3]. LDL-cholesterol has long been known to play a central role in the development of ASCVD and thus the primary therapeutic option for people at risk of ASCVD is reducing LDL-cholesterol using statins [3], which block the synthesis of cholesterol in the liver, which in turn increases LDL uptake and decreases LDL in circulation. However, despite a significant reduction in LDL cholesterol, some patients still have ASCVD [161]. In recent years, it has been established that elevated plasma triglycerides are related to this residual risk [6]. This is evident from epidemiological and genetic studies, which have shown the independent and causal role of plasma triglycerides in the development of ASCVD [6,162]. Moreover, a recent study suggested that all apoB-containing lipoproteins – TRLs, their remnants and LDL – have the same effect on the risk of cardiovascular disease per particle [28].

The condition in which plasma triglyceride levels are higher than normal is called hypertriglyceridemia. It can be mild-to-moderate (fasting plasma triglyceride

concentration > 2 mM or 180 mg/dl) or severe (fasting plasma triglyceride concentration > 10 mM or 885 mg/dl) [121]. While an unhealthy lifestyle can cause mild-to-moderate hypertriglyceridemia, it is most commonly related to many diseases, including metabolic syndrome, diabetes and obesity [163,164]. This type of hypertriglyceridemia can cause the development of ASCVD. Severe hypertriglyceridemia can be caused by rare biallelic monogenic mutations in the five essential genes in the plasma triglyceride metabolism, such as LPL, apoC-II, GPIHBP1, apoA-V and LMF1 [121]. However, severe hypertriglyceridemia is more often caused by polygenic factors, where mutations are found in several plasma triglyceride metabolism related genes, which individually have a small effect, but collectively have a large effect [121]. Additionally, environmental factors can play a role in an increase in triglycerides in the case of polygenic severe hypertriglyceridemia. While severe hypertriglyceridemia can also cause ASCVD, it primarily causes acute pancreatitis, which is a life-threatening condition [19,25]. Severe hypertriglyceridemia affects one out of 600 people (0.1-0.2% of the total population) [121], whereas mild-to-moderate hypertriglyceridemia is relatively common and affects about one in four people (25% of the total population) in modern westernized societies [165].

While statins are also used to lower triglycerides in individuals with hypertriglyceridemia and at risk of ASCVD, most commonly fibrates are used as an additional therapy [3,166]. Other therapies include omega-3 fatty acids [166]. Statins lower triglycerides by 22-45%, fibrates by 30-50% and omega-3 fatty acids by 30-50% [3]. Fibrates interact with a transcription factor peroxisome proliferator-activated receptor-α (PPAR-α), which increases the expression of many lipid-related genes, including LPL and apoA-I [166]. Omega-3 fatty acids lower triglycerides via multiple mechanisms, including increased fatty acid catabolism, decreased triglyceride synthesis in the liver and increased triglyceride clearance from circulation [166]. How statins reduce plasma triglycerides is poorly understood. Although all of these therapies lower triglycerides, they do not seem to be efficient enough to lower the risk of ASCVD. Several clinical trials have demonstrated inconsistent results [3,166].

Novel therapies are being actively developed to reduce higher amounts of plasma triglycerides and the risk of ASCVD. Many novel therapeutic approaches are aimed at stimulating the LPL-mediated lipolysis [29]. This is supported by several human genetic studies showing the correlation between increased LPL activity and reduced risk of ASCVD [26–28].

LPL activity can be increased directly by activating the enzyme or indirectly by suppressing the effect of LPL's endogenous inhibitors. Because human genetic studies have shown that reduction in ANGPTL3 [167] or apoC-III [113,114] concentration causally protects against ASCVD, pharmaceutical companies are developing therapies to lower the concentration of these proteins. In fact, an antisense oligonucleotide targeting apoC-III mRNA to prevent its production was approved in May 2019 in the European Union for treatment of severe hypertriglyceridemia in patients with familial chylomicronemia syndrome who are at risk for pancreatitis [168]. This drug, called Waylivra (volanesorsen), has been shown to reduce both fasting and fed triglycerides more than 70% [168]. Moreover, antisense oligonucleotides that block ANGPTL3 protein synthesis were recently shown in a Phase 1 clinical study to reduce plasma triglyceride levels in humans by 85% [169] and, in another very recent Phase 1 clinical study, monoclonal antibodies against ANGPTL3 were demonstrated to lower triglycerides more than 80% in subjects with hypertriglyceridemia [170]. However, because of the possible

side effects and high costs of novel therapeutics, there is a need for more alternatives to efficiently treat hypertriglyceridemia in patients at risk of ASCVD or pancreatitis.

Another way to lower plasma triglycerides is direct LPL activation. An apoC-II mimetic peptide was recently designed as a potential LPL activator and triglyceride-lowering drug [158]. This peptide contains a synthetic N-terminal helix designed for binding lipoproteins with high affinity and a C-terminal helix of apoC-II, which is needed for LPL activation. The peptide was shown to activate LPL *in vitro* and to lower triglycerides in various animal models of hypertriglyceridemia [158,171–173].

2 AIMS OF THE STUDY

- To elucidate the role and contribution of GPIHBP1's N-terminal and Ly6 domain in interaction with LPL

- To develop a method that makes it possible to study the regulation of LPL activity near physiological conditions, i. e. in undiluted human plasma

- To apply the developed method for testing drug candidates that target LPL activity or stability in human plasma

3 MATERIALS AND METHODS

More detailed descriptions of the materials and methods used in the thesis can be found in the corresponding publications.

Publication I

- Recombinant mouse GPIHBP1, mutant Q114P GPIHBP1, and a variant of GPIHBP1 containing a thrombin cleavage site between the N-terminal and the Ly6 domain were expressed in CHO cells. To obtain the Ly6 domain, the GPIHBP1 variant containing the thrombin recognition site was treated with thrombin. All GPIHBP1 variants lacked the GPI-anchor.
- Synthetic peptides corresponding to the full-length or various parts of the sequence of the human, mouse or bovine GPIHBP1 N-terminal domain were purchased from different companies.
- Bovine LPL was purified from milk by using heparin chromatography.
- SPR, fluorescence anisotropy and LPL activity and stability measurements were used to investigate the interaction between LPL and GPIHBP1 or individual domains of GPIHBP1.
- Chemical cross-linking combined with mass spectrometry was used to localize the binding region of GPIHBP1's N-terminal domain on LPL.

Publication II

- Isothermal titration calorimetry (ITC) was applied to measure LPL activity in human plasma.
- Purified bovine LPL or human post-heparin plasma, obtained from Prof. Gunilla Olivecrona's group (Umeå University, Sweden), was used as an exogenous source of LPL for enzymatic activity measurements.
- Human plasma was purchased from the Tallinn Blood Centre.
- Recombinant human N-terminal coiled-coil domain of ANGPTL4, human apoC-II and human apoA-V with a His-tag were expressed in *Escherichia coli* cells. The proteins were purified from cell lysates using Ni-affinity, anion-exchange and size-exclusion chromatography (SEC).
- ApoC-III was purified from human plasma.
- Full-length human ANGPTL3, expressed in *Sf21* cells, was obtained from R&D Systems (USA).

Publication III

- ApoC-II mimetic peptides were obtained from Dr. Alan T. Remaley (National Institutes of Health, USA).
- Human plasma was separated using SEC. Additionally, chylomicron, VLDL, LDL, and HDL fractions were isolated from human plasma by density gradient ultracentrifugation.
- As an alternative assay to ITC, LPL activity was determined by measuring the generation of fatty acids after the lipolysis of human lipoproteins using NEFA kit (FUJIFILM Wako Diagnostics, USA).

4 RESULTS AND DISCUSSION

4.1 Roles of GPIHBP1 domains in interaction with lipoprotein lipase (Publication I)

The aim of this study was to investigate the role and contribution of GPIHBP1's two domains in the interaction with LPL. This study was the first to quantitatively compare the binding of LPL to the N-terminal domain of GPIHBP1 (a synthetic peptide corresponding to the sequence of this domain), to the Ly6 domain of GPIHBP1 (recombinant GPIHBP1 lacking the N-terminal domain and GPI-anchor) and to the GPIHBP1 that consisted of both these domains, lacking only the GPI-anchor. Previous studies had shown the importance of both domains of GPIHBP1 in the interaction with LPL, but the exact role and function was unclear. We demonstrated that the two domains of GPIHBP1 represent two distinct binding sites for LPL and both domains bind to LPL with a similar nanomolar affinity. Our results suggested that LPL can bind independently to the N-terminal domain of GPIHBP1 or to the Ly6 domain of GPIHBP1, or two LPL molecules can be bound to these sites at the same time. It was not possible to determine from our experiments, however, if dimeric or monomeric LPL is bound to the two binding sites on GPIHBP1.

Methodological considerations in studying the interactions between LPL and GPIHBP1 or individual domains of GPIHBP1

Our conclusions were based on experiments conducted with various biophysical techniques, including SPR, fluorescence anisotropy, enzyme activity and stability measurements and chemical cross-linking combined with mass spectrometry. The main results were obtained by using SPR. This technique is based on the detection of a change in SPR response, which is linearly proportional to the mass concentration close to the surface of a sensor chip [174]. In our experiments, the full-length GPIHBP1, its N-terminal domain or Ly6 domain was immobilized on the surface of the sensor chip, and LPL was injected in the flow phase over this surface (Figure 5A). This setup mimicked the *in vivo* conditions of LPL, where GPIHBP1 is anchored to the endothelial cells and LPL can be in a dynamic interaction with GPIHBP1's domains (Figure 5B). SPR made it possible to determine both the affinity and the dynamics of the interactions and calculate the life-times of the complexes.

Purified LPL is known to be extremely unstable *in vitro* at physiological temperatures and NaCl concentrations, losing its activity within minutes [15,60]. In a plasma environment, however, LPL is more stable, probably because of the stabilizing effects of albumin, heparan sulfate chains, GPIHBP1 and lipoproteins (Publication II of this thesis). LPL activity can be preserved *in vitro* by adding albumin, heparin, GPIHBP1, high concentrations of NaCl at low temperatures or detergents to buffers (e. g. sodium deoxycholate or TritonX-100) [40,84,150]. Additionally, previous SPR experiments have shown that LPL is prone to bind non-specifically to the negatively charged SPR sensor matrix via ionic interactions [40]. Therefore, we took great care in this study to find the conditions in which LPL preserves its activity and where non-specific binding is minimal. It was found that in the presence of 2 mg/ml bovine serum albumin and a minimum of 0.4 M NaCl at 4 °C, LPL is active and absolutely stable, and non-specific binding was very low.

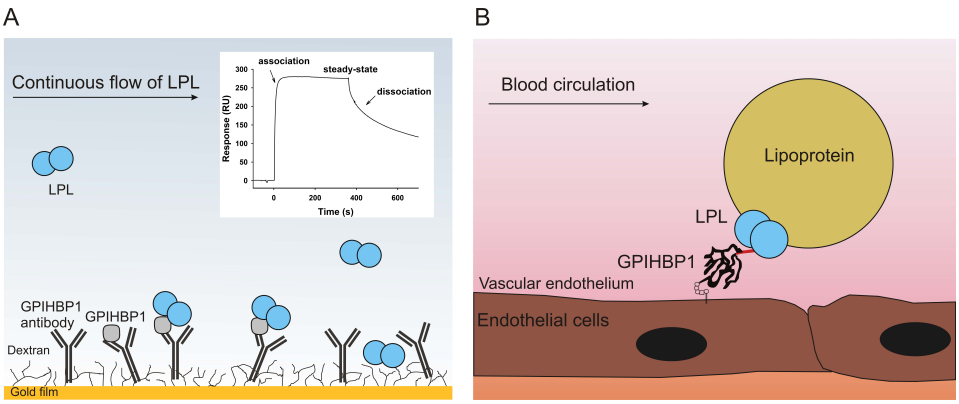

Figure 5. *Comparison of the SPR system and endothelial lipolysis. (A) Illustration of an SPR model system for studying interactions between LPL and GPIHBP1 or individual domains of GPIHBP1. (B) Illustration of LPL-mediated TRL hydrolysis at vascular endothelium. LPL is bound to GPIHBP1, which is anchored to the endothelial cells. Figure 5B is created partially based on a drawing in [42].*

The N-terminal and the Ly6 domain of GPIHBP1 represent two independent binding sites for LPL

The two independent binding sites for LPL on GPIHBP1 were evident from the binding curve obtained using SPR in the presence of 0.4 M NaCl (Figure 6A). This curve could only be fitted to a two-binding site model, assuming two independent LPL binding sites on GPIHBP1. Moreover, when the NaCl concentration in the running buffer was increased to 0.6 M, only the binding of LPL to one site on GPIHBP1 was observed (Figure 6A). The affinity of this interaction was little affected by the increase in the NaCl concentration. LPL did not bind to the second binding site on GPIHBP1 in the presence of 0.6 M NaCl.

The two-site model was confirmed with analogous binding experiments conducted with LPL and individual domains of GPIHBP1. Firstly, LPL bound to the N-terminal domain of GPIHBP1 with an affinity (K_d=3.9 µM) similar to the second binding site on full-length GPIHBP1 (K_d=4.2 µM) in the presence of 0.4 M NaCl (Figures 6A and 6B). Similarly to the second binding site, LPL did not interact with the N-terminal domain of GPIHBP1 in the presence of 0.6 M NaCl (Figure 6B). Secondly, LPL bound to the Ly6 domain of GPIHBP1 with an affinity (K_d=93 nM) comparable to the first binding site on full-length GPIHBP1 (K_d=6.4 nM) in the presence of 0.4 M NaCl (Figures 6A and 6C), and this interaction was little affected by the NaCl concentration, as in the case of the first binding site on GPIHBP1. For comparison, SPR binding experiments were also performed with the naturally occurring Ly6 domain mutant GPIHBP1 Q114P (mouse sequence) [86]. Carriers of this mutation have hypertriglyceridemia due to the inability of the GPIHBP1 Q114P to transport LPL to the vascular endothelium. Our measurements showed that LPL bound to this mutant GPIHBP1 with an affinity and NaCl concentration dependency similar to the N-terminal domain of GPIHBP1, suggesting that the Ly6 domain of this GPIHBP1 variant was not functional. Taken together, these measurements suggested that the first binding site for LPL corresponded to the Ly6 domain and the second binding site to the N-terminal domain of GPIHBP1.

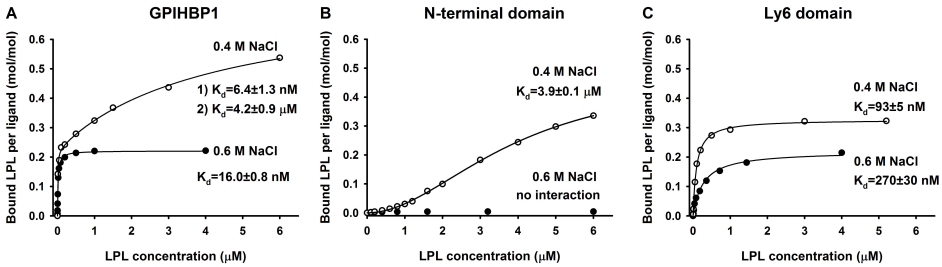

Figure 6. *Binding curves for LPL interactions with GPIHBP1 or individual domains of GPIHBP1 as measured by SPR. Binding of LPL to immobilized (A) full-length GPIHBP1, (B) the N-terminal domain of GPIHBP1 or (C) the Ly6 domain of GPIHBP1 when the running buffer contained 0.4 M or 0.6 M NaCl. In panel A, the binding of LPL to GPIHBP1 in the presence of 0.4 M NaCl was characterized by a two-binding site model, whereas the binding of LPL to GPIHBP1 in the presence of 0.6 M NaCl was characterized by one-binding site model. In panel B, LPL bound to the N-terminal domain of GPIHBP1 with an affinity and NaCl concentration dependency similar to the second binding site in panel A. In panel C, LPL bound to the Ly6 domain of GPIHBP1 with an affinity and NaCl concentration dependency comparable to the first binding site in panel A.*

The two -binding site model was further proved by heparin displacement experiments using SPR (Figure 7). It appeared that heparin was able to completely displace LPL from the N-terminal domain of GPIHBP1 (Figure 7B) but was not able to release LPL from the Ly6 domain of GPIHBP1 (Figure 7C). In the case of full-length GPIHBP1, heparin displaced about 50% of the LPL, suggesting that free heparin eluted only that fraction of LPL which was bound to the N-terminal domain of GPIHBP1 (Figure 7A). Finally, the two-binding site model was tested in the following SPR experiments: full-length GPIHBP1, its N-terminal domain or Ly6 domain was immobilized on the sensor chip surface and the solution containing LPL and the N-terminal domain of GPIHBP1 was injected over the surfaces. These measurements revealed that while associated with the N-terminal domain, LPL could still bind to the Ly6 domain and also to GPIHBP1. Moreover, the kinetic parameters of these interactions were not affected by the presence of the N-terminal domain. In the case of one binding site consisting of two binding regions, the LPL/N-terminal domain complex should have bound to full-length GPIHBP1 with different kinetics and with a lower affinity than LPL alone.

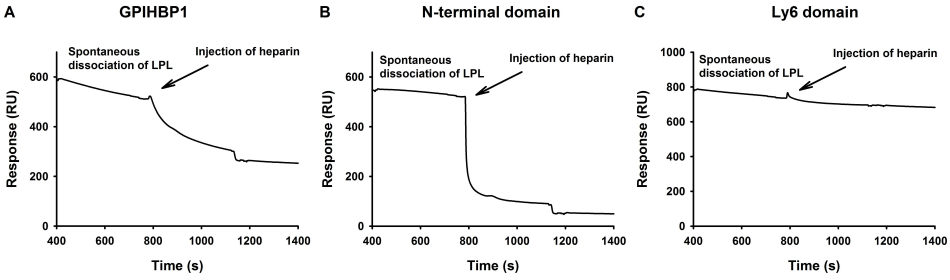

Figure 7. *LPL displacement by heparin from GPIHBP1 or individual domains of GPIHBP1. The ability of heparin to dissociate LPL from immobilized (A) full-length GPIHBP1, (B) the N-terminal domain of GPIHBP1 or (C) the Ly6 domain of GPIHBP1. Heparin dissociated LPL almost completely from the N-terminal domain of GPIHBP1 but did not affect the interaction between LPL and the Ly6 domain of GPIHBP1. In the case of full-length GPIHBP1, heparin probably dissociated only the part of LPL which was bound to the N-terminal domain of GPIHBP1.*

LPL binds to both domains of GPIHBP1 with a similar high affinity, but the dynamics of the interactions are different

As the affinity of the interaction between LPL and the N-terminal domain of GPIHBP1 was very sensitive to the NaCl concentration, it was reasonable to assume that this interaction would be stronger at physiological NaCl concentration. We used LPL activity stabilization and fluorescence anisotropy measurements to study this interaction in buffers containing 0.15 M NaCl. It was not possible to conduct SPR measurements at physiological NaCl concentration due to the high non-specific binding of LPL to the sensor chip matrix. The N-terminal domain of GPIHBP1 stabilized LPL in a concentration-dependent manner. It was possible to calculate the K_d from these measurements, which was shown to be 2 nM. The binding curve obtained by fluorescence anisotropy measurements gave a K_d equal to 60 nM (Figure 8, unpublished results). It is likely that labeling the N-terminal domain of GPIHBP1 with a fluorescent tag affected the affinity for LPL. Overall, both methods showed that the N-terminal domain of GPIHBP1 bound to LPL with a nanomolar affinity in the presence of 0.15 M NaCl. This was similar to the affinity of the interaction between LPL and the Ly6 domain at higher NaCl concentrations. Taking into account the small dependence on the NaCl concentration of the interaction between LPL and the Ly6 domain, we concluded that at physiological NaCl concentration, both domains bind to LPL with a nanomolar affinity. In the case of the Ly6 domain, the nanomolar affinity at physiological salt concentration was later also demonstrated by others [78]. Because of the similar affinities of GPIHBP1 domains for LPL, two separate binding events are indistinguishable in the binding curves at physiological NaCl concentration. Two-step binding could only be seen in the binding curve at higher NaCl concentrations because the binding site on the N-terminal domain of GPIHBP1 had a lower affinity at higher NaCl concentrations.

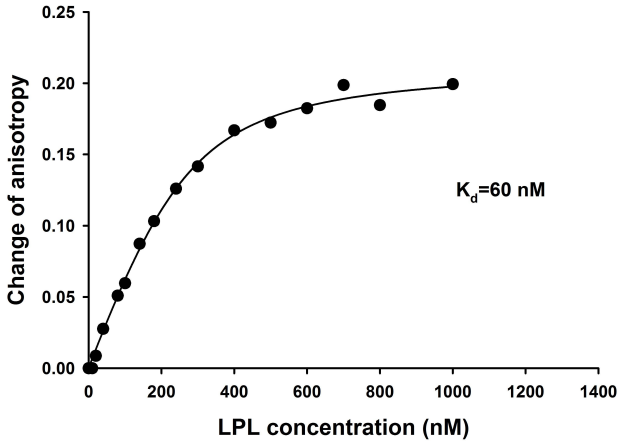

Figure 8. *Binding curve for LPL interaction with the N-terminal domain of GPIHBP1 as measured by fluorescence anisotropy. LPL bound to the N-terminal domain of GPIHBP1 with a nanomolar affinity when the measurements were carried out in a buffer that contained 0.15 M NaCl.*

Kinetic analysis revealed that the dynamics of the interactions between LPL and the two domains of GPIHBP1 were very different at physiological NaCl concentration. We combined results from SPR, LPL activity stabilization and fluorescence anisotropy experiments to come to this conclusion. While LPL associated with the N-terminal domain very rapidly (k_{on} = 7.7 x 10^7 $M^{-1}s^{-1}$), it also dissociated from this domain rapidly (k_{off} = 0.15 s^{-1}). The calculated average life-time of the LPL/N-terminal domain complex was 7 s. The complex between LPL and the Ly6 domain formed more slowly (k_{on} = 2.9 x 10^4 $M^{-1}s^{-1}$, in the presence of 0.4 M NaCl) but was also more stable (k_{off} = 1.9 x 10^{-3} s^{-1}, in the presence of 0.15 M NaCl). The average life-time of this complex was 9 min. Subsequent studies have also investigated the kinetics of the interaction between LPL and GPIHBP1 using SPR. Two consecutive papers by Michael Ploug's group studied the interactions of GPIHBP1 and the Ly6 domain, but not the individual N-terminal domain, with LPL [38,78]. The authors first reported that the association rate constant was 10-fold higher for intact GPIHBP1 than for the Ly6 domain in interaction with LPL [78]. In the second study, they reported that the difference in association rates was 250-fold [38]. It was concluded that the N-terminal domain plays an important role for the association of LPL to GPIHBP1. Similar to our results, the dissociation rate constants did not differ between intact GPIHBP1 and the Ly6 domain in interaction with LPL. Although the authors proposed a different model than we did for the LPL/GPIHBP1 interaction (more thoroughly discussed below), both studies agreed with the observation that the N-terminal domain is very dynamic in the interaction with LPL and the interaction with the Ly6 domain is more stable. An illustration of the two distinct binding site model, where the dynamics of the interactions are different, is presented in Figure 9.

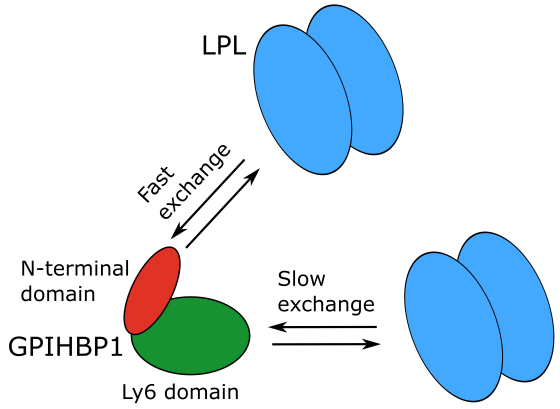

Figure 9. *Illustration demonstrating the proposed model for the interaction between LPL and two domains of GPIHBP1. The N-terminal domain and LPL form a tight but short-lived complex, characterized by fast on- and off-rates. The complex between the Ly6 domain and LPL forms slower and remains together for a longer time. It was not possible to determine the oligomeric state of LPL when it interacted with GPIHBP1's domains. LPL is drawn here as a dimer because active LPL can form dimers.*

It has long been speculated that the N-terminal domain of GPIHBP1 may play a role in capturing LPL in the subendothelial space from HSPGs [80,175]. Because the interaction between the N-terminal domain and LPL was characterized by a very high association rate constant due to the ionic nature of this interaction, it is likely that the N-terminal domain of GPIHBP1 attracts LPL from long distances in the subendothelial space and brings LPL close to the surface of the basolateral side of the endothelial cells. However,

LPL also dissociates rapidly from the N-terminal domain binding site on GPIHBP1. It is possible that once LPL is no longer bound to the N-terminal domain and finds its way to the more stable binding site on the Ly6 domain, it is then transported to the vascular endothelium. It is clear that the Ly6 domain is needed for the transport function of GPIHBP1. Most of the human mutations in GPIHBP1 that cause hypertriglyceridemia are in the Ly6 domain and these variants of GPIHBP1 are unable to transport LPL across the endothelial cells [85].

LPL can only interact with lipoproteins when it is bound to the N-terminal domain of GPIHBP1

We also investigated whether the localization of LPL on GPIHBP1 affects its ability to bind lipoproteins. These measurements were carried out with triglyceride-poor human LDL to avoid the effects of triglyceride hydrolysis by LPL. However, it has been shown that LPL binds to LDLs and VLDLs in similar ways via protein-lipid interactions [176,177]. Our SPR binding studies demonstrated that an LDL was able to bind to the complex LPL/N-terminal domain but not to the complex LPL/Ly6 domain (Figure 10).

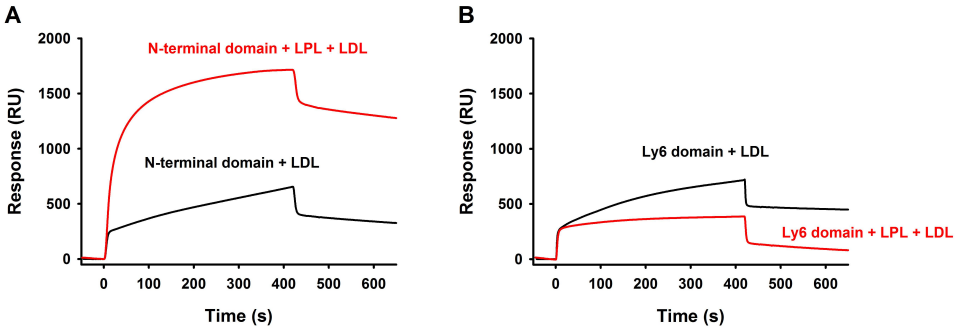

Figure 10. *Binding of lipoproteins to (A) the N-terminal domain of GPIHBP1 or to (B) the Ly6 domain of GPIHBP1 in the presence or absence of LPL. An LDL bound avidly to LPL which was attached to the N-terminal domain of GPIHBP1, whereas an LDL did not bind to LPL which was attached to the Ly6 domain of GPIHBP1.*

Specificity of the interaction between LPL and the N-terminal domain of GPIHBP1

The interaction between LPL and the N-terminal domain of GPIHBP1 was studied more thoroughly using SPR in the presence of 0.4 M NaCl. Experiments were performed with synthetic peptides that corresponded to GPIHBP1's N-terminal domain sequences of different species or to various regions of this domain (Table 3). Additionally, two artificial peptides were generated to study the specificity of the interaction between the N-terminal domain and LPL (Table 3, peptides 6 and 7). We proposed in our paper that the last 19 residues at the C-terminal part of the N-terminal domain (residues 33-51 in the human GPIHBP1 sequence; sequence of peptide 5 in Table 3) are crucial in the interaction with LPL. This was because peptide 5 (Table 3), where 10 residues from the N-terminus of the human native peptide were deleted, had almost the same affinity for LPL as the human N-terminal domain of GPIHBP1. However, the sequences of the N-terminal domains vary among species and they all still bind to LPL with a similar affinity

(Table 3). Also peptide 7 had a similar affinity for LPL, although its sequence was randomly generated based on a human N-terminal domain of GPIHBP1.

We have now studied different peptides corresponding to different regions of the human N-terminal domain of GPIHBP1 containing the same amount of negatively charged residues (peptides 1,2,3 in Table 3, unpublished results). These peptides all interacted with LPL with similar affinities. Therefore, we can now conclude that the affinity of the interaction between LPL and the N-terminal domain of GPIHBP1 does not depend on the exact sequence of the N-terminal domain. More likely, the amounts of negatively charged residues are important. The interaction between LPL and peptide 1, 2 or 3 was more than 10-fold weaker than with the full-length human N-terminal domain of GPIHBP1. These peptides had 10 negatively charged residues, while the human N-terminal domain of GPIHBP1 had 21 negatively charged residues. Peptide 5 had 13 negatively charged residues and this peptide had nearly the same affinity in the interaction with LPL as a full-length N-terminal domain of GPIHBP1, suggesting that this is the optimal amount of negatively charged residues needed for binding to LPL with high affinity.

To examine whether only the amount of negatively charged residues is important, we designed peptide 6, which was partially based on the sequence of the human N-terminal domain of GPIHBP1, but where the negative charges were more evenly distributed within the sequence. This peptide contained 13 negatively charged residues, like peptide 5, but by inserting alanine residues into the sequence of this peptide, negatively charged residues were not clustered. This peptide did not bind to LPL (Table 3, unpublished results). Thus, negatively charged residues have to form a cluster of at least 13 residues to bind LPL with a high affinity, based on current results. Determining the exact number of negatively charged residues in a cluster required for a high affinity requires further investigation.

Table 3*. The affinities for the interactions between LPL and different peptides as determined by SPR. Peptides 1-5 sequences correspond to the various regions of the human N-terminal domain of GPIHBP1. Peptides 6 and 7 sequences are partially based on the human N-terminal domain of GPIHBP1. The number of negatively charged amino acid residues in each peptide are shown in parentheses. The measurements were conducted in the presence of 0.4 M NaCl.*

Peptide	**Sequence**	**K_d (μM)**
Human N-terminal domain	QQEEEEEDEDHGPDDYDEEDEDEVEEEETC (21)	18±3
Peptide 1	QQEEEEEDEDHGPDDY (10)	338±44
Peptide 2	EDHGPDDYDEEDED (10)	257±13
Peptide 3	EEDEDEVEEEETC (10)	230±41
Peptide 4	EVEEEETC (5)	no binding
Peptide 5	HGPDDYDEEDEDEVEEEETC (13)	52±5
Peptide 6	QQEAEAAEEAHDGPEDYAEADADEVAEAETC (13)	no binding
Peptide 7	VDQDYEDDHETEDDEEQDPEEEEGEEEEEC (21)	63±4
Mouse N-terminal domain	AQEDGDADPEPENYNYDDDDDEEEEEETC (17)	22±2
Bovine N-terminal domain	AQEDEDDDPDAGREGYDDEDEEEEEA (17)	33±5

Localizing the binding region of the N-terminal domain of GPIHBP1 on LPL

Chemical cross-linking in combination with mass spectrometry was used to localize the binding region of the synthetic peptide corresponding to the human N-terminal domain of GPIHBP1 on LPL. Because this peptide contains only one reactive amine at its

N-terminus, it was possible to localize the position of the N-terminus of the peptide on LPL. All identified cross-linked lysines were on the positively charged side of LPL, which also contains regions responsible for heparin- and HSPG-binding. The identified cross-linked lysines on the LPL/peptide 1:1 complex were as follows: 272, 300, 407, 414, 422 and 437 (numbering based on the human LPL sequence). Although most of the cross-linked lysines on LPL were identified in the C-terminal domain, some cross-linked lysines were also identified in the N-terminal domain of LPL. This demonstrated that the N-terminal domain of GPIHBP1 could bind to LPL in different orientations, suggesting that there might be multiple binding sites on LPL for this domain. Our results are in line with a subsequent study by Mysling et al [78]. The authors used zero-length cross-linker to reveal that the N-terminal domain is directly linked to lysines 296, 414, 422 and 428 in LPL. HDX-MS measurements in the same study demonstrated that residues 279-290 in LPL, which are located at the interface between the C- and N-terminal domain of LPL, form a binding site for the N-terminal domain.

Based on recently reported crystal structures of LPL/GPIHBP1 complexes [51,52], heparin-binding motifs form a large continuous positively charged patch, which covers the N-terminal domain, the C-terminal domain and the hinge region of LPL. Because the N-terminal domain of GPIHBP1 was not identified in the crystal structures, the exact binding site of this domain on LPL remains to be determined. However, it was suggested that the positively charged patch on LPL also forms a binding site for the N-terminal domain of GPIHBP1. Our results are consistent with that and suggest that binding sites for heparin/HSPG and for the N-terminal domain of GPIHBP1 on LPL may at least partly overlap.

Two distinct binding sites or two regions of one binding site on GPIHBP1 for LPL?

Shortly after our study was published, another model for the LPL/GPIHBP1 interaction was proposed [78]. By using SPR and HDX-MS experiments, the authors of this study concluded that LPL binds to GPIHBP1 via a two-step mechanism: in the first step, LPL binds to the N-terminal domain of GPIHBP1 and in the second step LPL moves to the Ly6 domain, forming a more stable complex, where a single LPL molecule interacts at the same time with the N-terminal domain and the Ly6 domain. According to their model, the Ly6 domain is responsible for the high affinity binding of LPL to GPIHBP1, while the N-terminal domain only plays an accessory role, having a small effect on the overall affinity of the interaction between LPL and GPIHBP1. It is important to note, however, that such a two-step mechanism should have been evident in the SPR sensorgrams. In contrast, the SPR sensorgrams of this study followed a simple 1:1 interaction model. The authors also claimed that their SPR experiments were conducted under more physiological conditions than ours. In their SPR experiments, LPL was immobilized on the surface via a monoclonal antibody, whereas GPIHBP1 was in the flow phase. This is opposite to our experimental setup. Importantly, *in vivo* LPL can freely interact with different ligands and finds its way from HSPGs to GPIHBP1, which is anchored to endothelial cells. Furthermore, these authors used monoclonal antibody 5D2 to capture LPL on the sensor chip. Although the authors showed that LPL was bound as homodimers to the surface, previous studies have shown that LPL is not active when complexed with 5D2 [178,179]. Additionally, the presence of the detergent P20 in their experiments can also influence the LPL/GPIHBP1 interaction.

Even the recently published crystal structures of LPL/GPIHBP1 complexes do not give an unambiguous answer to the question of whether the domains of GPIHBP1 interact independently or simultaneously with LPL. In these structures, only the contacts of LPL with the Ly6 domain were identified. It was proposed that the interaction between LPL and the N-terminal domain was not visible due to the lack of a defined secondary structure and flexibility in the N-terminal domain. However, the possibility that LPL did not interact with the N-terminal domain in these crystals cannot be ruled out.

Functional LPL at the vascular endothelium

Recent studies by Stephen Young's group suggested that LPL at vascular endothelium is only bound to GPIHBP1 *in vivo* [14]. According to their model, LPL is simultaneously bound to both domains of GPIHBP1 [31]. However, the intravenous injection of standard heparin, or even heparin decasaccharides, into animals or humans rapidly releases active LPL from its endothelial binding sites [44]. Most of the LPL is released within a few minutes [180]. Because LPL binds to the Ly6 domain of GPIHBP1 via hydrophobic contacts, it is puzzling why heparin should release LPL from this domain. Indeed, we showed that heparin dissociated LPL only from the N-terminal domain of GPIHBP1, and not from the Ly6 domain of GPIHBP1. The same was observed by Kristensen et al., who conducted SPR experiments similar to ours [38]. In their experiments, LPL was attached to the pre-immobilized heparin and the ability of GPIHBP1 or the Ly6 domain to dissociate LPL from this surface was studied. In line with our results, the Ly6 domain was unable to dissociate LPL from the heparin surface, while GPIHBP1 containing the N-terminal domain eluted LPL from the sensor chip surface. Moreover, we showed that only LPL that is bound to the N-terminal domain of GPIHBP1 is capable of binding lipoproteins. These observations suggest that functional LPL is bound to the N-terminal domain of GPIHBP1 at the vascular endothelium (Figure 11).

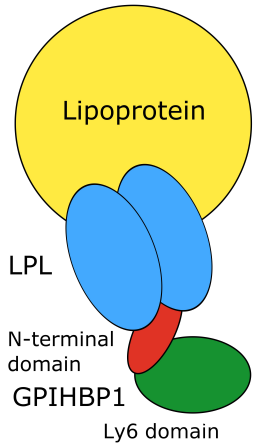

Figure 11. *Proposed model for the interaction of lipoproteins with GPIHBP1-bound LPL at the vascular endothelium. Our data suggest that only LPL that is bound to the N-terminal domain of GPIHBP1 can bind to lipoproteins. It was not possible to determine the oligomeric state of LPL when it was bound to the N-terminal domain of GPIHBP1 and to a lipoprotein. LPL is drawn here as a dimer because active LPL can form dimers.*

4.2 A novel calorimetric approach for studying lipoprotein lipase in human plasma (Publication II)

In this study we developed a novel ITC-based approach which makes it possible to study exogenously added LPL activity on natural lipoproteins directly in human plasma. The main advantage of our approach over the previously used methods (e. g. methods discussed in chapter 1.5 “Assays for determining lipoprotein lipase activity”) is the possibility of conducting real-time measurements in undiluted human plasma. Real-time measurements make it possible to investigate the kinetics of lipoprotein degradation, as well as the activation or inactivation of LPL by potential drug candidates or physiological LPL regulators. To the best of our knowledge, the ITC-based approach is currently the only assay which makes it possible to perform continuous measurements in undiluted plasma.

Description of the ITC assay

An ITC sample cell is filled with human plasma which does not have to be diluted (Figure 12A). The plasma sample can be mixed with different ligands. Human plasma does not contain endogenous LPL activity. Therefore, purified LPL or post-heparin plasma as a source of active LPL is injected into the sample cell from a syringe-stirrer. This starts the exothermic enzymatic reaction (Figure 12B). Heat is produced because triglycerides and phospholipids in lipoproteins are hydrolyzed by LPL. This results in an increase in the heat rate (Figure 12B). Although several other factors (the binding of LPL to lipoproteins, the exchange of apolipoproteins and lipids between lipoproteins, structural changes in lipoproteins, the binding of fatty acids to albumin etc.) besides the hydrolysis of ester bonds of triglycerides and phospholipids probably contribute to the total heat rate, it was shown in this publication that the change in heat rate was proportional to the LPL reaction rate. This was evident from the linear relationship between injected LPL concentration and the change in heat rate. Furthermore, total heat production due to the action of LPL was proportional to the amount of released fatty acids.

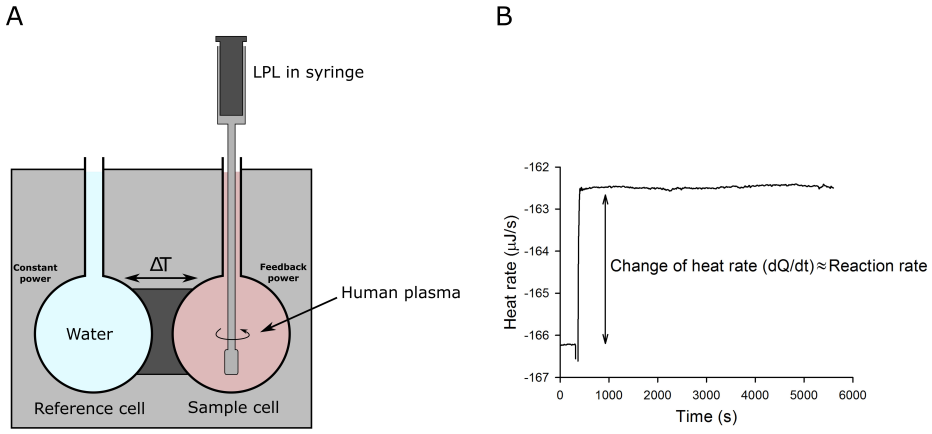

Figure 12. *Illustration of the novel calorimetric approach for measuring LPL activity in human plasma. (A) Schematic model of the ITC system. The ITC cell is filled with undiluted human plasma and LPL is injected into the cell from the syringe-stirrer. (B) Heat rate change as a result of a single injection of LPL into an ITC cell containing human plasma. The change of heat rate is proportional to the reaction rate.*

Plasma as an environment for studying LPL

The use of plasma as a substrate source and environment for studying LPL is an advantage in several ways.

Firstly, plasma is the physiological environment where LPL acts and it contains natural lipoprotein substrate in an intact form for LPL. During the LPL-catalyzed degradation of TRLs, properties of the substrate lipoproteins change. Lipoprotein particle size decreases, the surface pressure increases and there is an exchange of lipids and apolipoproteins between TRLs and other lipoproteins [181]. Furthermore, lipolysis products, such as monoglycerides and fatty acids, may accumulate on the surface of lipoproteins [182]. All of the changes in lipoproteins are sensed by LPL and can affect the activity of LPL.

Secondly, as discussed in the literature review, LPL activity is regulated by many plasma proteins, such as apoC-I, C-II, C-III, A-V and ANGPTL3, 4 and 8. These regulators are present at physiological concentrations in plasma. Little is known about how these ligands collectively affect LPL in plasma. They can influence each other's functionality. For example, it was recently demonstrated that ANGPTL8's inhibitory effect is greater when it is complexed with ANGPTL3 but the effect is reduced when it forms a complex with ANGPTL4 [145]. Also, since plasma is a complex fluid containing thousands of proteins and other components, it is possible that some ligands of LPL may not have been identified yet.

Thirdly, plasma protein concentration is estimated to be 80 g/l, which can cause crowding effects [183]. Indeed, our unpublished data indicate that the affinity of the N-terminal domain of GPIHBP1 for LPL is 10-fold lower in lipoprotein free plasma than in regular assay buffer. Furthermore, ANGPTL4's ability to inactivate LPL was enhanced in a PBS buffer containing 50 g/l bovine serum albumin compared to regular PBS buffer.

Effects of physiological regulators of LPL on its activity in human plasma

The ITC assay allowed us to study how different physiological regulators of LPL influence its activity and stability in human plasma. The possibility of continuously monitoring the reaction rate of LPL enabled us to accurately determine true initial reaction rates under zero-order conditions. In the case of apoC-III, we found that it decreased the initial reaction rate of LPL but did not affect the stability of the enzyme. We also found that the same amount of lipoproteins was degraded by LPL when the apoC-III concentration was increased in plasma but the kinetics of the complete hydrolysis of the available substrate was slower. This indicated that apoC-III reduced the number of LPL molecules that simultaneously acted on TRLs. This is in accordance with studies showing that apoC-III inhibits LPL activity by displacing the enzyme from lipoproteins, meaning that the presence of apoC-III decreases the amount of active LPL on a lipoprotein [116]. Compared to previous results obtained with isolated chylomicrons [116], the effect of apoC-III on LPL activity was relatively low in plasma: an addition of 50 µM apoC-III to plasma reduced LPL activity by about 30%. These results support the view that *in vivo* the larger effect of apoC-III on plasma triglyceride levels is the inhibition of clearance of TRLs through LDL receptors rather than the direct inhibition of LPL activity [117]. Interestingly, using isolated TRLs as a substrate, it was recently demonstrated that GPIHBP1-bound LPL was more susceptible to the inhibitory effect of apoC-III than was free LPL [184]. In future studies, it would be interesting to test the effect of apoC-III on the activity of GPIHBP1-bound LPL in human plasma using ITC.

We also tested the effect of apoA-V on LPL activity in human plasma. It has been proposed that apoA-V may lower plasma triglyceride levels by stimulating LPL activity [126]. However, we found that apoA-V did not directly increase the catalytic activity of LPL in plasma, and high concentrations (100 nM) of apoA-V even inhibited LPL activity. Nevertheless, *in vivo* apoA-V may still enhance LPL-catalyzed triglyceride hydrolysis by increasing the binding of TRLs to HSPG or GPIHBP1 on endothelial cells.

Finally, we studied the effects of ANGPTL3 and ANGPTL4 on LPL activity in plasma. A two-step mechanism for the inhibition of LPL activity was observed. With lower concentrations, both ANGPTL3 and ANGPTL4 decreased only the initial reaction rate of LPL. With higher concentrations of ANGPTLs, LPL activity initially dropped more and continued to decrease after the first drop. As discussed in the literature review, two different mechanisms have been proposed for how ANGPTL3 and ANGPTL4 might suppress LPL activity [147,149]. It is possible that the first step in our experiments involves a complex formation between ANGPTLs and LPL which causes reduced activity of such a complex. This may lead to the second step, involving continuous irreversible inactivation of LPL, where dimers are converted to monomers. Overall, our findings suggested that both mechanisms, inhibition and inactivation of LPL by ANGPTL3 and ANGPTL4, may be relevant *in vivo*. We also tested the ability of the N-terminal domain of GPIHBP1 to protect LPL from the inhibitory effect of ANGPTL4, and found that it had no effect. It would be interesting to test the effect of full-length GPIHBP1 in a similar experiment because many publications have now demonstrated that GPIHBP1 protects LPL from inactivation by ANGPTL4 [83,84,145].

In addition to plasma, other sources of triglycerides can be used as a substrate for LPL in an ITC assay. We demonstrated that there is a linear relationship between heat rate and LPL concentration when plasma, Intralipid or purified VLDLs were used as a substrate. Intralipid was used as a substrate to study the activation of LPL by apoC-II. Through real-time measurements, we were able to see how apoC-II stabilized LPL. Unlike in plasma, LPL was unstable in the intralipid system in the absence of apoC-II.

It should be noted that our studies with LPL regulators were performed with plasma samples obtained from one or two donors. Future studies should examine the effects of ligands on LPL activity in many different plasma samples with various triglyceride concentrations.

Determination of post-heparin plasma LPL activity

We demonstrated that ITC can be used to determine the endogenous LPL activity of human post-heparin plasma by simply injecting post-heparin plasma into an ITC cell containing normal plasma or purified VLDL as a substrate. Although heparin also releases hepatic lipase and endothelial lipase into plasma, our measurements indicated that the heat rate detected with the injection of post-heparin plasma into the ITC cell was almost fully due to LPL. For hepatic lipase, specific antibodies that inhibited its activity in post-heparin plasma were used to come to this conclusion. In the case of endothelial lipase, we did not have specific inhibitors but it has been shown that heparin increases its plasma concentration only three-fold [185] (for comparison, LPL is increased more than 100-fold). By injecting post-heparin plasma into the ITC cell, it is diluted 50-fold. Thus, it is likely that the concentration of endothelial lipase was not significantly increased in the ITC cell after the injection of post-heparin plasma. It is important to note, however, that our measurements were done with a post-heparin plasma sample

obtained from only one individual. Therefore, future ITC studies with post-heparin plasma should explore the effects of hepatic lipase and endothelial lipase on the total heat rate by using specific inhibitor antibodies and plasma samples from a wide variety of individuals. Because of the simple set up of the ITC experiment and the opportunity to automate the assay, the determination of post-heparin plasma LPL activity using ITC could serve as an alternative to the standard radioisotope or fluorometric assays used in clinical laboratories currently.

Properties of different plasma samples as substrates for LPL

The ITC assay was also shown to be a valuable tool to study properties of different plasma samples as substrates for LPL. In other words, with ITC it was possible to evaluate how efficiently LPL hydrolyzes lipids in different plasma samples. This was done by monitoring the kinetics of complete hydrolysis of available lipids for LPL in a plasma sample. Two random plasma samples, which differed in their initial triglyceride concentrations, were compared and it was shown that the efficiencies of lipid hydrolysis by LPL were different in these plasmas. Future studies should investigate the compositional variances between different plasma samples to find out the cause of the differences in the efficiency of lipolysis.

The information about plasma properties obtained by ITC analysis could also be useful in clinical laboratories to examine if hypertriglyceridemia in patients is caused by unfavorable plasma composition for LPL.

Applications of the ITC-based approach in drug development

Since ITC makes it possible to study how LPL activity is influenced under various conditions directly in human plasma, it could serve as an additional tool in drug development with the aim of affecting the LPL system. The ITC assay could help determine how pharmaceuticals that directly or indirectly affect LPL activity by acting on its regulators (antibodies against LPL inhibitors, mimetic peptides activating LPL etc.) influence LPL activity in undiluted human plasma. Furthermore, ITC could help investigate how drugs affect LPL activity in plasma samples obtained from different patients with hypertriglyceridemia. These results could help to develop a plasma profile in which the effect of a certain drug is most effective.

4.3 Activation of lipoprotein lipase by a new triglyceride-lowering drug candidate in human plasma (Publication III)

In this study, the ITC-based approach was applied on a new potential triglyceride-lowering drug candidate apoC-II mimetic peptide called 18A-CII-a (Figure 13). The triglyceride-lowering effect of the peptide was previously shown in different animal models for hypertriglyceridemia [158,171–173]. It was not known, however, how the peptide works in undiluted human plasma. As previous studies had shown that the peptide activates LPL similarly to full-length apoC-II [158,171], we also studied the effect of apoC-II on LPL activity in human plasma.

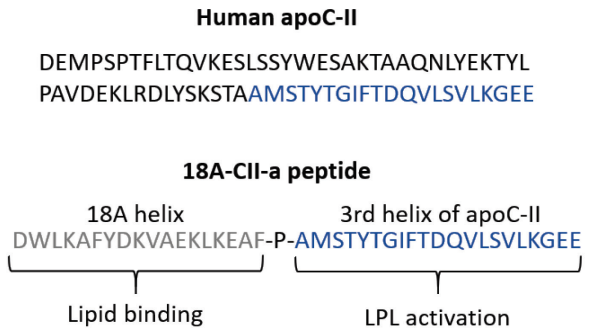

Figure 13. *Sequences of human apoC-II and apoC-II mimetic peptide (18A-CII-a). The 18A-CII-a peptide contains 18-residue long amphipathic α-helix called 18A in its N-terminus, which binds to lipoproteins with a high affinity. 18A is linked via proline residue to another amphipathic α-helix containing residues 59-79 of human apoC-II that are needed for LPL activation. The peptide is described in more detail in the publication by Amar et al [158].*

Comparison of 18A-CII-a peptide and full-length apoC-II

Our main finding was that the peptide efficiently enhanced LPL activity in nearly undiluted human plasma samples obtained from both normolipidemic and hypertriglyceridemic individuals. The effect of the 18A-CII-a on LPL activity varied between plasma samples from different individuals but tended to be larger in plasma samples obtained from normolipidemic or moderately hypertriglyceridemic subjects. The effect of 18A-CII-a on LPL activity was different from that of full-length apoC-II. We found that the 18A-CII-a peptide was 3.5-fold more efficient than full-length apoC-II in stimulating LPL activity in normolipidemic human plasma that was only diluted 1.2-fold (Figure 14). Unlike apoC-II, high concentrations of 18A-CII-a did not inhibit LPL activity. These results were different from those of the previous study, where the peptide activated LPL similarly to full-length apoC-II when synthetic emulsion of triglycerides or 50-250-fold diluted plasma samples were used as a substrate [158]. This disparity can be explained by an earlier observation, where it was shown that the activation factor of apoC-II for LPL is greater at lower triglyceride concentrations [186]. This could be the reason why the differences in the effects of apoC-II and 18A-CII-a on LPL activity were not seen in previous assays using lower triglyceride concentrations. The ITC assay makes possible measurements in nearly undiluted human plasma, and thus such differences can be picked up.

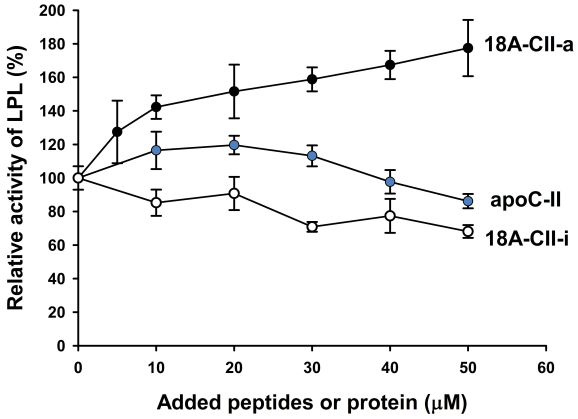

Figure 14. *Effects of apoC-II mimetic peptide (18A-CII-a), its inactive analog (18A-CII-i) and apoC-II protein on LPL activity in nearly undiluted normolipidemic human plasma sample as measured by ITC. While 18A-CII-a increased LPL activity in a concentration-dependent manner, its inactive analog 18A-CII-i decreased the activity. ApoC-II protein increased LPL activity when its concentration in plasma was increased by 20 μM. Higher concentrations of apoC-II inhibited LPL activity.*

Both 18A-CII-a and apoC-II enhanced LPL activity in plasma by increasing the amount of available substrate for LPL. In other words, some substrate molecules in lipoproteins were not available for LPL without the addition of 18A-CII-a or extra apoC-II and became available only after addition of the peptide or extra apoC-II. It is likely that some of these unavailable lipoproteins simply did not contain enough apoC-II for full LPL activity. An analysis of the reaction rate dependency on substrate concentration revealed that the "new available substrate" was hydrolyzed less efficiently than the substrate molecules that were available for LPL before the addition of the peptide or extra apoC-II.

Effect of 18A-CII-a peptide on the lipolysis of different lipoproteins

Experiments conducted with lipoproteins isolated by SEC or gradient density ultracentrifugation revealed that the relative LPL activation effect by the 18A-CII-a peptide was larger in smaller size lipoproteins, such as remnant lipoproteins, LDLs and HDLs. The relative effect of the peptide on LPL activity in isolated TRLs was small. This was different from studies performed with mouse models with hypertriglyceridemia, where the peptide helped to lower the VLDL-triglyceride concentration [158,171]. However, our unpublished data indicate that the relative effect of the peptide on TRL hydrolysis is enhanced when TRLs are present in a plasma environment compared to isolated TRLs. It is possible that the peptide assisted reduction of VLDL-triglycerides does not only involve enhanced LPL activity against VLDLs in a plasma environment. It may be that a significant fraction of triglyceride hydrolysis actually occurs on smaller size lipoproteins. In plasma, triglycerides rapidly equilibrate between lipoproteins by CETP. After triglycerides in smaller size lipoproteins are hydrolyzed by LPL, CETP may transfer more triglycerides from TRLs to other lipoproteins to enhance the overall lipolysis. However, it is important to note that the hypertriglyceridemic mouse models that were used in the previous studies lacked apoE [158] or contained a defective form of apoC-II

that did not bind to TRLs in plasma [171]. Therefore, TRLs in these mouse models were modified and less crowded by apolipoproteins. It is known that exchangeable apolipoproteins compete for surface on lipoproteins. Thus, a lack of apoE or apoC-II can influence the effect of different apolipoproteins, or their mimetics, on the lipolysis of TRLs. Further investigations are needed to clarify the mechanism of how apoC-II mimetic peptides help to hydrolyze TRLs *in vivo*.

The effect of the 18A-CII-a on LPL activity was the highest in remnant lipoproteins and LDLs. Elevated triglyceride concentrations in remnants and in LDLs are associated with increased cardiovascular mortality [187]. Moreover, a recent study suggested that TRL remnants, enriched in apoC-III and apoE, are particularly atherogenic [188]. Future studies should investigate the properties of remnant lipoproteins and LDLs after treatment with the peptide and LPL. It would be interesting to study the changes in protein content in these particles and binding to liver receptors before and after treatment with the peptide and LPL. Eventually, it is important to examine whether the peptide also lowers remnant and LDL-triglyceride content *in vivo* and if this reduction reduces ASCVD risk.

Unexpectedly, 18A-CII-a also increased LPL activity in an isolated HDL fraction. An earlier study found that while LPL has some activity against isolated HDLs, LPL activity against HDLs is minimal when plasma components are present [189]. Whether 18A-CII-a also has an effect on the hydrolysis of HDL lipids *in vivo* and, if so, whether such HDLs are less efficient in their function in reverse cholesterol transport remains to be determined. Interestingly, a recent study suggested that an elevation in triglyceride levels in HDLs is a biomarker of ASCVD risk [190]. Thus, the degradation of HDL-triglycerides may actually be beneficial.

When interpreting the effect of 18A-CII-a on isolated lipoproteins, several aspects should be considered. Firstly, we studied the effects of the peptide and apoC-II on isolated lipoproteins. Future studies should also study the effects in a plasma environment. Secondly, while the relative effects of the peptide and apoC-II on LPL activity were greater in remnant lipoproteins, LDLs and HDLs, much more triglycerides are present in CMs and VLDLs. Therefore, the small activation effect of the peptide seen in TRL lipolysis may have a larger impact than the effect on triglyceride-poor lipoproteins, on the total reduction of triglycerides *in vivo*. Thirdly, as discussed above, LPL can also hydrolyze phospholipids besides triglycerides. This was not studied in the current work, but future studies should investigate the effect of apoC-II mimetic peptides on LPL substrate specificity. However, it has been demonstrated that LPL activity against chylomicron-phospholipids was 20-fold lower than for chylomicron-triglycerides [50]. Additionally, it was shown that LPL-catalyzed phospholipid hydrolysis is less dependent on apoC-II than triglyceride hydrolysis [191]. Nevertheless, it is still possible that some substrate lipids which became available for LPL after the addition of 18A-CII-a peptide were phospholipids.

Overall, while the relative effects of 18A-CII-a and apoC-II on LPL activity were greater in smaller lipoproteins, such as remnant lipoproteins, LDLs and HDLs, 18A-CII-a also slightly enhanced LPL activity in isolated TRLs. Thus, it is likely that a fraction of the lipids in all lipoprotein classes was not available for LPL and became available only after the addition of 18A-CII-a. Therefore, our data suggest that a 18A-CII-a peptide not only helps to hydrolyze more TRLs, as seen in animal models of hypertriglyceridemia, but also makes otherwise poorly hydrolyzable remnant lipoproteins, LDLs and HDLs available for LPL.

Is there enough apoC-II in human plasma for full activation of LPL?

The finding that the addition of apoC-II or 18A-CII-a to human plasma increased LPL activity was somewhat unexpected. Several observations suggest that plasma contains enough apoC-II for full LPL activity. Firstly, heterozygotes for apoC-II deficiency typically have normal plasma triglyceride concentrations [105]. Secondly, apoC-II plasma levels are elevated in subjects with hypertriglyceridemia [106] and have been shown to be correlated to triglyceride concentration [192]. Thirdly, studies with synthetic triglyceride emulsions and isolated lipoproteins have revealed that 10-50-fold lower apoC-II concentrations than the mean human plasma apoC-II concentration (4.5 μM) are sufficient for the maximal activation of LPL (Publication II) and [156]. Finally, mice that overexpress human apoC-II have hypertriglyceridemia [109]. Our results show, however, that in some cases LPL is not saturated by apoC-II in human plasma. The addition of 10-30 μM apoC-II to normolipidemic human plasma, which initially contained 7 μM apoC-II, increased the activity of LPL. The maximum increase was 20%. In this plasma sample, LPL activity was the highest when the apoC-II concentration was 17-27 μM. These results are in line with a study by Li et al., who demonstrated that the depletion of long non-coding RNA, called lncLSTR, increased plasma apoC-II levels and LPL activity in mice, which resulted in a decreased plasma triglyceride concentration [193]. Additionally, Lee et al. showed that the injection of recombinant apoC-II into mice slightly lowered plasma triglyceride levels [194]. Furthermore, a recent study found that endogenous apoC-II levels are insufficient in mice to rapidly clear triglycerides after the intravenous injection of triglyceride-rich emulsion [173]. The injection of apoC-II mimetic peptide (18A-CII-a) into these mice helped to overcome the insufficiency of apoC-II and the triglyceride-rich emulsion was rapidly cleared. Overall, these findings suggest that, in some states, apoC-II may be rate limiting (discussed also in ref [13]) and some increase in apoC-II levels may have a beneficial effect on LPL activity and lower triglyceride concentration *in vivo*.

At the same time, it has long been known that high concentrations of apoC-II inhibit LPL activity [156]. Using synthetic emulsions of triglycerides as a substrate for LPL, it has been demonstrated that 100 nM apoC-II fully activates LPL (Publication II of this thesis) and concentrations in the range of 2-15 μM decrease LPL activity almost to a minimum level [156,195]. Much higher apoC-II concentrations (>40 μM) were needed in our experiments with undiluted human plasma to inhibit LPL activity. This demonstrates that the effect of ligands on LPL activity can be different depending on the substrate system used. Our measurements conducted in nearly undiluted human plasma suggest that high levels of apoC-II reported in mice (apoC-II concentration was 30 μM) [109] and humans (apoC-II concentration was 17 μM) [108] probably do not cause hypertriglyceridemia by directly inhibiting the interaction between LPL and lipoproteins and hence the rate of lipolysis. For this to happen, much higher concentrations of apoC-II than reported are probably needed in a plasma environment. Our results support the previously proposed model, according to which high levels of apoC-II on lipoproteins may block the movement of lipoproteins close to the endothelial cell surface where LPL is complexed with GPIHBP1 and thereby inhibit lipolysis [109]. The latter mechanism is based on the observation that apoC-II enriched lipoproteins have decreased binding to heparin, and cell surfaces are covered by HSPGs [109].

Future perspectives of apoC-II mimetic peptides

Our results support the future development of apoC-II mimetic peptides as a new therapeutic approach for treatment of hypertriglyceridemia. Further investigations are needed to clarify how apoC-II mimetic peptides help to hydrolyze lipoproteins in different subclasses *in vivo* and if these changes lower the risk of ASCVD.

We have shown that ITC is a valuable tool to study the effects of mimetic peptides on LPL activity in nearly undiluted human plasma, meaning that the effects can be studied close to the physiological conditions of LPL. In future studies, ITC could be used to study the effects of apoC-II mimetic peptides in plasma samples from different individuals with hypertriglyceridemia to develop a plasma profile where the effects of apoC-II mimetic peptides are the highest.

CONCLUSIONS

- The two domains of GPIHBP1, the N-terminal and the Ly6 domain, interact with LPL as two distinct binding sites. Although LPL binds to both domains with similar nanomolar affinity, the kinetics of the interactions are different.

- Heparin dissociates LPL from the N-terminal domain of GPIHBP1 but not from the Ly6 domain. Only LPL that is bound to the N-terminal domain of GPIHBP1 is able to interact with lipoproteins. These observations suggest that LPL is bound to the N-terminal domain of GPIHBP1 at the vascular endothelium.

- An isothermal titration calorimetry (ITC) assay of LPL overcomes the limitations of previously used techniques and makes it possible to investigate LPL activity, stability and ligand interactions in real time in undiluted human plasma. In a plasma environment, concentrations and proportions of lipoproteins and LPL regulators, as well as other components that collectively influence LPL activity, remain physiological.

- The ITC-based approach revealed that the apoC-II mimetic peptide is a potent activator of LPL in human plasma. Therefore, our results support the contention that the apoC-II mimetic peptide could be a new potential triglyceride-lowering drug candidate.

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ABSTRACT

New Aspects in Stabilization and Activation Mechanisms of Lipoprotein Lipase

Epidemiological and genetic studies have now established that elevated triglyceride concentrations in human blood plasma – clinically called hypertriglyceridemia – are an independent and causal risk factor of cardiovascular disease, a leading cause of death globally. Because lipoprotein lipase (LPL) is the key enzyme in the plasma triglyceride metabolism, whose activity determines plasma triglyceride concentration, the modulation of LPL activity has recently received great attention from pharmaceutical companies striving to reduce the risk of atherosclerotic cardiovascular disease in patients with hypertriglyceridemia. LPL activity is regulated by numerous extracellular proteins, which transport, stabilize, activate or inhibit the enzyme, depending on the physiological state of the body. While this provides many therapeutic targets to affect LPL activity, the complex regulation mechanism also needs to be thoroughly studied and understood to avoid unexpected negative side effects of different therapies.

After LPL is secreted from adipocytes or myocytes, it first binds to cell surface heparan-sulfate proteoglycans (HSPG). Next, HSPG-bound LPL is attracted by glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1), which transports the enzyme across the capillary endothelial cells to the luminal surface of the endothelium, where GPIHBP1-bound LPL catalyzes the hydrolysis of circulating plasma triglycerides within triglyceride-rich lipoproteins. In plasma, LPL activity is controlled by two groups of proteins: angiopoietin-like proteins (ANGPTLs), which inhibit LPL activity, and various apolipoproteins, which are components of the substrate lipoproteins and act as LPL activators or inhibitors. Apolipoprotein (apo) C-II is an essential activator of LPL, whose deficiency results in undetectable LPL activity and massive hypertriglyceridemia. ApoA-V also stimulates LPL-mediated lipolysis. In contrast, apoC-I and apoC-III reduce the activity of LPL.

The current thesis is divided into three parts. In the first part, the interaction between LPL and its transporter GPIHBP1 was investigated using various biophysical techniques. More specifically, the roles of GPIHBP1's two domains – the N-terminal and the Ly6 domain – were studied. It was demonstrated that the two domains act as two independent functional units, which have similar high affinities for LPL, but the dynamics of the interactions are different. While the interaction between LPL and the N-terminal domain of GPIHBP1 was characterized by high association and high dissociation rate constants, the complex between LPL and the Ly6 domain of GPIHBP1 formed more slowly but also stayed together for a longer time. These results were in line with a previous speculation according to what the N-terminal domain is responsible for attracting HSPG-bound LPL from long-distances in the subendothelial space. This model was also supported by cross-linking experiments, which showed that the N-terminal domain binds to the region in LPL which is known to form a binding site for HSPGs. Interestingly, it was found that LPL was able to interact with lipoproteins only when the enzyme was bound to the N-terminal domain of GPIHBP1. LPL did not interact with lipoproteins while bound to the Ly6 domain. Moreover, heparin displacement experiments demonstrated that only GPIHBP1's N-terminal domain-bound LPL was eluted by heparin. In the case of Ly6 domain-bound LPL, heparin was unable to dissociate LPL from this binding site. Because the intravenous injection of heparin is known to dissociate LPL from its *in vivo* endothelial

cell binding sites into blood circulation and because it was recently demonstrated that LPL is mainly bound to GPIHBP1 at the vascular endothelium, our results suggest that *in vivo* functional LPL is bound to the N-terminal domain of GPIHBP1 at the vascular endothelium.

In the second part of the thesis, an isothermal titration calorimetry (ITC)-based approach was developed, which enabled us to measure exogenously added LPL activity in undiluted human plasma. Because LPL is attached to the vascular endothelium, human plasma does not contain circulating active LPL. Therefore, purified bovine LPL or human post-heparin plasma LPL was used as a source of the enzyme. The assay is based on determination of the heat rate accompanying the LPL hydrolysis of lipids. The change in heat rate was shown to be proportional to the LPL concentration and to the released fatty acids. The novel assay allowed us to study the effects of different endogenous and exogenous regulators on LPL activity and stability near physiological conditions. Several known LPL activators and inhibitors, including apoA-V, apoC-III, ANGPTL3 and ANGPTL4, were studied in human plasma. It was shown that apoC-III inhibits the initial rate of hydrolysis by LPL in plasma, but the effect was much lower than reported in previous studies conducted with isolated lipoproteins. ANGPTL3 and ANGPTL4 were shown to reduce LPL activity in plasma via a two-step mechanism. ApoA-V did not increase LPL activity. The ITC assay was also shown to be a valuable tool to evaluate properties of plasma samples obtained from different donors as substrates for LPL. This could be used in clinical laboratories to examine if hypertriglyceridemia in patients is caused by unfavorable plasma composition for LPL.

Finally, the ITC-based approach was used to study the efficiency of a new potential triglyceride-lowering drug candidate – apoC-II mimetic peptide – in human plasma. It was demonstrated that unlike apoC-II protein, the peptide efficiently increased LPL activity in both normolipidemic and hypertriglyceridemic plasma samples. Although the peptide increased lipolysis in all lipoprotein classes, the relative effect was larger in smaller-size lipoproteins, such as remnant lipoproteins, low-density lipoproteins and high-density lipoproteins. Additionally, while previous studies have suggested that there is enough apoC-II in human plasma for maximal activity of LPL, it was shown that, in some cases, LPL is not saturated by apoC-II and the cofactor concentration can be rate limiting. In summary, our results support the future development of apoC-II mimetic peptides as a new therapeutic approach for the treatment of hypertriglyceridemia. Additionally, the ITC assay can be used to study other drug candidates developed to affect the LPL system in human plasma.

KOKKUVÕTE

Uudsed aspektid lipoproteiinlipaasi stabiilsuse ja aktiivsuse regulatsioonis

Epidemioloogilised ja geneetilised uuringud on näidanud, et kõrgenenud triglütseriidide tase inimeste vereplasmas – hüpertriglütserideemia – on sõltumatu ja põhjuslik südame-veresoonkonna haiguste riski faktor. Viimane on peamine surmapõhjustaja üle kogu maailma. Vereplasma triglütseriidide metabolismi võtmeensüüm on lipoproteiinlipaas (LPL), mille aktiivsus määrab vere triglütseriidide kontsentratiooni. Seetõttu on viimasel ajal mitmed ravimifirmad asunud välja töötama ravimikandidaate eesmärgiga tõsta LPL-i aktiivsust, et vähendada südame-veresoonkonna haiguste riski patsientidel, kellel on hüpertriglütserideemia.

LPL-i aktiivsus on veres reguleeritud mitmete valkude poolt, mis osalevad ensüümi transportimisel, stabiliseerimisel, aktiveerimisel ja inhibeerimisel, et tagada energiarikaste triglütseriidide liikumine õigetesse kudedesse vastavalt inimkeha füsioloogilistele vajadustele. Ühest küljest pakub suur hulk LPL-i regulaatorvalke mitmeid terapeutilisi sihtmärke, mille kaudu on võimalik ensüümi aktiivust mõjutada. Teisest küljest on vajalik põhjalikult uurida kõiki aspekte sellises komplekses regulatsioonivõrgustikus, et vältida erinevate ravivõimaluste võimalikke kõrvalmõjusid.

LPL sünteesitakse rasva-, skeletilihas- ja südamelihasrakkudes. Pärast ensüümi sekreteerimist nendest rakkudest interakteerub LPL rakupinnal asetsevate heparaansulfaat proteoglükaanidega (HSPG). Seejärel seostub ensüüm glükosüülfosfatidüülinositool-ankurdatud kõrge tihedusega lipoproteiine siduva valk 1-ga (GPIHBP1), mis transpordib LPL-i läbi endoteelirakkude vaskulaarsele endoteeli kihile. Seal jääb LPL seotuks GPIHBP1-ga ning katalüüsib triglütseriidide hüdrolüüsi, mis on veres ringlevate triglütseriidide-rikaste lipoproteiinide komponendiks. Vereplasmas on LPL-i aktiivsus reguleeritud angiopoietiini-sarnaste valkude (ANGPTL) poolt, mis on ensüümi inhibiitorid, ning apolipoproteiinide poolt, mis olles substraat-lipoproteiinide komponendid, käituvad nii LPL-i aktivaatoritena kui ka inhibiitoritena. Apolipoproteiin (apo) C-II on hädavajalik LPL-i aktivaator, mille puudumisel LPL ei ole aktiivne. Lisaks stimuleerib lipolüüsi apoA-V. Seevastu apoC-I ja apoC-III inhibeerivad LPL-i aktiivsust.

Antud doktoritöö koosneb kolmest osast. Esimeses osas uuriti LPL-i ja ensüümi transportimisel kriitilist tähtsust omava valgu GPIHBP1 vahelist interaktsiooni, kasutades erinevadi biofüüsikalisi meetodeid. Täpsemalt uuriti GPIHBP1 N-terminaalse ja Ly6 domeeni funktsioone. Töös näidati, et domeenid käituvad kui kaks eraldiseisvat üksust, millel on küll sarnane kõrge afiinsus LPL-i suhtes, kuid interaktsioonide dünaamikad on erinevad. LPL seostus N-terminaalse domeeniga kiirelt, kuid samas ka dissotsieerus kiiresti. Seevastu LPL-i ja Ly6 domeeni vaheline kompleks moodustus aeglasemalt, kuid püsis kauem koos. Need tulemused on kooskõlas varasema spekulatsiooniga, mille kohaselt võiks N-terminaalsel domeenil olla oluline roll LPL-i sidumisel HSPG-de küljest rakkudevahelises ruumis. Seda mudelit toetasid ka keemilise ristsidumise katsed, mille põhjal leiti, et N-terminaalne domeen seostub LPL-il lähedasse piirkonda, mis on oluline HSPG-de sidumisel. Lisaks näidati, et LPL on võimeline interakteeruma lipoproteiinidega, olles seotud GPIHBP1 N-terminaalse domeeniga, kuid GPIHBP1 Ly6 domeeniga seotud LPL lipoproteiinidega ei interakteerunud. Veelgi enam, hepariini toimel dissotsieerus ainult N-terminaalse domeeni külge seotud LPL ning Ly6 domeeni külge seotud LPL ei dissotsieerunud. Kuna on teada, et veenisisene hepariini süst vabastab

endoteelirakkudele seotud aktiivse LPL-i vereringesse ning ühes hiljutises töös näidati, et endoteelirakkudel on LPL peamiselt seotud GPIHBP1 külge, siis antud töö tulemused viitavad, et *in vivo* on funktsionaalne LPL endoteelirakkudele seotud GPIHBP1 N-terminaalse domeeni kaudu.

Doktoritöö teises osas töötati biofüüsikalise meetodi isotermilise tiitrimis-kalorimeetria baasil välja uudne lähenemine LPL-i aktiivsuse määramiseks füsioloogilistele lähedastel tingimustel ehk lahjendamata inimese vereplasmas. *In vivo* on LPL seotud vaskulaarsele endoteelile ja veres aktiivset LPL ei ringle. Seetõttu kasutati ensüümi allikana lehma piimast puhastatud LPL-i või inimese plasmat, mis oli kogutud pärast hepariini manustamist (post-hepariin plasma). Antud kalorimeetriline meetod põhineb LPL-i poolt katalüüsitud lipiidide hüdrolüüsil vabaneva soojusvoo detekteerimisel. Töös näidati, et soojusefekt korreleerub nii LPL-i kontsentratsiooniga kui ka lipolüüsi tagajärjel triglütseriididest vabanevate rasvhapete kontsentratsiooniga. Antud uudne lähenemine võimaldas uurida erinevate füsioloogiliste LPL-i regulaatorite mõju ensüümi aktiivsusele ja stabiilsusele inimese vereplasma keskkonnas. Töös uuriti mitme olulise LPL-i aktivaatori ja inhibiitori toimemehhanismi. Näiteks näidati, et apoC-III vähendas LPL-i katalüüsitud lipolüüsi algkiirust, kuid efekt oli oluliselt väiksem, kui varasemalt teistes töödes on demonstreeritud, kasutades substraadina isoleeritud lipoproteiine. Lisaks leiti, et ANGPTL3 ja ANGPTL4 inaktiveerivad LPL-i kahe-etapilise mehhanismi kaudu. Olulise aspektina näidati, et antud meetodiga on võimalik võrrelda, kui hästi erinevate inimeste vereplasma triglütseriide hüdrolüüsitakse ehk meetod võimaldas uurida plasmade lipolüütilisi omadusi. Antud lähenemist võiks tulevikus kasutada näiteks haiglates, et uurida, kas hüpertriglütserideemia on põhjustatud plasma halbade omaduste tõttu või on põhjuseks madal hepariiniga vabastatav LPL-i hulk.

Viimases doktoritöö osas kasutati väljatöötatud kalorimeetrilist meetodit, et uurida uudse triglütseriide alandava ravimikandidaadi omadusi inimese vereplasmas. Antud ravimikandidaat, apoC-II analoogpeptiid, osutus oluliselt efektiivsemaks LPL-i aktivaatoriks kui apoC-II valk. Peptiid tõstis LPL-i aktiivsust nii normaalse triglütseriidide tasemega kui ka kõrgenenud triglütseriidide tasemega plasmaproovides. Lisaks leiti vastupidiselt varasematele tulemustele, et inimese vereplasmas ei pruugi alati olla piisavalt apoC-II valku ning apoC-II kontsentratsioon võib olla seega teatud juhtudel lipolüüsi kiirust limiteerivaks. Kokkuvõttes toetasid antud töö tulemused edasist apoC-II analoogpeptiidide arendamist triglütseriide alandavaks ravimiks. Lisaks näidati, et kalorimeetriline meetod sobib hästi ravimikandidaatide testimiseks inimese vereplasmas ning seda võiks tulevikus kasutada ka teiste LPL-i aktiivsust mõjutavate ravimite uurimiseks.

PUBLICATION I

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Evidence for two distinct binding sites for lipoprotein lipase on glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1 (GPIHBP1)

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Evidence for Two Distinct Binding Sites for Lipoprotein Lipase on Glycosylphosphatidylinositol-anchored High Density Lipoprotein-binding Protein 1 (GPIHBP1)*

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Background: GPIHBP1 is crucial for the transport and localization of lipoprotein lipase (LPL) at the vascular endothelium.
Results: The N-terminal and Ly6 domains of GPIHBP1 interact with LPL independently and with different kinetics.
Conclusion: The domains of GPIHBP1 act as two independent binding sites for LPL.
Significance: The partition of LPL between the binding sites on GPIHBP1 may influence its function in lipoprotein metabolism.

GPIHBP1 is an endothelial membrane protein that transports lipoprotein lipase (LPL) from the subendothelial space to the luminal side of the capillary endothelium. Here, we provide evidence that two regions of GPIHBP1, the acidic N-terminal domain and the central Ly6 domain, interact with LPL as two distinct binding sites. This conclusion is based on comparative binding studies performed with a peptide corresponding to the N-terminal domain of GPIHBP1, the Ly6 domain of GPIHBP1, wild type GPIHBP1, and the Ly6 domain mutant GPIHBP1 Q114P. Although LPL and the N-terminal domain formed a tight but short lived complex, characterized by fast on- and off-rates, the complex between LPL and the Ly6 domain formed more slowly and persisted for a longer time. Unlike the interaction of LPL with the Ly6 domain, the interaction of LPL with the N-terminal domain was significantly weakened by salt. The Q114P mutant bound LPL similarly to the N-terminal domain of GPIHBP1. Heparin dissociated LPL from the N-terminal domain, and partially from wild type GPIHBP1, but was unable to elute the enzyme from the Ly6 domain. When LPL was in complex with the acidic peptide corresponding to the N-terminal domain of GPIHBP1, the enzyme retained its affinity for the Ly6 domain. Furthermore, LPL that was bound to the N-terminal domain interacted with lipoproteins, whereas LPL bound to the Ly6 domain did not. In summary, our data suggest that the two domains of GPIHBP1 interact independently with LPL and that the functionality of LPL depends on its localization on GPIHBP1.

Hydrolysis of triglyceride-rich lipoproteins at the vascular surface of endothelial cells, a process catalyzed by lipoprotein lipase (LPL),[2] is a central step in the turnover of triglycerides in plasma lipoproteins (1, 2). LPL is not synthesized by endothelial cells but mainly by parenchymal cells in adipose and muscle tissue. Glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1 (GPIHBP1), a membrane protein of endothelial cells, is crucial for the transport and localization of LPL at the vascular side of the endothelial cell plasma membrane (3, 4). The interaction between LPL and GPIHBP1 is prevented by heparin (3, 5) explaining that intravenous injections of heparin cause release of LPL to blood (1). A number of mutations have been identified in the GPIHBP1 gene that dramatically reduce the amount of heparin-releasable LPL and that cause massive hypertriglyceridemia (6–12). Recently, it was shown that the LPL·GPIHBP1 complex determines binding (called margination) of triglyceride-rich particles at the vascular endothelium (13). Another important effect of GPIHBP1 is that it protects LPL from inactivation by angiopoietin-like protein 4 (14).

Based on molecular modeling, mature GPIHBP1 contains two principal domains: an acidic N-terminal domain and a central cysteine-rich Ly6 domain (15). Both domains contribute to the interaction of GPIHBP1 with LPL as evidenced by deletion, mutation, or replacement of one of the two domains that significantly reduce the ability of GPIHBP1 to associate with LPL (5, 16, 17). Beigneux *et al.* (17) systematically examined the effects of mutations in the Ly6 domain on the interaction of LPL in cell culture experiments. In this study, 12 amino acid residues, aside from the cysteines, were identified in the Ly6 domain to be essential for the interaction with LPL. Single replacements of each of these residues lead to the substantially lowered ability of GPIHBP1 to bind to LPL, indicating high structural complementarity between the interacting regions of the proteins. In addition, most of the known natural GPIHBP1 mutations that cause chylomicronemia are located in the Ly6 domain (18). The mutation Q115P in the Ly6 domain of human GPIHBP1, corresponding to Q114P in the mouse sequence, is the first discovered and well characterized example (6). Both of these mutants were unable to bind LPL in cell culture experi-

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[1] To whom correspondence should be addressed: Akadeemia tee 15, Tallinn 12618, Estonia. Tel.: 372-620-4378; E-mail: aivar.lookene@ttu.ee.
[2] The abbreviations used are: LPL, lipoprotein lipase; GPIHBP1, glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1; GPI, glycosylphosphatidylinositol; Ly6, lymphocyte antigen 6; SPR, surface plasmon resonance; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; DGGR, 1,2-*O*-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresofurin) ester; DST, disuccinimidyl tartrate; LTQ, linear ion trap.

ments (6). The binding of LPL to the N-terminal acidic domain was presumable, as LPL associates tightly with negatively charged biopolymers such as heparin, heparan sulfate, dermatan sulfate, and even polynucleotides (19, 20). The fact that heparin reduces the association of LPL with GPIHBP1 suggests that the binding sites for heparin/heparan sulfate and GPIHBP1 on LPL are partially overlapping. The GPIHBP1 binding site does not completely coincide with the heparin/heparan sulfate binding site, as the mutations C418Y and E421K in LPL do not affect heparin affinity but abolish binding to GPIHBP1 (21). A cluster of negatively charged amino acid residues in the N-terminal domain is invariably present in all known GPIHBP1 sequences, but the number of negatively charged residues varies from 17 to 22 between different mammalian species (22). The sequence variation and the high proportion of negatively charged residues of the N-terminal domain of GPIHBP1 raises questions about the role of the N-terminal domain for interaction with LPL. It has been speculated that this domain may function as a magnet to attract LPL at a long distance, whereas the more specific interaction may occur between LPL and the Ly6 domain (5, 18).

Several aspects of the LPL/GPIHBP1 interaction remain unclear. For example, what is the contribution of the two domains of GPIHPB1 to the interaction? Do the domains of GPIHBP1 act as two independent binding sites or as two regions of one binding site? Is the N-terminal domain just needed for tethering LPL to the more specific interaction with the Ly6 domain or does the N-terminal domain play a more specific role on its own? Can kinetic studies performed *in vitro* explain why LPL preferentially binds to GPIHBP1 and not to other polyanions like heparan sulfate? To address these questions, we have investigated the mechanism of the interaction of LPL with GPIHBP1 using surface plasmon resonance (SPR), fluorescence anisotropy, enzyme activity measurements, and a combination of chemical cross-linking with mass spectrometry. The experiments were performed with mouse GPIHBP1, the Ly6 domain mutant GPIHBP1 Q114P (mouse sequence) (6), the Ly6 domain (GPIHBP1 lacking the N-terminal domain), synthetic peptides corresponding to the N-terminal domain of GPIHBP1 from mouse, human, or bovine sequence (N-terminal peptide), and with bovine LPL.

Experimental Procedures

Reagents—Bovine LPL was purified from milk (23) and stored at −80 °C as a stock solution of 0.5–1 mg/ml in 1.5 M NaCl, 10 mM BisTris buffer, pH 6.5. Synthetic peptides corresponding to the N-terminal sequence of human, mouse, or bovine GPIHBP1 or a composition of human GPIHBP1 sequences were bought from GeneCust (Luxembourg) or Storkbio (Estonia). To allow specific modification by biotinylation or by DyLight 488 Sulfhydryl-Reactive dye, an extra cysteine residue was added to the C-terminal end of the peptides. The sequences of the synthetic peptides were as follows: QQEEEEEDEDHGPDDYDEEDEDEVEEEETC (human N-terminal peptide, human GPIHBP1 residues 23–51), AQEDGDADPEPENYNYDDDDDEEEEEETC (mouse N-terminal peptide, mouse GPIHBP1 residues 22–49), AQEDEDDDPDAGREGYDDEDEEEEEA (bovine N-terminal peptide, bovine GPIHBP1 residues 22–47), HGPDDYDEEDEDEVEEEETC (peptide 1, human GPIHBP1 residues 33–51), EEDEDEVEEEETC (peptide 2, human GPIHBP1 residues 40–51), EVEEEETC (peptide 3, human GPIHBP1 residues 45–51) and VDQDYEDDHETEDDEEQDPEEEEGEEEEEC (peptide 4, a random sequence of human GPIHBP1 residues 23–51). The sequence of peptide 4 was generated using Microsoft Excel's functions RAND and RANK. The synthetic peptides were biotinylated at their C-terminal cysteines in 20 mM phosphate buffer, pH 7.5, with 0.8 mM peptide, and 0.8 mM biotin maleimide (Sigma). The reaction mixtures were incubated at room temperature for 30 min. Unreacted biotin was removed by dialysis. Heparan sulfate was biotinylated at amino groups as previously described (24). Labeling of human N-terminal peptide by DyLight 488 Sulfhydryl-Reactive dye (Pierce) was performed as follows: a 0.1 mM solution of the peptide in 20 mM phosphate, pH 7.2, was incubated with a 7-fold molar excess of DyLight 488 Sulfhydryl-Reactive dye (8.8 mM stock in *N*,*N*-dimethylformamide) for 4 h at room temperature in the dark. Unreacted dye was removed with excessive washing on Amicon Ultra 3 MWCO Centrifugal Filter Units (Millipore). Extinction coefficients at 280 nm for determination of peptide/protein concentrations were as follows: LPL, 70440 M^{-1} cm^{-1}; human N-terminal peptide, bovine N-terminal peptide, peptide 1 and peptide 4, 1480 M^{-1} cm^{-1}; mouse N-terminal peptide, 2960 M^{-1} cm^{-1}. The extinction coefficients were calculated according to Gill and von Hippel (25). Concentrations of LPL were calculated using its monomer molecular mass of 55 kDa.

A rat monoclonal antibody, 11A12, against mouse GPIHBP1 (16) was a generous gift from Dr. Stephen G. Young (University of California, Los Angeles). The epitope of this antibody is located at the C-terminal part of GPIHBP1 (16). VLDL and LDL isolated from human plasma by ultracentrifugation (26) was a kind gift from Dr. Elena Makoveichuk (Umea University). Heparin was obtained from LEO Pharma (Denmark). 1,2-*O*-Dilauryl-rac-glycero-3-glutaric acid-(6′-methylresofurin) ester (DGGR, 30058 lipase substrate) was purchased from Sigma.

Expression of GPIHBP1, the Mutant Q114P, and the Cleavable Form of GPIHBP1—pTriEx4 plasmid, containing either sequence of mouse GPIHBP1 or the Q114P mutant without the GPI-anchor (residues 1–198) (16), was a kind gift from Dr. Anne Beigneux (University of California, Los Angeles). A thrombin recognition site was generated in the linker region of GPIHBP1 (amino acids 49–55 in the mouse sequence) using the QuikChange Lightning Site-directed Mutagenesis Kit (Agilent Technologies) according to the protocol from the manufacturer using the forward primer, GATGAAGAGGAAGAGGAGGAGACCTTAGTGCCCCGTGGAAGCAGGGACAGAGCACCTCT and reverse primer, AGAGGTGCTCTGTCCCTGCTTCCACGGGGCACTAAGGTCTCCTCCTCTTCCTCTTCATC. The mutation was verified by DNA sequencing using BigDye 3.1 with TriEx DOWN (TCGATCTCAGTGGTATTTGTG) and TriEx UP (GGTTATTGTGCTGTCTCATCA) primers. The plasmid was amplified using DH5α cells and purified using the E.Z.N.A.® Fastfilter Plasmid Midi Kit according to manufacturer's protocol. CHO-K1 cells were grown in Ham's F-12K (Kaighn's) medium, supplemented with 10% (v/v) FCS and 1:200 penicillin. Cells were transfected using

Amaxa® Cell Line Nucleofector® Kit T and a Nucleofector™ 2b Device (Lonza) according to the manufacturer's protocol optimized for CHO-K1 cells. Proteins were expressed in ProCHO-AT medium in the presence of 5% medium (F-12K, 10% (v/v) FCS, 1:200 penicillin) and 1:200 protease inhibitor mixture (Sigma). Expression media were harvested and concentrated 10 times using Amicon Ultra 10 MWCO Centrifugal Filter Units (Millipore). Protein expression was verified by Western blot using 300 ng/ml of GPIHBP1 antibody 11A12, followed by detection with a 1:20000 dilution of horseradish peroxidase-conjugated goat anti-rat antibody. To obtain the Ly6 domain, the GPIHBP1 variant incorporating the thrombin cleavage site was treated with thrombin (0.1 unit of restriction grade thrombin (Novagen) per 50 μl of concentrated expression medium) for 18 h at room temperature under shaking. The obtained protein was analyzed using Western blot under the same conditions as described above (data not shown).

SPR Measurements—SPR experiments were performed on a Biacore 3000 instrument using CM5 sensorchips (GE Healthcare). GPIHBP1, the Ly6 domain, or the mutant Q114P were bound to the surface of the sensorchip via antibody 11A12 that was covalently preimmobilized using the amino coupling kit (GE Healthcare). Biotinylated N-terminal peptide or biotinylated heparan sulfate was attached to covalently pre-bound NeutrAvidin (Sigma). The surface densities of non-covalently immobilized ligands were between 0.6 and 1.2 ng/mm^2, except for the determination of rate constants. Then the surface densities were lower, between 0.06 to 0.12 ng/mm^2, to avoid mass transport limited association and rebinding effects. Measurements were carried out at 4 °C in running buffer that contained 20 mM phosphate, pH 7.4, 2 mg/ml of BSA, and the indicated concentrations of NaCl unless otherwise stated. Under these conditions LPL was stable and its nonspecific binding to the sensorchip matrix was low. For steady-state measurements sequential injections (120 μl or 60 μl, 20 μl/min) of LPL at increasing concentrations were made over the surface. In parallel, to evaluate nonspecific binding, the same solutions were injected over a surface with only the antibody or NeutrAvidin. For data analysis, nonspecific binding was subtracted. The sensorchip surface was regenerated after each injection using 1.5 M NaCl. When the Ly6 domain was used, the regeneration was not successful and the interaction was therefore studied using single cycle experiments. The amount of proteins and peptides at the sensorchip surface was calculated using the relationship: 1 resonance unit corresponds to protein/peptide surface concentration (1 pg/mm^2) (Biacore Assay Handbook). Bound LPL per ligand (mol/mol) was calculated based on the monomer molecular masses of the proteins, 55 kDa for LPL, 25 kDa for GPIHBP1, 21 kDa for the Ly6 domain, 3.8 kDa for biotinylated mouse N-terminal peptide, and 25 kDa for the mutant Q114P.

For the interaction between GPIHBP1 and LPL at 0.4 M NaCl, data were fitted to a two-binding site model (Equation 1),

$$f = \frac{a \cdot L_0}{K_{d1} + L_0} + \frac{b \cdot L_0}{K_{d2} + L_0} \quad \text{(Eq. 1)}$$

where f is bound LPL per GPIHBP1 (mol/mol) at steady-state (equilibrium), L_0 is the concentration of LPL in the injected solution, K_{d1} is the equilibrium dissociation constant for binding site 1, "a" is the maximal binding capacity of site 1, K_{d2} is the equilibrium dissociation constant for binding site 2, "b" is the maximal binding capacity of site 2.

For the interactions of GPIHBP1 with LPL at 0.6 M NaCl, the Ly6 domain with LPL at 0.4 and 0.6 M NaCl, respectively, the mutant Q114P with LPL at 0.4 M NaCl and heparan sulfate with LPL at 0.4 M NaCl data were fitted using Equation 2.

$$f = \frac{a \cdot L_0}{K_d + L_0} \quad \text{(Eq. 2)}$$

Data for the interaction of LPL with the mouse N-terminal peptide at 0.4 M NaCl were fitted using the Hill's equation,

$$f = \frac{a \cdot [L_0]^c}{(K_d)^c + [L_0]^c} \quad \text{(Eq. 3)}$$

where c is the Hill coefficient.

For determination of the affinities of the different N-terminal peptides for LPL, SPR competition experiments were performed at 0.4 M NaCl. Biotinylated human N-terminal peptide was attached to the sensorchip via NeutrAvidin. LPL (800 nM) was mixed with different concentrations of peptides for 90 s at 4 °C to reach binding equilibrium. Then these solutions were injected over sensorchips. Binding of LPL was registered at the end of each injection near the equilibrium. The affinities of the peptides for LPL were calculated by Equation 4,

$$\Delta R = \frac{a \cdot K_d}{K_d + P_0} \quad \text{(Eq. 4)}$$

where P_0 is the concentration of peptide in the injected solution, K_d is the equilibrium dissociation constant, ΔR is the change in SPR at equilibrium, and a is the change in SPR for binding of LPL to the surface when the solution did not contain free peptide.

Determination of Kinetic Constants—Kinetic data were analyzed using standard evaluation software from Biacore (BIAevaluation 4.0.1). For the interaction between binding site 1 of GPIHBP1 and LPL, studied at 0.4 M NaCl, a simple 1:1 Langmuir interaction model was used to calculate kinetic parameters. It was not possible to get reliable fits for the interaction between binding site 2 of GPIHBP1 and LPL studied under the same conditions. For the interactions of the N-terminal peptide with LPL, heparan sulfate with LPL and mutant Q114P with LPL at 0.4 M NaCl, a bivalent analyte interaction model was used that assumes that bivalent analyte A binds to monovalent ligand B ($A + B \leftrightarrow AB$; $AB + B \leftrightarrow AB2$; where A represents LPL and B represents immobilized ligand). In this model, k_{on1} and k_{off1} are the forward and reverse rate constants for formation of a 1:1 LPL·ligand complex, and k_{on2} and k_{off2} are the forward and reverse rate constants for binding of the LPL·ligand complex to another ligand molecule on the surface. For the interaction between LPL and the Ly6 domain at 0.4 M NaCl only the dissociation rate constant (k_{off}) was determined experimentally. Data were analyzed using a monoexponential

decay equation. The corresponding association rate constant was calculated from the equation $k_{on} = k_{off}/K_d$, where K_d is the equilibrium dissociation constant obtained from SPR steady-state analysis. Similarly, dissociation rate constants for the interactions of the Ly6 domain or the N-terminal peptide with LPL at 25 °C in the presence of 0.15 M NaCl were determined using monoexponential decay equation.

Stability Measurements of LPL Activity—For this analysis LPL (10 nM) was preincubated alone or with the bovine N-terminal peptide at 25 °C in 20 mM HEPES, pH 7.4, in the presence of 0.15, 0.23, or 0.3 M NaCl. Remaining LPL activity at the indicated time points was measured using DGGR as a substrate as described previously (27). The curves for inactivation of LPL were analyzed by simple monoexponential decay kinetics. The obtained inactivation rate constants (k_i) were used for K_d determinations. At 0.15 M NaCl, the concentration of LPL was in the same order of magnitude as the N-terminal peptide. Equation 5 was used for calculation of the K_d values,

$$k_i = k_0(L_0 + P_0 + K_d - \sqrt{(L_0 + P_0 + K_d)^2 - 4L_0P_0}) + k_\infty \quad \text{(Eq. 5)}$$

where k_i is the rate constant for inactivation of LPL, k_0 is the rate constant for inactivation of LPL in the absence of peptide, L_0 is the concentration of LPL, P_0 is the concentration of the peptide, K_d is the equilibrium dissociation constant, and k_∞ is the rate constant for inactivation of the LPL·peptide complex.

At 0.23 or 0.30 M NaCl, the concentration of LPL was much lower than the stabilizing N-terminal peptide, and in these cases the K_d values could be calculated by Equation 6.

$$k_i = \frac{k_0 \cdot K_d}{K_d + P_0} + k_\infty \quad \text{(Eq. 6)}$$

Fluorescence Anisotropy Measurements—Fluorescence anisotropy experiments were performed on a Hitachi F-7000 (Hitachi High-Tech, Japan) fluorescence spectrophotometer. The excitation wavelength was 493 nm and the emission wavelength was 518 nm. Measurements were carried out at 4 °C in 20 mM phosphate, pH 7.4, 0.3 M NaCl. DyLight 488 maleimide-labeled N-terminal peptide (100 nM) was mixed with different concentrations of LPL. After binding equilibrium had been reached, within 90 s, fluorescence was measured when excitation and emission polarizer were oriented vertically and when excitation polarizer was oriented vertically and emission polarizer was oriented horizontally. From this data the fluorescence anisotropy was calculated using the Equation 7,

$$r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} \quad \text{(Eq. 7)}$$

where r is fluorescence anisotropy, $I_\parallel$ is the observed fluorescence intensity when the emission polarizer was oriented parallel to the direction of the polarized excitation, $I_\perp$ is the observed fluorescence intensity when the emission polarizer was oriented perpendicular to the direction of the polarized excitation.

To calculate the corresponding K_d for this interaction, the dependence between the change of anisotropy and LPL concentration was fitted with Hill's equation,

$$r = \frac{r_{max} \cdot [L_0]^b}{(K_d)^b + [L_0]^b} \quad \text{(Eq. 8)}$$

where r is the change of fluorescence anisotropy, r_{max} is the value of anisotropy of the complex of LPL with the N-terminal peptide, L_0 is the concentration of LPL, K_d is the equilibrium dissociation constant, and b is the Hill's coefficient.

Chemical Cross-linking Combined with Mass Spectrometry—The bovine N-terminal peptide was chemically cross-linked to LPL using the homobifunctional amino reactive cross-linker disuccinimidyl tartarate (DST) (Pierce). The reaction was carried out for 18 h at 4 °C in 20 mM HEPES buffer, pH 7.4, in the presence of 0.4 M NaCl. The concentrations of LPL and the N-terminal peptide were 7.3 and 73 μM, respectively. A freshly prepared stock solution of DST (2.3 mg/ml in dimethyl sulfoxide) was added to a final concentration of 300 μM. The reaction was stopped by adding 1 M ethanolamine-HCl, pH 8.5, to a final concentration of 50 mM. The mixture was subjected to SDS-PAGE on an 8% gel. Bands with molecular masses of ~59 and ~71 kDa that corresponded to the complexes of LPL·N-terminal peptide with stoichiometries 1:1 and 1:4, respectively, were excised. After reduction by DTT and alkylation by iodoacetic acid, the complex was in-gel digested by trypsin (28) and the extracted peptides were desalted using C18 StageTips (29). The cross-linked peptide mixture was separated on an Agilent 1200 series nano-LC with in-house packed (3-μm 100-Å ReproSil-Pur C18AQ particles, Dr. Maisch, Entringen, Germany) 15-cm 75-μm inner diameter (tip diameter 8 μm) emitter columns (New Objective, Woburn, MA) using 3–50% (90 min) separating gradient. Buffer A was 0.5% acetic acid in water and buffer B was 0.5% acetic acid in 80% acetonitrile (ACN). Separated peptides were eluted at 200 nl/min (nano-ESI spray voltage 2.0 kV) to an LTQ Orbitrap mass spectrometer (Thermo Electron, Waltham, MA) operating with a top-5 MS/MS strategy. Briefly, one high-resolution MS scan 350–2000 m/z was taken with a resolution setting of $r = 60{,}000$ at 400 m/z, and the 5 most intense precursors were subjected to collision-induced dissociation fragmentation combined with Orbitrap MS/MS detection ($r = 15{,}000$). Full scan (target value 1E6 ions) and MS/MS (3E4 ions) maximum injection times were 500 and 2,000 ms. Dynamic exclusion (list size: 500) was set to 90 s and only charge states $>+2$ were subjected to MS/MS. The cross-links were identified using pLink software (30). The search parameters were as follows: cross-linker, DST; enzyme, trypsin; maximum missed sites, 2; fixed modification, carbamidomethylation of cysteines; variable modification, oxidation of methionines, formation of pyroglutamate of an amino terminus glutamine, N-terminal protein acetylation; mass accuracy for precursor and fragment ions were 5 and 20 ppm, respectively.

Homology Modeling of Bovine LPL—A homology model of bovine LPL was obtained from the protein structure prediction service Phyre (31) using horse pancreatic lipase (32) as a template. The resulting structure was visualized using Chimera from the University of California San Francisco (33).

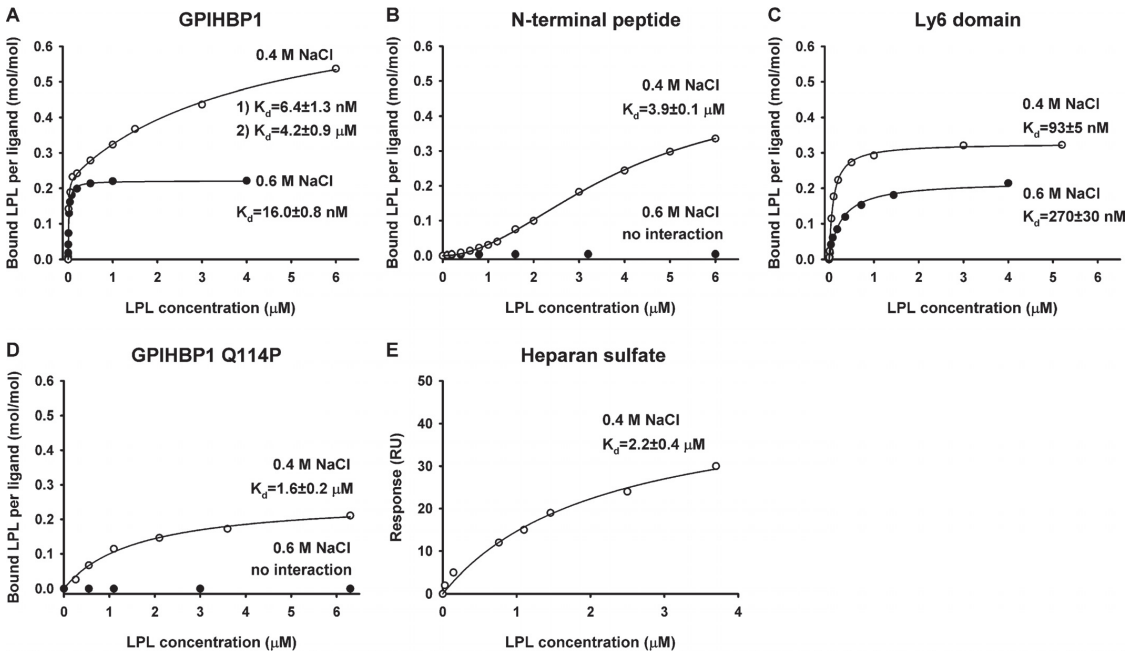

FIGURE 1. **Steady-state SPR analysis of binding of LPL to GPIHBP1 variants and heparan sulfate.** Binding of LPL to GPIHBP1 (*A*), mouse N-terminal peptide (*B*), the Ly6 domain (*C*), the mutant GPIHBP1 Q114P (*D*), and heparan sulfate (*E*) is shown. GPIHBP1, the Ly6 domain, and the mutant GPIHBP1 Q114P were bound to the surface of the sensorchip via the 11A12 monoclonal antibody. The N-terminal peptide and heparan sulfate were both biotinylated and immobilized via biotin/NeutrAvidin interaction. Solutions of LPL were injected to the flow cells with the immobilized ligands. Data in *panels A–D* are presented as the mole ratio of bound LPL (monomer) to the immobilized ligand at steady-state/equilibrium as function of the injected LPL concentration. In *panel E*, the amount of bound LPL is presented in response units, because the amount of immobilized heparan sulfate was known only in response units. Measurements were performed at 4 °C in 20 mM phosphate, pH 7.4, 2 mg/ml of BSA containing 0.4 or 0.6 M NaCl. Corresponding sensorgrams are shown in Fig. 2. Errors shown are S.D. of the fitting.

Results

SPR Experiments for Studies of the Interaction between GPIHBP1 and LPL—Solutions containing different concentrations of LPL were injected to flow cells with immobilized GPIHBP1, the mouse N-terminal peptide, the Ly6 domain, or the mutant GPIHBP1 Q114P. For comparison, binding of LPL to heparan sulfate was performed under the same conditions. LPL associated with all the ligands, but the observed binding characteristics varied largely and were dependent on the concentration of NaCl. Binding curves for these interactions at equilibrium conditions and corresponding sensorgrams are presented in Figs. 1 and 2, respectively. We started the analysis with the equilibrium data at 0.4 and 0.6 M NaCl. In the case of the interaction of LPL with GPIHBP1 at 0.4 M NaCl, a nanomolar high affinity binding and a micromolar low affinity binding was evident (Fig. 1*A*). The data were fitted to a two-binding site model (Equation 1), which assumed two independent LPL binding sites on GPIHBP1. Calculated K_d values for the sites differed more than 600-fold: 6.4 ± 1.3 nM for the high affinity binding site (site 1) and 4.2 ± 0.9 μM for the low affinity binding site (site 2). In contrast, data collected at 0.6 M NaCl could be fitted to a simple 1:1 binding model (Equation 2) with a K_d = 16.0 ± 0.8 nM. At this concentration of NaCl, the capacity of the immobilized GPIHBP1 to bind LPL was reduced to the level comparable with the capacity of binding site 1 recorded at 0.4 M NaCl. This suggests that the binding of LPL to binding site 2, observed at 0.4 M NaCl, was not detectable at 0.6 M NaCl.

The high affinity of LPL for binding site 1 of GPIHBP1 was not observed for binding of LPL to the N-terminal peptide when studied at 0.4 M NaCl. This binding became detectable when the concentration of LPL in the flow phase was in the micromolar range (Fig. 1*B*). The binding curve was slightly upward, suggesting cooperativity. Therefore, the data were fitted to the Hill's equation (Equation 3). The obtained K_d of 3.9 ± 0.1 μM was comparable with the binding of LPL to site 2 of GPIHBP1 at 0.4 M NaCl (K_d = 4.2 ± 0.9 μM). No measurable binding of LPL to the N-terminal peptide was detected at 0.6 M NaCl, indicating a crucial role of ionic forces for the interaction.

Binding of LPL to the Ly6 domain was observed already at nanomolar concentrations of LPL and the corresponding binding curve was well described by a simple 1:1 model (Equation 2) at both 0.4 and 0.6 M NaCl (Fig. 1*C*). The K_d values at these salt concentrations (93 ± 5 and 270 ± 30 nM, respectively) were 15-fold higher than that for the interaction of LPL with site 1 of GPIHBP1. It should be noted that this difference in the K_d values is not very significant. Addition of the thrombin recognition site into the sequence of GPIHBP1 already lowered the apparent site 1 affinity for LPL by 5-fold (K_d = 35 nM, data not shown). Similar to the characteristics of binding site 1, the interaction of LPL with the Ly6 domain was only slightly influ-

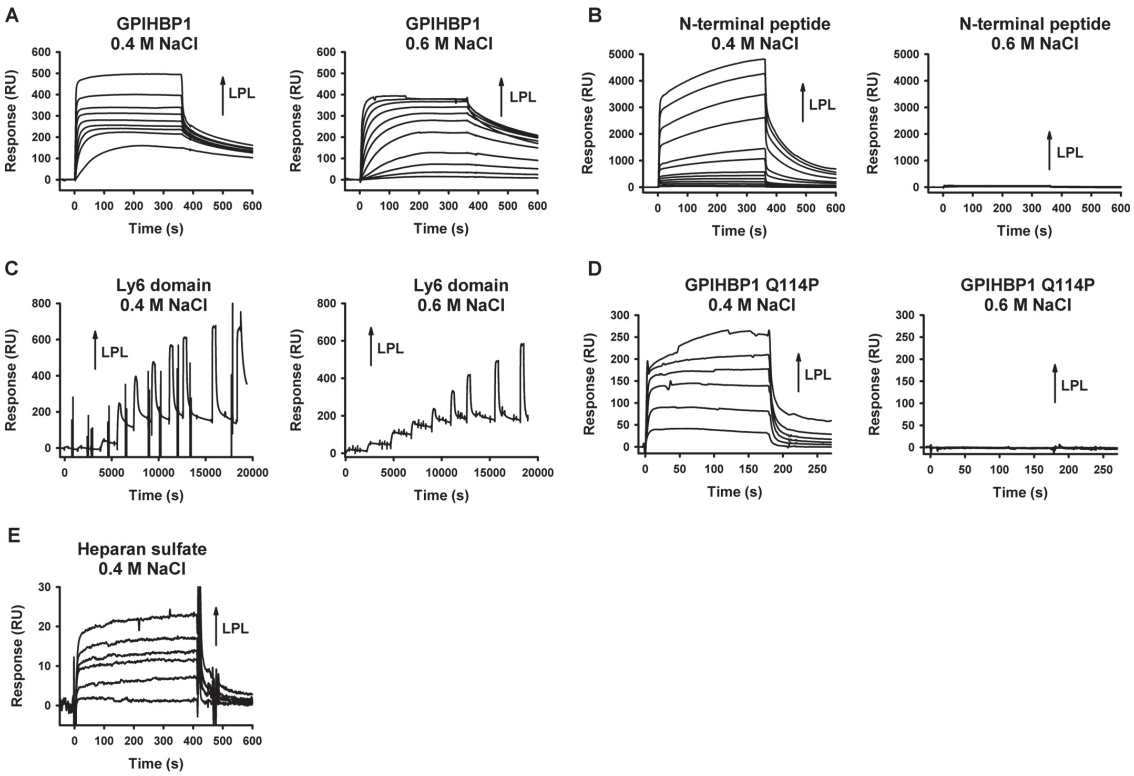

FIGURE 2. **SPR sensorgrams that show binding of LPL to GPIHBP1 variants and heparan sulfate.** Binding of LPL to GPIHBP1 (*A*), mouse N-terminal peptide (*B*), the Ly6 domain (*C*), the mutant GPIHBP1 Q114P (*D*), and heparan sulfate (E) at 0.4 M (*left*) or 0.6 M NaCl (*right*) is shown. In the case of the Ly6 domain, single cycle analysis was performed. Concentrations of LPL were as follows: 10 nM, 50 nM, 100 nM, 200 nM, 500 nM, 1 μM, 1.5 μM, 3 μM, and 6 μM in *A* (*left panel*), all at 0.4 M NaCl; 1 nM, 2 nM, 4 nM, 8 nM, 20 nM, 40 nM, 80 nM, 200 nM, 500 nM, 1 μM, and 4 μM in *A* (*right panel*), all at 0.6 M NaCl; 100 nM, 200 nM, 400 nM, 600 nM, 800 nM, 1 μM, 1.2 μM, 1.6 μM, 2 μM, 3 μM, 4 μM, 5 μM, and 6 μM in *B* (*left panel*), all at 0.4 M NaCl; 100 nM, 200 nM, 400 nM, 800 nM, 1.6 μM, 3.2 μM, and 6 μM in *B* (*right panel*), all at 0.6 M NaCl; 1 nM, 4 nM, 10 nM, 50 nM, 100 nM, 200 nM, 500 nM, 1 μM, 3 μM, and 5.2 μM in *C* (*left panel*), all at 0.4 M NaCl; 10 nM, 20 nM, 40 nM, 80 nM, 180 nM, 360 nM, 720 nM, 1.44 μM, and 4 μM in *C* (*right panel*), all at 0.6 M NaCl; 260 nM, 550 nM, 1.1 μM, 2.1 μM, 3.6 μM, and 6.3 μM in *D* (*left panel*), all at 0.4 M NaCl; 550 nM, 1.1 μM, 3 μM, and 6.3 μM in *D* (*right panel*), all at 0.6 M NaCl; 29 nM, 146 nM, 760 nM, 1.1 μM, 1.46 μM, and 2.5 μM in *E*, all at 0.4 M NaCl.

enced by salt. The data therefore suggest that site 1 of GPIHBP1 corresponds to the Ly6 domain and site 2 to the N-terminal domain.

To further explore the contributions of different parts of GPIHBP1 to the interaction with LPL we used the Ly6 domain mutant GPIHBP1 Q114P (6). LPL bound to this mutant with much lower affinity than to site 1 of GPIHBP1 and more similar to the affinity of site 2 (Fig. 1*D*). The affinity of the Q114P mutant for LPL at 0.4 M NaCl ($K_d = 1.6 \pm 0.2$ μM) was similar to that of LPL for the N-terminal peptide ($K_d = 3.9 \pm 0.1$ μM). In accordance, the Q114P mutant did not bind LPL at 0.6 M NaCl. These observations suggest that the Q114P mutant is unable to interact with LPL via the Ly6 domain.

For comparison we studied binding of LPL to immobilized heparan sulfate and found an affinity of 2.2 $\pm$ 0.4 μM at 0.4 M NaCl (Fig. 1*E*). This is comparable with the affinity of LPL for the N-terminal peptide of GPIHBP1.

Effects of Heparin and the N-terminal Peptide on the Binding of LPL to GPIHBP1—Based on the results in Fig. 1 we hypothesized that the interaction of LPL with GPIHBP1 includes two separate binding events: one that occurs to the N-terminal domain of GPIHBP1 and another that engages the Ly6 domain. To test this model, SPR displacement experiments were performed by injecting heparin (10 IU/ml) or the N-terminal peptide (100 μM) to the flow cells in which LPL was bound to GPIHBP1, the N-terminal peptide, or the Ly6 domain (Fig. 3). Heparin was able to partly dissociate LPL from GPIHBP1 (Fig. 3*A*) and almost completely from the N-terminal peptide (Fig. 3*B*). In contrast, heparin did not dissociate LPL from the immobilized Ly6 domain (Fig. 3*C*). The N-terminal peptide behaved similar to heparin with regard to dissociation of LPL, but the effect was less pronounced (data not shown). These observations suggest that heparin and the N-terminal peptide were only able to elute LPL from the N-terminal domain of GPIHBP1.

The proposed two-binding site model was tested using competition experiments in which LPL was mixed with the competing N-terminal peptide before injection to the flow cells with immobilized GPIHBP1, N-terminal peptide, or the Ly6 domain. At a concentration of 100 μM the N-terminal peptide blocked about 75% of the association of LPL to immobilized N-terminal peptide (Fig. 4*B*) but only 25% of the association of

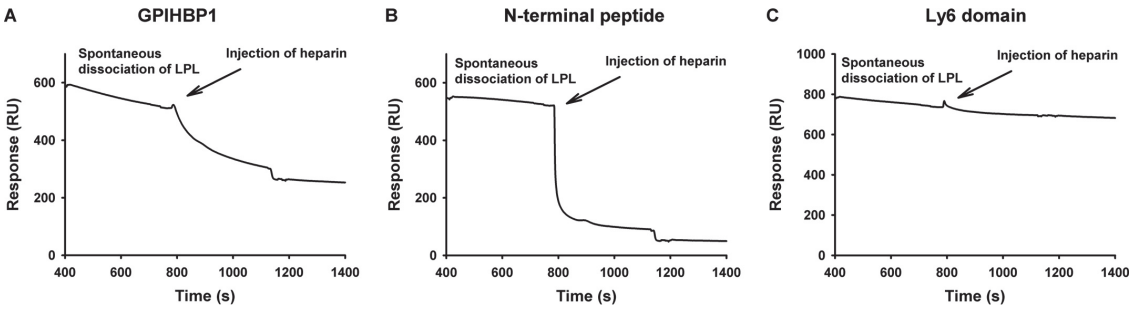

FIGURE 3. **Effect of heparin on the dissociation of LPL from GPIHBP1 (*A*), the mouse N-terminal peptide (*B*), and the Ly6 domain (*C*).** LPL was first bound at 0.4 M NaCl. The spontaneous dissociation of LPL from the ligands was studied at 0.15 M NaCl. The *arrow* shows the start of the heparin injection (10 IU/ml, 0.15 M NaCl). Experiments were run at 4 °C in 20 mM phosphate, pH 7.4, 2 mg/ml of BSA, 0.15 M NaCl.

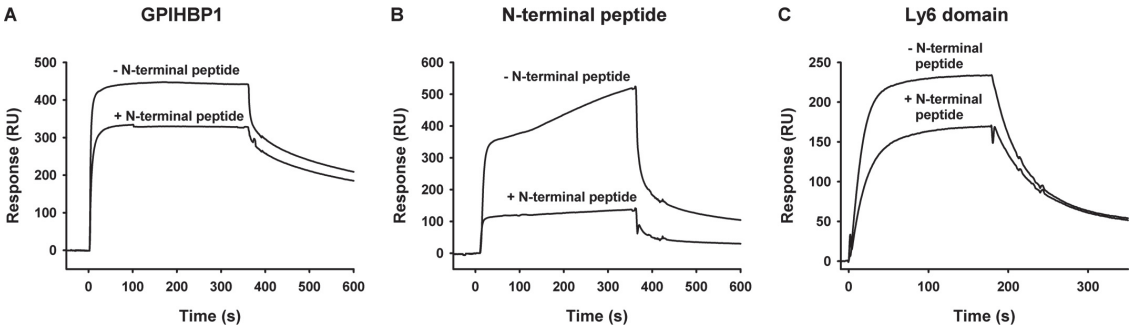

FIGURE 4. **Effect of the addition of mouse N-terminal peptide on the association of LPL with immobilized GPIHBP1 (*A*), mouse N-terminal peptide (*B*), or Ly6 domain (*C*).** For this experiment 1 μM LPL without (− *N-terminal peptide*) or with preincubation (+ *N-terminal peptide*) with 100 μM of the N-terminal peptide were injected to the flow cells with the immobilized ligands. Experiments were run at 4 °C in 20 mM phosphate, pH 7.4, 2 mg/ml of BSA, 0.4 M NaCl.

LPL to GPIHBP1 (Fig. 4*A*) or the Ly6 domain (Fig. 4*C*). Interestingly, in the experiments with immobilized GPIHBP1 or the Ly6 domain the kinetic constants were not affected by the presence of the peptide. More than 80% of LPL was calculated to be bound to the competing N-terminal peptide in the injection mixture when using the K_d value of 22 μM that was obtained for the interaction of LPL with the N-terminal peptide by SPR in competition experiments at 0.4 M NaCl (Table 3). The results in Fig. 4 indicate that the soluble complex of LPL with the N-terminal peptide is able to associate with the immobilized Ly6 domain or GPIHBP1 via the Ly6 domain.

Combined Analysis of Binding Kinetics Using SPR, Fluorescence Anisotropy, and Stabilization Experiments—Due to high nonspecific binding of LPL to sensorchip matrix, it was not possible to use SPR at NaCl concentrations lower than 0.3 M. Therefore we studied effects of the N-terminal peptide on the stability of LPL by measurements of enzymatic activity for estimations of K_d values for the interaction between LPL and the peptide. The N-terminal peptide stabilized LPL from thermal inactivation in a concentration-dependent manner (Fig. 5). The inactivation curves were fitted to the monoexponential decay equation. To derive the K_d value, the dependence between inactivation rate constants for LPL and the concentrations of the N-terminal peptide were fitted to Equation 5. At 0.15 M NaCl, the K_d value for the interaction between LPL and the N-terminal peptide was 2.0 ± 0.6 nM. Analogous experiments were done in a buffer containing 0.23 or 0.30 M NaCl (inactivation curves not shown). In these cases, higher concentrations of the N-terminal peptide were needed to stabilize LPL and K_d calculations were performed using Equation 6. The corresponding K_d values were 180 ± 14 nM and 2.4 ± 0.7 μM, respectively.

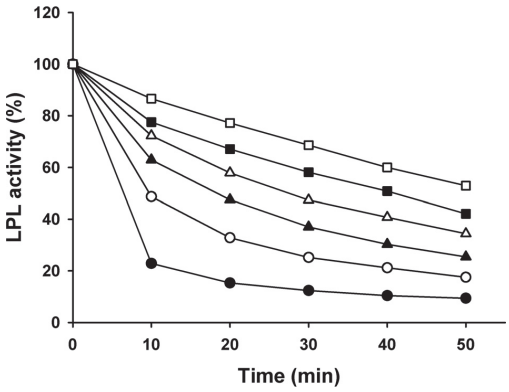

FIGURE 5. **Stabilization of LPL activity by the N-terminal peptide.** Time-dependent inactivation of LPL was measured in the absence or presence of various concentrations of the bovine N-terminal peptide in 20 mM HEPES, pH 7.4, 0.15 M NaCl at 25 °C. At the times indicated, aliquots of the reaction mixtures were withdrawn for activity measurements. The LPL activities are expressed as percent remaining of the initial activity at time 0. Values are the mean of triplicate determinations. The LPL concentration was 10 nM. The concentrations of the N-terminal peptide were (●) 0 M, (○) 10 nM, (▲) 25 nM, (△) 50 nM, (■) 100 nM, and (□) 300 nM.

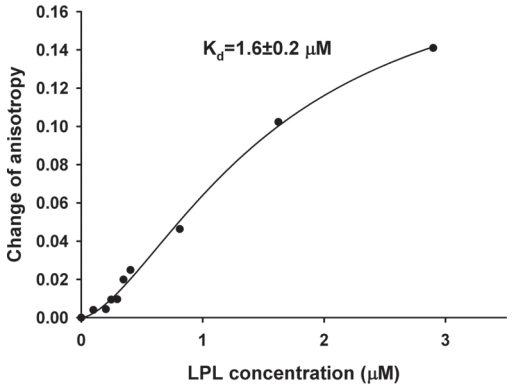

FIGURE 6. **Measurement of fluorescence anisotropy to study the interaction between the N-terminal peptide and LPL.** The change of fluorescence anisotropy of Dylight 488 maleimide-labeled human N-terminal peptide was measured at different concentrations of LPL. The increase in the change of anisotropy shows the increasing number of complexes of LPL with the N-terminal peptide. Fluorescence anisotropy was calculated using Equation 7. The corresponding K_d was calculated using Equation 8. The concentration of labeled N-terminal peptide was 100 nM. Experiment was carried out at 4 °C in 20 mM phosphate, pH 7.4, 0.3 M NaCl.

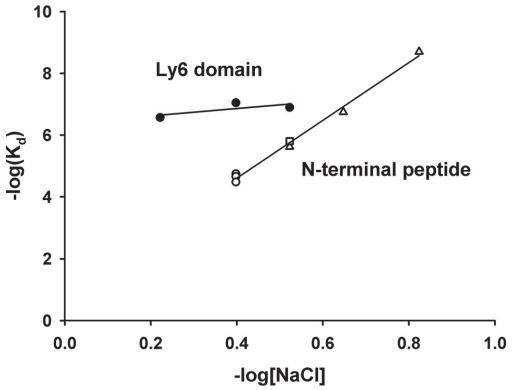

FIGURE 7. **Effect of salt on the interaction of LPL with the Ly6 domain or the N-terminal peptide.** The dependence of the K_d values on the NaCl concentration in logarithmic scale is presented. The K_d values for the interaction of LPL with the Ly6 domain were calculated from the steady-state SPR data (Fig. 1) (●). For the interaction of LPL with the N-terminal peptide, data from the SPR competition experiments using mouse N-terminal peptide, bovine N-terminal peptide, and human N-terminal peptide (Table 3) (○) were combined with stabilization of LPL activity (Fig. 5) (△) and data from measurement of anisotropy (Fig. 6) (□).

The K_d values for the interactions between LPL and the Ly6 domain and between LPL and the N-terminal peptide determined at different concentrations of NaCl were obtained by a combination of results from fluorescence anisotropy (Fig. 6), SPR (Fig. 1, Table 3), and measurements of LPL stabilization (Fig. 5). Results of this analysis are presented in Fig. 7. As indicated, binding of LPL to the N-terminal peptide was highly influenced by salt, whereas the interaction between LPL and the Ly6 domain was only slightly influenced by salt. To estimate the K_d value for the interaction of LPL with the Ly6 domain at 0.15 M NaCl, the line of the relationship -log K_d *versus* -log[NaCl] was extrapolated to this salt concentration. The K_d value was ~45 nM using this technique. The combined data analysis revealed that LPL binds to both the Ly6 domain and the N-terminal peptide with nanomolar affinity at 0.15 M NaCl. This affinity is comparable with the affinity of LPL for heparan sulfate (20).

To further characterize the interaction of LPL with GPIHBP1 and the GPIHBP1 domains, binding dynamics was examined by determination of rate constants, k_{on} and k_{off}. For comparison, kinetic parameters were determined for binding of LPL to heparan sulfate and to the mutant GPIHBP1 Q114P. At 0.15 M NaCl, only k_{off} values were determined by SPR. For these experiments, LPL was first associated with the immobilized ligands in the presence of 0.4 M NaCl, whereas the dissociation was monitored at 0.15 M NaCl. In the case of the N-terminal peptide, 10 IU/ml of heparin was injected during the dissociation phase to avoid rebinding of dissociated LPL to the immobilized N-terminal peptide (20). SPR sensorgrams and their analysis are presented in Fig. 8. Calculated parameters obtained in the presence of 0.4 or 0.15 M NaCl are summarized in Tables 1 and 2, respectively. As can be seen in Table 1, the N-terminal peptide/LPL and the heparan sulfate/LPL interactions had similar binding dynamics characterized by moderately fast association (k_{on} ~10^4 M^{-1} s^{-1}) and fast dissociation (k_{off} ~0.1 s^{-1}) at 0.4 M NaCl. The interaction of the mutant Q114P with LPL was characterized by similar rate constants supporting our previous observations that the Q114P mutant interacts with LPL via the N-terminal domain. When SPR data were combined with measurements of stabilization of LPL activity at 0.15 M NaCl, very fast association rate ($k_{on} = 7.7 \times 10^7$ M^{-1} s^{-1}) was estimated for the binding of LPL to the N-terminal peptide (Table 2). Because of a fast dissociation rate ($k_{off} = 0.15$ s^{-1}), resulting in a calculated average lifetime of 7 s for the N-terminal peptide·LPL complex, the affinity of this interaction was not significantly stronger than the interaction between LPL and the Ly6 domain. Thus, the high affinity for the binding of LPL to the N-terminal peptide is determined by the very high k_{on} value. The complex between LPL and the Ly6 domain was formed more slowly and the proteins stayed together for a longer time. The determined off-rate values were similar at both concentrations of NaCl (0.4 and 0.15 M), giving an average lifetime of 9 min for the complex between LPL and the Ly6 domain (Table 2). However, the k_{on} value over 10^4 M^{-1} s^{-1} is still high, suggesting a diffusion-controlled association mechanism (34). Interestingly, the k_{off} values for the interaction of LPL with binding site 1 of GPIHBP1 or the Ly6 domain at 0.4 M NaCl were similar (~10^{-3} s^{-1}), whereas k_{on} values for association of LPL to site 1 of GPIHBP1 was measured to be 62-fold faster than that to the Ly6 domain ($k_{on} = 1.8 \times 10^6$ M^{-1} s^{-1} and $k_{on} = 2.9 \times 10^4$ M^{-1} s^{-1}, respectively). This indicated that to some extent, the N-terminal domain influenced the interaction between LPL and the Ly6 domain.

Interaction with Lipoproteins—Previous studies have shown that LPL can interact with lipoproteins while being bound to GPIHBP1 (13, 35). Here we examined whether this important ability was affected by the type of complex LPL had formed with GPIHBP1. To minimize effects of triglyceride lipolysis by LPL, these experiments were first performed with triglyceride-poor, human LDL. GPIHBP1, the Ly6 domain, or the N-terminal pep-

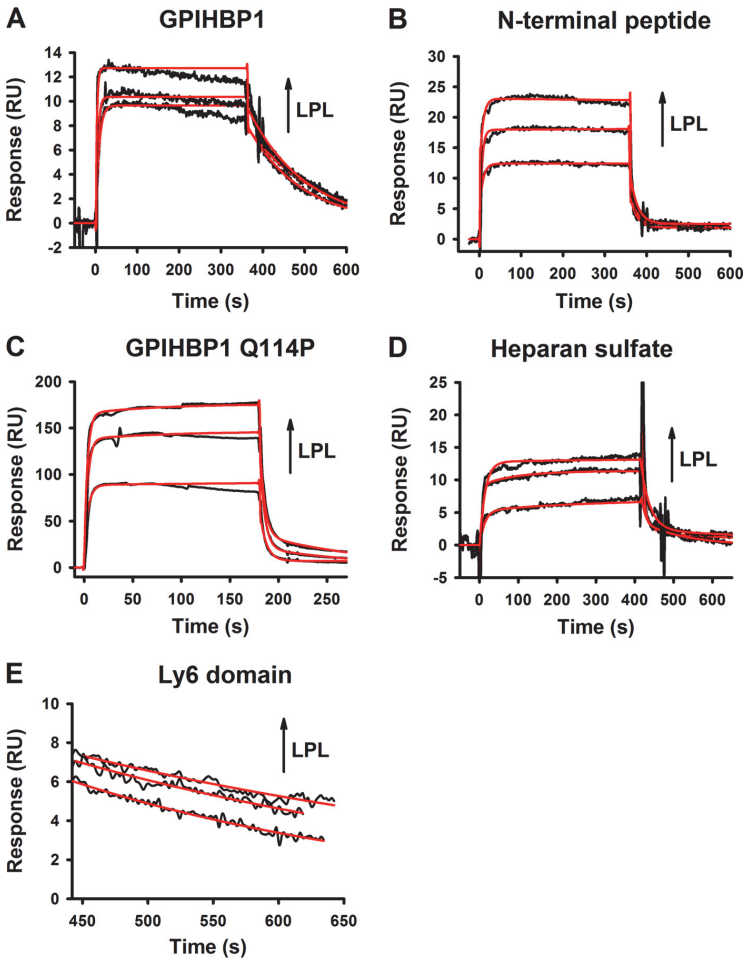

FIGURE 8. **Kinetic analysis of SPR sensorgrams.** Presented are interactions between LPL and GPIHBP1 (*A*), LPL and mouse N-terminal peptide (*B*), LPL and the mutant GPIHBP1 Q114P (*C*), LPL and heparan sulfate (*D*), LPL and the Ly6 domain (*E*). In the case of the Ly6 domain, only the dissociation phase was analyzed. *Black lines* represent raw data, *red traces* are the fits to the experimental data curves. Kinetic rate constants obtained from these fits are given in Table 1. Concentrations of LPL were as follows: 50, 100, and 500 nM in *A*; 600 nM, 800 nM, and 1 μM in *B*; 550 nM, 1.1 μM, and 2.1 μM in *C*; 146 nM, 760 nM, and 1.1 μM in *D*; and 500 nM, 1 μM, and 3 μM in *E*. Measurements were performed at 4 °C in 20 mM phosphate, pH 7.4, 2 mg/ml of BSA, 0.4 M NaCl.

TABLE 1

Kinetic parameters for the interactions between LPL and different ligands at 0.4 M NaCl as determined by SPR

Corresponding fitted sensorgrams are shown in Fig. 8. K_d(eq) express equilibrium dissociation constants obtained from steady-state SPR analysis (Fig. 1). Experiments were run at 4 °C in 20 mM phosphate, pH 7.4, 2 mg/ml of BSA, 0.4 M NaCl. The constants are mean ± S.D. of three different determinations.

Ligand	k_{on}	k_{off}	K_d	K_d(eq)
	$M^{-1}\,s^{-1}$	s^{-1}	k_{off}/k_{on}	
GPIHBP1				
Binding site 1	$(1.8 \pm 0.8) \times 10^6$	$(7.1 \pm 0.8) \times 10^{-3}$	4.0 nM	6.4 nM
Binding site 2				4.2 μM
Ly6 domain	$(2.9 \pm 0.7) \times 10^4$	$(2.7 \pm 0.7) \times 10^{-3}$		93 nM
N-terminal peptide	$(4.8 \pm 1.1) \times 10^4$	0.07 ± 0.02	1.5 μM	3.9 μM
Heparan sulfate	$(2.9 \pm 1.5) \times 10^4$	0.10 ± 0.07	3.4 μM	2.2 μM
GPIHBP1 Q114P	$(4.9 \pm 2.6) \times 10^4$	0.18 ± 0.03	3.7 μM	1.6 μM

TABLE 2

Kinetic parameters for the interaction of LPL with the Ly6 domain or with the mouse N-terminal peptide at 0.15 M NaCl

Dissociation rate constants (k_{off}) were determined by SPR. Association rate constant (k_{on}) for the interaction of LPL with the N-terminal peptide was calculated from the equation $k_{on} = k_{off}/K_d$, where K_d is the equilibrium dissociation constant obtained from stability measurements of LPL activity in the presence of 0.15 M NaCl (Fig. 5, K_d = 2 nM). Experiments were run at 25 °C in 20 mM phosphate, pH 7.4, 0.5 mg/ml of BSA, 0.15 M NaCl at 25 °C. The constants are mean ± S.D. of three different determinations.

Ligand	k_{on}	k_{off}	Average lifetime
	$M^{-1}\,s^{-1}$	s^{-1}	
N-terminal peptide	$(7.7 \pm 1.5) \times 10^7$	0.15 ± 0.03	7 s
Ly6 domain		$(1.9 \pm 1.1) \times 10^{-3}$	9 min

tide were immobilized to sensorchips. LPL was then added and noncovalently bound at 0.4 M NaCl. Studies of binding of LDL to LPL or to the variants of GPIHBP1 were then performed by addition of LDL to running buffer containing 0.15 M NaCl. As can be seen in Fig. 9*A*, binding of LDL to the sensorchip containing the N-terminal peptide was markedly increased when

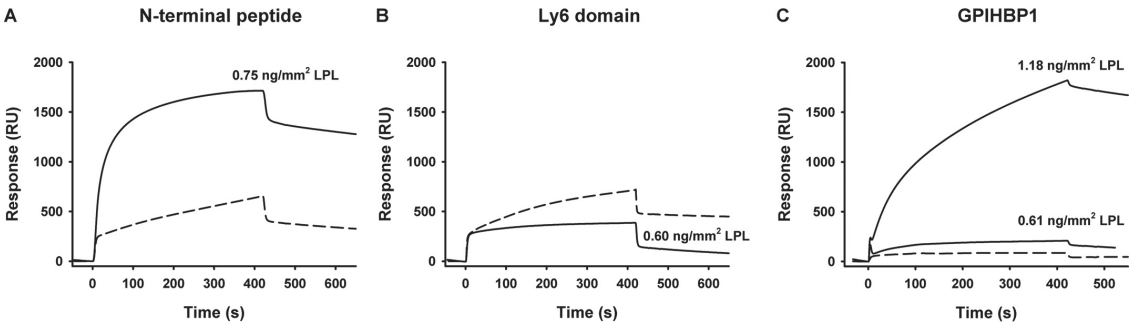

FIGURE 9. **Binding of LDL to GPIHBP1 variants in the presence or absence of LPL.** Binding of LDL to the mouse N-terminal peptide (*dashed line*) or the complex of LPL with the N-terminal peptide (*solid line*) (*A*), the Ly6 domain (*dashed line*), the complex of LPL with Ly6 domain (*solid line*) (*B*) and GPIHBP1 (*dashed line*) or to the complex of LPL with GPIHBP1 (*solid lines*) (*C*). The N-terminal peptide, the Ly6 domain, or GPIHBP1 were bound to the surface of the sensorchip. LPL was then noncovalently attached to the ligands in the presence of 0.4 M NaCl. A solution of 17 μg/ml of LDL (protein concentration) was injected to the flow cells. Experiments were run at 4 °C in 20 mM phosphate, pH 7.4, 2 mg/ml of BSA, 0.15 M NaCl.

there was LPL present on the chip. In contrast, LPL did not stimulate binding of LDL to the Ly6 domain, but rather decreased the interaction (Fig. 9*B*). When complexed with GPIHBP1, LPL is distributed between binding sites 1 and 2. The occupancy of the sites depends on the concentration of LPL in the flow phase (Fig. 1*A*). Therefore it was possible to examine whether localization of LPL on GPIHBP1 affects binding of LDL. Calculations of binding site occupancy from the data in Fig. 1*A* and using Equation 1 indicated that the occupancy of binding site 2 on GPIHBP1 was increased from 13 to 42% when the concentration of LPL in flow phase was increased from 0.6 to 3 μM, whereas the occupancy of binding site 1 was near complete at both LPL concentrations. As can be seen in Fig. 9*C*, the increase of the occupancy of binding site 2 resulted in a more than 12-fold increased LDL binding. These observations indicate that when LPL is bound to site 2 of GPIHBP1, or to the acidic N-terminal peptide, the enzyme is able to simultaneously associate with LDL. Analogous binding studies were performed with VLDL. Unfortunately the data obtained with the Ly6 domain or GPIHBP1 were not completely reliable, although a similar tendency to the results of LDL was observed. The high nonspecific binding of VLDL to the immobilized GPIHBP1 antibody made interpretation of the data difficult. However, when LPL was bound to the N-terminal peptide, which was in turn immobilized via NeutrAvidin, the LPL/VLDL interaction was reliably detectable (Fig. 10).

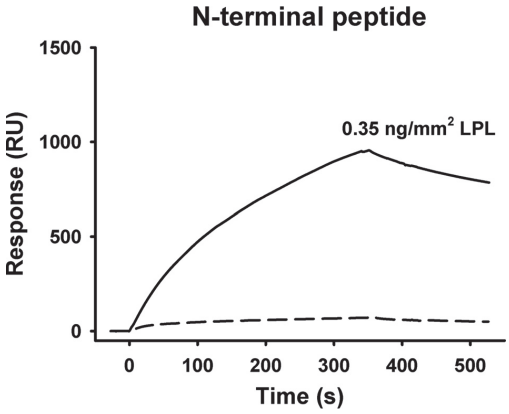

FIGURE 10. **Binding of VLDL to the mouse N-terminal peptide (*dashed line*) or the complex of LPL with the N-terminal peptide (*solid line*).** The N-terminal peptide was bound to the surface of the sensorchip. LPL was then noncovalently attached to the peptide in the presence of 0.4 M NaCl. A solution of 6 μg/ml of VLDL (protein concentration) was injected to the flow cells. Experiments were run at 4 °C in 20 mM phosphate, pH 7.4, 2 mg/ml of BSA, 0.15 M NaCl.

Specificity of the Interaction between LPL and the N-terminal Peptide from GPIHBP1—The observations that LPL binds at two distinct binding sites on GPIHBP1, and that binding to site 2 in the N-terminal domain may be compatible with binding of LPL also to lipoproteins, whereas binding to the Ly6 domain is not, prompted us to focus on the interaction of LPL with the acidic N-terminal domain. To investigate the specificity of this interaction, experiments were performed with synthetic peptides that corresponded to various regions of the human N-terminal domain of GPIHBP1 and with a peptide that was composed of the same amino acids as the whole N-terminal domain but whose sequence was randomly generated. The affinity of these peptides for LPL at 0.4 M NaCl was studied in the solution by SPR competition experiments. Corresponding K_d values are shown in Table 3. No significant differences in the K_d values were observed for the human, bovine, or mouse N-terminal peptide, despite variations in their length and number of negatively charged residues. Deletion of 10 residues from the N-terminal end (peptide 1) slightly decreased the affinity in comparison with that of the intact peptide. Subsequent deletion of the next 7 residues (peptide 2) resulted in a 13-fold increased K_d value. The shortest peptide tested contained 8 amino acid residues (peptide 3) and was unable to compete with the immobilized human N-terminal peptide, demonstrating that a longer stretch is required for formation of the binding site for LPL. The K_d value for the interaction of LPL with the random-sequence peptide (peptide 4) was only 3-fold higher than that of the corresponding native peptide. The high affinity peptides (human, mouse, bovine, peptide 1, and peptide 4) with K_d values lower than 70 μM shared similarities at their C-terminal parts. In contrast, the N-terminal parts of the peptides were different with regard to the number of negatively charged residues and dis-

TABLE 3

Equilibrium dissociation constants (K_d) for the interactions between LPL and different peptides as determined by competition experiments using SPR.

Affinities of the interactions were calculated by the Eq. 4. Negatively charged residues are shown in red. Experiments were run at 4 °C in 20 mM phosphate, pH 7.4, 2 mg/ml of BSA, 0.4 M NaCl. Errors shown are S.D. of the fitting.

Peptide	Sequence	K_d (μM)
Human N-terminal peptide	QQEEEEEDEDHGPDDYDEEDEDEVEEEETC	18±3
Peptide 1	HGPDDYDEEDEDEVEEEETC	52±5
Peptide 2	EEDEDEVEEEETC	230±41
Peptide 3	EVEEEETC	no binding
Peptide 4	VDQDYEDDHETEDDEEQDPEEEEGEEEEEC	63±4
Mouse N-terminal peptide	AQEDGDADPEPENYNYDDDDDEEEEEETC	22±2
Bovine N-terminal peptide	AQEDEDDDPDAGREGYDDEDEEEEEA	33±5

played low sequence similarities. The observation that peptide 2 showed only 7–13 times lower affinity than the full-length N-terminal domain peptides suggests that the affinity for LPL is largely determined by the sequence stretch that corresponded to this peptide in the intact peptides (residues 40–51 in the human GPIHBP1 sequence). This assumption is supported by the reasonable sequence similarities in the respective regions in all high affinity peptides used in the current study. Because the affinity of peptide 1 almost matched that of the intact N-terminal peptides, we propose that the last 19 residues at the C-terminal part of the N-terminal domain (residues 33–51 in the human GPIHBP1 sequence) are crucial in the interaction with LPL.

Chemical Cross-linking of LPL and the N-terminal Peptide—To localize the binding region of the N-terminal peptide on LPL, chemical cross-linking was combined with mass spectrometry. The experiment was performed using the homobifunctional amino group-specific chemical cross-linker DST. Because the N-terminal amino group is the only reactive amine in this peptide, it was possible to localize its position at the surface of LPL. DST bridged covalent complexes between LPL and the N-terminal peptide resulting in molecular masses ~59 or ~71 kDa, corresponding to LPL monomers with one (1:1) or four (1:4) bound peptides (Fig. 11*A*). In the 1:1 complex several cross-linked lysines (residues 410, 417, 440, and 425 in bovine LPL) were identified in the C-terminal domain of LPL. Only two cross-linked lysines, at positions 275 and 303, were identified in the N-terminal domain of LPL. In the case of the 1:4 complex, also lysines 151, 263, 270, 295, and 444 were cross-linked with the peptide, in addition to the cross-linked residues of the 1:1 complex. The abundance of the cross-links with lysine residues in the C-terminal domain of LPL was higher than that of the N-terminal domain in all performed experiments, indicating a higher probability for formation of the complex in which the N-terminal amino group of the peptide is localized at the C-terminal domain of LPL. For localization of the cross-linked lysine residues on the three-dimensional structure of bovine LPL, we first modeled the structure of this lipase using the protein structure prediction service Phyre (31). The final LPL structure was very similar to that obtained in previous studies (36, 37). The cross-linked lysines on the three-dimensional model of bovine LPL are shown in Fig. 11*B*. As can be seen, all of the identified cross-linked lysines are located on the same side of the enzyme subunit. Based on the distances between the cross-linked residues on the model of LPL, and taken in account that the length of the spacer arm of the cross-linker is 6.4 Å, it was obvious that the peptide could be bound to LPL in various orientations. In addition to the cross-links between LPL and the peptide, we were able to detect several intramolecular LPL cross-links. All of them were consistent with the model of bovine LPL, if the distances between α-carbon atoms in the model were compared with the maximum cross-linkable distance (6.4 Å DST spacer arm + 2 × 6 Å lysine side chain = 18.4 Å). Identified intramolecular cross-linked lysine pairs and their α-carbon distances were Lys^{70}-Lys^{77} (Cα-Cα 10.1 Å), Lys^{270}-Lys^{303} (Cα-Cα 9.7 Å), Lys^{270}-Lys^{307} (Cα-Cα 9.8 Å), Lys^{275}-Lys^{307} (Cα-Cα 13.0 Å), Lys^{410}-Lys^{417} (Cα-Cα 4.6 Å), and Lys^{431}-Lys^{433} (Cα-Cα 7.0 Å).

Discussion

In the present study we demonstrate that LPL binds to two distinct binding sites on GPIHBP1: one within the acidic N-terminal domain and one within the Ly6 domain. The contribution of both GPIHBP1 domains for the interaction with LPL have been demonstrated in several previous studies (5, 16, 17), but we have here shown that the two domains act as two distinct and functionally different binding sites. Our measurements suggest that LPL can be independently bound to the N-terminal domain or to the Ly6 domain, whereas occupancy of either site does not prevent binding to the other site. This conclusion is based on several observations. The first was that the binding of LPL to wild type GPIHBP1 could only be fitted to a two-binding site model. The second observation was that a salt-dependent and salt-insensitive binding component could be clearly distinguished. Binding of LPL to the Ly6 domain appeared similar to binding to the salt-insensitive site of GPIHBP1 (binding site 1), whereas binding to the acidic N-terminal domain resembled binding to the salt-dependent site of GPIHBP1 (binding site 2). The third observation was that the capacity of wild type GPIHBP1 to bind LPL was about two times higher than that of the N-terminal or Ly6 domain. The fourth observation was that heparin could nearly completely dissociate LPL from the N-terminal peptide, but was totally ineffective with regard to release LPL from the Ly6 domain. In the case of the interaction with GPIHBP1, heparin eluted only a fraction of the bound LPL, indicating that the binding was not homogenous. The fifth observation was that while associated with the N-terminal peptide, LPL could still bind to the Ly6 domain and also to GPIHBP1. Moreover, the kinetic parameters of the interactions were not influenced by the presence of the peptide. We conclude that the previously observed lowered or abolished binding of LPL to modified variants of GPIHBP1, with one of the two domains mutated or deleted (5, 16, 17), was likely caused by a reduced number of binding sites leading to a reduced overall binding capacity, rather than a complete prevention of the interaction.

The observation that heparin efficiently eluted LPL only from the N-terminal domain of GPIHBP1 is intriguing. It is assumed, but seldom demonstrated, that heparin removes essentially all LPL from the capillary endothelium in tissues (1, 38). Determination of LPL activity in post-heparin plasma has been used for many years as a surrogate measure of the functional pool of LPL on the capillary bed in the body. Intravenous

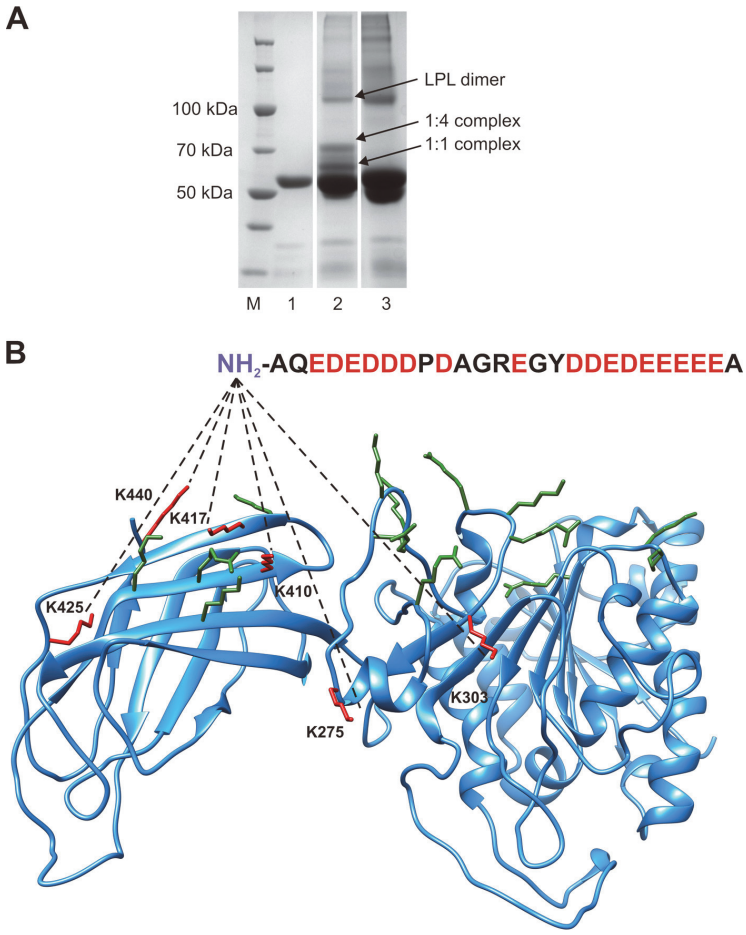

FIGURE 11. **Chemical cross-linking of bovine N-terminal peptide to LPL.** Analysis of cross-linked products by SDS-PAGE. *M*, molecular weight marker; *1*, LPL without modifications; *2*, N-terminal peptide cross-linked to LPL; *3*, LPL cross-linked without N-terminal peptide. *Arrows* show the 1:1 LPL·N-terminal peptide complex, the 1:4 LPL·N-terminal peptide complex and the LPL dimer (*A*). Localization of residues in LPL cross-linked with the N-terminal peptide analyzed in the 1:1 complex. Cross-linked lysines are shown in *red*. Heparin binding clusters are shown in *green*, except lysines 303, 410, and 417 that were cross-linked and shown in *red* (*B*).

administration of heparin into humans and animals releases LPL from the endothelial binding sites, and most likely also from the subendothelial tissue but at a slower rate, leading to more than a 100-fold increase of LPL activity in plasma (39). According to our study, heparin dissociates LPL only from the N-terminal domain of GPIHBP1 (binding site 2). This observation suggests that LPL is bound mainly to the N-terminal domain of GPIHBP1 at the luminal side of the endothelium. Interestingly, our data also indicate that only the fraction of LPL that is bound to the N-terminal domain of GPIHBP1 is able to interact with lipoproteins, as concluded from the binding studies with LDL. Analogous binding experiments with VLDL were possible to conduct only with the N-terminal domain. In the case of the Ly6 domain or wild type GPIHBP1, the data were difficult to interpret due to nonspecific effects. However, previous studies indicate that the nature of the interaction of LPL with LDL is similar to that with VLDL (40–42). It is therefore reasonable to assume that LDL can be used as a surrogate model for analysis of the interaction of LPL with apoB100-containing lipoproteins, but details in composition of lipids and apolipoproteins in the surface layer of the particles are likely to affect the binding parameters in each case.

From the data obtained by SPR, fluorescence anisotropy, and stabilization measurements, it can be concluded that LPL binds to both of the sites on GPIHBP1 with nanomolar affinity, but the nature of these interactions are quite different. Although LPL and the N-terminal domain formed a complex with a relatively short lifetime, the complex between LPL and the Ly6 domain formed more slowly, but stayed stable for a longer time. The average lifetimes were 7 s and 9 min, respectively. The high affinity of the binding of LPL to the N-terminal domain was explained by a very high k_{on} value, which stems from the strong ionic interaction between the highly negatively charged peptide and the well known positively charged clusters on LPL. Accord-

ing to the concept of electrostatic steering, polyelectrolytic biopolymers with opposite charge attract each other from a long distance, resulting in a very high association rate (34). In contrast, the slower association of LPL with the Ly6 domain was relatively insensitive to the concentration of NaCl, and therefore not primarily electrostatic. This is in accordance with that hydrophobic residues in the Ly6 domain are important for the interaction with LPL, as previously demonstrated by site-directed mutagenesis (17). The observation that the affinity of LPL for the Ly6 domain was somewhat lower than its affinity for binding site 1 of the wild type GPIHBP1 (Ly6 domain in the intact protein) indicates that the two binding sites do not act completely independently and suggests cooperativity in their interaction with LPL. If cooperativity exists, binding of LPL to the N-terminal domain should increase the affinity of LPL for the Ly6 domain in the intact GPIHBP1. However, the LPL·N-terminal peptide complex and LPL alone bound to the Ly6 domain or GPIHBP1 with similar kinetic parameters. We can therefore exclude the possibility of cooperativity between the two domains. A more likely explanation is that the N-terminal domain influences the conformation of the Ly6 domain. An effect of the two domains of GPIHBP1 on each other was supported by our observation that insertion of the thrombin cleavage site between the domains slightly increased the K_d value for binding of LPL to GPIHBP1. However, under non-equilibrium conditions, as *in vivo*, it is possible that long range electrostatic steering effects of the N-terminal domain increase the association rate of LPL for binding to the Ly6 domain.

Analysis of our SPR data suggested that the Q114P mutant (representing the mouse sequence) was unable to bind to LPL via the Ly6 domain, but that binding to LPL via the N-terminal domain was still possible. Previous studies in cell cultures have demonstrated that neither mouse GPIHBP1 Q114P nor the corresponding human GPIHBP1 Q115P showed detectable binding to LPL (6, 17). In contrast, the human mutants Q115A, Q115K, and Q115E bound to LPL as wild type GPIHBP1 (17). These observations, together with our results, suggest that the reduced affinity of the Q114P mutant for LPL is due to some conformational change that leads to a non-functional Ly6 domain. Also, a comparison by homology modeling of wild type GPIHBP1 and GPIHBP1 Q115P, using the protein structure prediction service Phyre (31), revealed that the structure of the Q115P mutant was different (data not shown). In the case of patients carrying the Q115P mutation, previous studies have shown that the amount of LPL in their post-heparin plasma is very low, demonstrating a crucial role of the Ly6 domain of GPIHBP1 in the transport of LPL to the luminal side of vascular endothelium (6). Together with our data this suggests that binding of LPL only to the N-terminal domain of GPIHBP1 is not sufficient to trigger the transport of LPL.

One question addressed in this study was what mechanism ensures the transfer of LPL from other extracellular binding sites, such as heparan sulfate proteoglycans, to GPIHBP1. It is possible that GPIHBP1 provides binding sites for LPL with considerably higher affinity than those of heparan sulfate or other extracellular ligands, but it is also possible that the distribution of LPL on the endothelium is controlled by kinetics. As discussed in a number of excellent recent reviews, association and dissociation rate constants are often more relevant parameters than equilibrium constants to describe interactions *in vivo* (34, 43). Our study demonstrates, however, that the interaction of LPL with GPIHBP1 is not stronger than the interaction with heparan sulfate. Even the association rates of these interactions were comparable. Furthermore, heparan sulfate proteoglycans can be assumed to be much more abundant on cell surfaces than GPIHBP1 and they should therefore be the dominating binding partner. To some extent, the interaction of LPL with heparan sulfate proteoglycans is similar to the interaction with the N-terminal domain of GPIHBP1. Both interactions are characterized by very fast association and dissociation rates. We had previously described that LPL dissociates very slowly from sensorchips with heparin or heparan sulfate (20) and the same appears to be the case with sensorchips with immobilized N-terminal peptide. In both cases, injection of free heparin in the flow phase caused a tremendous increase in the observed dissociation rate, demonstrating that, in the absence of free heparin, avid rebinding of LPL to the sensorchip surface is the reason of the slow dissociation.

Because the N-terminal domain of GPIHBP1 is highly negatively charged, and its sequence varies largely among species (22), it is reasonable to assume that the interaction between LPL and the N-terminal domain is not perfectly specific, even though the affinity is in the nanomolar range. However, based on comparison of the affinities of the N-terminal domain peptides, we propose that the last 19 residues at the C-terminal part of the N-terminal domain (residues 33–51 in the human GPIHBP1 sequence) are crucial in the interaction with LPL. The cross-linking data, demonstrating that the amino group of the N-terminal peptide could be attached at different positions on the LPL subunit, indicated various possibilities for formation of the complex. This is likely to be an additional reason for the fast association rate. However, the binding of the peptide to LPL was not random, because the peptide preferentially bound to LPL in the orientation where the N-terminal group of the peptide was identified in the C-terminal domain of LPL. Interestingly, LPL and the N-terminal peptide formed complexes with two stoichiometries 1:1 and 1:4. It is possible that formation of the cross-linked 1:4 LPL·N-terminal peptide complex is due to nonspecific interactions. However, we cannot rule out the possibility that the 1:4 complex has physiological significance. First, GPI-anchored proteins are usually clustered in lipid rafts of plasma membranes (44, 45). The N-terminal domains of closely located GPIHBP1 proteins may be able to simultaneously interact with LPL, leading to formation of up to 1:4 complexes. Second, it is noteworthy that we were able to detect formation of 1:1 and 1:4 complexes but not 1:2 and 1:3 complexes. Third, based on the results of binding studies (Table 3), the cross-linking experiments were performed under conditions where not all LPL molecules were associated with the peptide (about 80%) to prevent nonspecific effects of oversaturation. Usually nonspecific binding is clearly weaker than specific binding. According to LPL models, the enzyme has an asymmetrical charge distribution with four clusters of positively charged amino acid residues that form a large positively charged area opposite to the active site (36). It has been shown that this area is involved in the interaction with heparin (46–

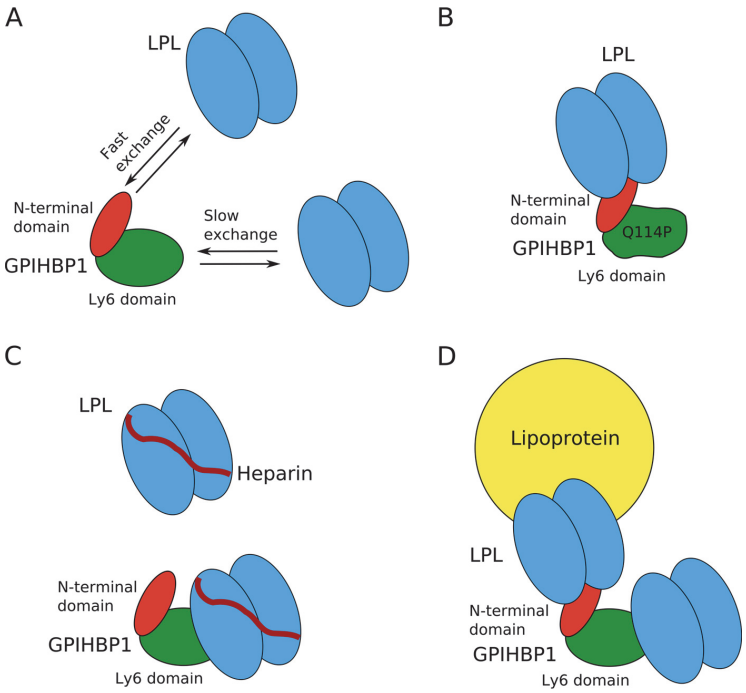

FIGURE 12. **Schematic models illustrating how the two domains of GPIHBP1 interact with LPL.** The N-terminal domain and LPL form a tight but short lived complex, characterized by fast on- and off-rates. The complex between the Ly6 domain and LPL forms slower and remains together for a longer time (*A*). The Ly6 domain mutant GPIHBP1 Q114P is unable to interact with LPL via the Ly6 domain and binds to LPL only via the N-terminal domain (*B*). Heparin disrupts the interaction between LPL and the N-terminal domain but is not able to brake the interaction between LPL and the Ly6 domain (*C*). Our data predict that only LPL that is bound to the N-terminal domain can bind to lipoproteins (*D*).

50). All cross-linked lysines in the 1:1 and 1:4 complexes were located on this side of the LPL molecule. Thus, it is likely that these clusters are also involved in the interaction with the acidic N-terminal domain of GPIHBP1. More detailed cross-linking studies would reveal more precisely how the 1:1 and 1:4 complexes are formed.

All of our experiments were performed with bovine LPL purified from milk. This was because production of recombinant mammalian LPL in amounts sufficient for biophysical studies has not been successful so far. However, we are quite convinced that the results would not be different if LPL from other sources had been used. First, because the degree of amino acid sequence identity between LPL from different mammalian species is more than 90% (51), and second, because bovine LPL has successfully been used in numerous previous model studies. Regardless of their origin all tested LPL ligands, such as apolipoprotein CII, lipoproteins, lipoprotein receptor-related protein (LRP), angiopoietin-like proteins 3 and 4, and GPIHBP1 interact similarly with bovine LPL.

The main observations of this study are summarized schematically in Fig. 12 where the interaction modes under different circumstances are proposed. We have demonstrated that two domains of GPIHBP1 act as two largely independent units. Our data also suggest that the role of LPL in lipoprotein metabolism may depend on how the enzyme is located on GPIHBP1.

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PUBLICATION II

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Lipoprotein lipase activity and interactions studied in human plasma by isothermal titration calorimetry

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*Equal contribution

methods

Lipoprotein lipase activity and interactions studied in human plasma by isothermal titration calorimetry

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Abstract **LPL hydrolyzes triglycerides in plasma lipoproteins. Due to the complex regulation mechanism, it has been difficult to mimic the physiological conditions under which LPL acts in vitro. We demonstrate that isothermal titration calorimetry (ITC), using human plasma as substrate, overcomes several limitations of previously used techniques. The high sensitivity of ITC allows continuous recording of the heat released during hydrolysis. Both initial rates and kinetics for complete hydrolysis of plasma lipids can be studied. The heat rate was shown to correspond to the release of fatty acids and was linearly related to the amount of added enzyme, either purified LPL or postheparin plasma. Addition of apoC-III reduced the initial rate of hydrolysis by LPL, but the inhibition became less prominent with time when the lipoproteins were triglyceride poor. Addition of angiopoietin-like protein (ANGPTL)3 or ANGPTL4 caused reduction of the activity of LPL via a two-step mechanism. We conclude that ITC can be used for quantitative measurements of LPL activity and interactions under in vivo-like conditions, for comparisons of the properties of plasma samples from patients and control subjects as substrates for LPL, as well as for testing of drug candidates developed with the aim to affect the LPL system.**—Reimund, M., O. Kovrov, G. Olivecrona, and A. Lookene. **Lipoprotein lipase activity and interactions studied in human plasma by isothermal titration calorimetry.** ***J. Lipid Res.*** **2017.** 58: **279–288.**

Supplementary key words lipolysis • apolipoproteins • angiopoietin-like proteins • triglycerides • very low density lipoprotein • enzymology

The hydrolytic breakdown of plasma triglycerides by LPL at the capillary endothelium is a crucial event that contributes to control of the levels of triglycerides in plasma (1, 2). Many recent studies support the view that an elevated level of triglycerides in plasma is an independent risk factor for development of atherosclerosis (3–5). Therefore, the LPL system is considered to be an interesting target for drug design (6, 7).

LPL is produced and secreted from parenchymal cells like adipocytes and myocytes for transport to the luminal side of the endothelium via interaction with the glycosylphosphatidylinositol-anchored high density lipoprotein binding protein 1 (GPIHBP1) (8). Several plasma components have been shown to directly or indirectly modulate the activity of LPL. apoC-II and apoA-V increase the activity of LPL, while apoC-I, apoC-III, angiopoietin-like protein (ANGPTL)3, ANGPTL4, and ANGPTL8 decrease the activity (1, 9). The expression of each of these proteins depends on nutritional and hormonal factors, so that lipid uptake in tissues to a large extent is regulated by posttranslational effects on LPL (1, 9). It is possible that the macromolecular environment in plasma itself may be an influence on the interaction of LPL with its ligands. The protein concentration of plasma (80 g/l) has been shown to cause significant crowding effects (10). It is also possible that some plasma regulators of LPL activity have not been identified yet.

LPL activity can be measured in vitro by using artificial, usually emulsified, systems of radiolabeled, fluorogenic, or chromogenic substrates, or isolated triglyceride-rich lipoproteins (TRLs). The reaction products are detected at certain time points by chemical quantification or by determination of radioactivity or fluorescence. These methods have been used to unravel important aspects of the action of LPL and also to quantitate the levels of LPL activity in cells and tissues. Only small amounts of LPL activity are normally present in the circulating blood (11). Therefore, intravenous injections of heparin are made to release LPL from its endothelial binding sites. Determination of LPL activity in postheparin plasma, using artificial substrate systems, is considered to give an estimation of the amount of active LPL at the vascular endothelium (12).

Lack of a suitable technique for continuous monitoring of triglyceride hydrolysis in plasma has hampered the understanding of the action of LPL under physiological

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Abbreviations: ANGPTL, angiopoietin-like protein; EL, endothelial lipase; GPIHBP1, glycosylphosphatidylinositol-anchored high density lipoprotein binding protein 1; ITC, isothermal titration calorimetry; TRL, triglyceride-rich lipoprotein.

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conditions. The properties of the substrate lipoproteins are likely to change during the lipolysis. When core triglycerides are removed, lipolysis products like monoglycerides and fatty acids may accumulate on the surface, the particle size will decrease, the surface pressure will increase and there will be an exchange of apolipoproteins between TRLs and other lipoproteins in plasma (13). Fatty acids can affect LPL either directly or through binding to some of its ligands, like ANGPTL4 (14, 15). Because the physical properties of the lipid substrate are sensed by LPL, there are several examples demonstrating that the activity of LPL on nonphysiological substrates is less affected by its regulator proteins (16) and is more resistant to thermal inactivation (17) or proteolytic cleavage (18) than when lipoproteins are used as substrates. Therefore, it is unlikely that determination of LPL activity in samples of postheparin plasma using artificial substrate systems will give a sufficiently good image of the lipolysis event in vivo.

In the present study, we demonstrate that isothermal titration calorimetry (ITC) overcomes a number of the limitations of other techniques for measurement of LPL activity. ITC provides a continuous assay using the observable heat rate that is directly proportional to the rate of the lipolysis (19). Raw data from ITC experiments are presented as thermograms in which the changes in the heat rate (also named heat flow, thermal power or heat flux) are monitored at a constant temperature. The method can be easily automated. We demonstrate that ITC can be used for determination of LPL activity on lipoproteins in human plasma. Both initial rates (zero-order kinetics) and kinetics for complete lipolysis can be measured. ITC can also be used for investigations of the effects of activating and inhibiting proteins on LPL activity. The ITC-based approach proposed in this report should be suitable for testing of drug candidates that are developed for targeting LPL activity.

MATERIALS AND METHODS

Reagents

Bovine LPL was purified from milk (20) and dialyzed to buffer containing 10 mM TRIS (pH 8.5, 4°C) and 4 mM sodium deoxycholate. Stock solutions of 0.5 mg LPL per milliliter were stored at −80°C. The N-terminal coiled-coil domain of human ANGPTL4, residues 26-184, was expressed in *Escherichia coli* and purified as described (14). Full-length human ANGPTL3, expressed in *Sf*21 cells, was obtained from R&D Systems (USA). apoC-III$_0$ was purified from human plasma (21). Human apoC-II and human apoA-V were expressed in *E. coli* and purified as described (22, 23). A synthetic peptide corresponding to the N-terminal domain of human GPIHBP1, residues 23-51, was purchased from Caslo (Denmark). The sequence of the peptide was as follows: QQEEEEEDEDHGPDDYDEEDEDEVEEEET. Antibodies to human HL were raised in a goat against HL isolated from human postheparin plasma (24). The IgG fraction was isolated using Protein-A columns and the final preparation contained 5 mg protein per milliliter in 20 mM Na-phosphate buffer and 0.15 M NaCl (pH 7.4).

Decoded samples of human plasma (treated with EDTA) were obtained from the Tallinn Blood Centrum. Blood was taken by forearm vein puncture from healthy 20- to 30-year-old volunteers 2 h after they had eaten a normal meal. Cells were removed from plasma by centrifugation for 30 min at 2,000 *g* at 4°C. The plasma samples were aliquoted and stored at −80°C and were only thawed once. EnzyChrom triglyceride assay kit (BioAssay Systems, USA) or Triglyceride Colorimetric assay kit (Cayman, USA) were used for determination of triglyceride concentrations. Fatty acids were quantified by the NEFA-HR (2) kit (Wako Chemicals). A sample of human postheparin plasma (used as LPL standard for many years in the Olivecrona group at Umeå University) was from a male volunteer that had received 100 IU heparin per kilogram body weight by intravenous injection in one forearm. After 15 min, blood was collected from the other arm into heparinized tubes and plasma was collected by centrifugation (11, 12). Human VLDLs were purified from normal plasma by floatation in the ultracentrifuge at d = 1.006 g/ml (25). The final preparation contained 1.3 mg protein per milliliter and 2.5 mM triglycerides. Commercial human VLDL was purchased from Kalen Biomedical (USA). This preparation contained 1 mg protein per milliliter and 4.2 mM triglycerides. Goat serum was from Invitrogen (product code 10000C). Intralipid (a 20% phospholipid-stabilized emulsion of soy bean triglycerides used for parenteral nutrition of patients) was obtained from Sigma. Heparin was purchased from LEO Pharma (Denmark).

Sample preparation

Before the experiments, the plasma samples were diluted 1.2 times with TRIS buffer (pH 7.4) or with the additions specified for each experiment. The final concentration of TRIS was 20 mM in all cases. The stock solution of bovine LPL was diluted in cold 10 mM TRIS (pH 8.5) containing 4 mM sodium deoxycholate. In this buffer, LPL is stable for a long period of time, even at low protein concentrations. The final concentration of deoxycholate during incubations with plasma or lipoproteins was 10 to 100 times lower than the initial. Control experiments showed that these levels had no influence on the enzymatic reaction. For inhibition of HL activity in postheparin plasma, 0.5 vol of goat anti-human HL IgG or the corresponding volume of PBS were added to the plasma. The mixture was then incubated for 2 h on ice prior to the experiments (24). All samples were degassed under vacuum for 15 min before the ITC experiments.

ITC measurements

Most of the experiments were performed on a Nano ITC model 5300 (TA Instruments, USA) at 25°C. A MicroCal Auto-iTC200 (GE Healthcare) instrument was used for experiments on the relationship between total heat production and released fatty acids. In a typical experiment performed by Nano ITC, the lipase substrate (plasma, Intralipid 20%, or VLDL) with or without added ligands was placed in the calorimetric cell (1,035 μl) and the syringe (250 μl) was filled with LPL-containing solution (bovine LPL or postheparin plasma) (see **Fig. 1A**). The reference cell contained MilliQ water (1,032 μl). The stirring speed in the sample cell was 400 rpm. The baseline was left to stabilize for at least 1 h before LPL or postheparin plasma was injected. The first injection was 2 μl and after that single or sequential injections of 10 to 25 μl were made. The interval between the injections was from 200 to 500 s. In experiments performed by MicroCal Auto-iTC200, 400 μl of lipase substrate (plasma), 400 μl of equilibration buffer (20 mM TRIS, pH 7,4), and a sufficient volume of LPL (0.8 μM) were placed on the loading plate. The calorimetric cell (200 μl) was rinsed with equilibration buffer and loaded with 200 μl of substrate in an automated fashion. The syringe was loaded with 40 μl of LPL-containing solution. The stirring speed in the sample cell was 600 rpm and the baseline was stabilized automatically. The first injection of LPL was 0.2 μl and the second injection was 2 μl. The experiment time was 1 h to allow full hydrolysis of

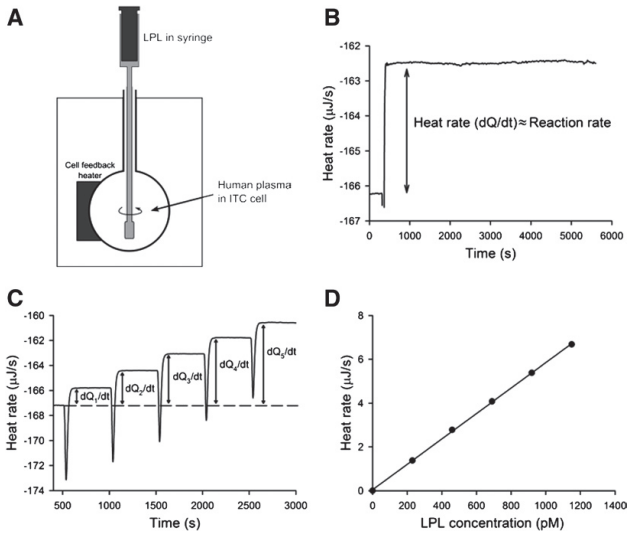

Fig. 1. Enzymatic activity of LPL recorded by ITC. A: Schematic model of the ITC system. The ITC cell is filled with human plasma. LPL is injected to the cell from the syringe-stirrer. B: Heat rate change as a result of a single injection of LPL into ITC cell containing 1 ml of human plasma. Triglyceride and LPL concentrations in the ITC cell were 2.7 mM and 500 pM, respectively. The change of heat rate, dQ/dt, is proportional to the reaction rate. C: Sequential injections of LPL into the same aliquot of plasma as in (B). Each injection increased LPL concentration in the ITC cell by 230 pM. D: Data obtained from (C) presented as relationship of heat rate versus LPL concentration.

plasma triglycerides. After each experiment the Nano ITC sample cell was washed with the following solutions, one after the other: MilliQ water, 2% SDS, 40 mM NaOH, and finally 95% ethanol. The MicroCal Auto-iTC200 was washed with MilliQ water, 10% Decon90 (Decon Laboratories Ltd.), and 100% methanol. Raw ITC data were analyzed using the NanoAnalyze (TA Instruments) or MicroCal Origin (GE Healthcare).

HL assay

Measurements of HL activity were performed using a gum arabic-stabilized emulsion of ^{3}H-triolein and soy bean triglycerides (24). For measurement of HL activity, samples corresponding to 10 μl of postheparin plasma were incubated (in triplicates) in a total volume of 200 μl emulsion mixture for 40 min at 25°C. One unit of enzyme activity corresponds to release of 1 μmol fatty acid per minute.

RESULTS

ITC can be used for measurements of LPL activity in human plasma

To evaluate whether ITC could be used for studies of LPL action, we first tested the stability of LPL during the ITC experiments. The ITC cell was filled with human plasma and LPL was injected (Fig. 1A). As can be seen in Fig. 1B, this resulted in an increase of the heat rate, which then remained constant for the duration of the experiment (5,500 s). This demonstrated, as expected, that the reaction was exothermic, and also that the catalytic activity of LPL was unchanged during the whole experiment. The reaction followed zero-order kinetics, meaning that consumption of the substrate (presumably triglycerides and phospholipids in plasma lipoproteins) or changes of the physical properties of the substrate due to lipolysis did not influence the reaction rate. Sequential injections of LPL into the ITC cell led to a step-wise increase of the heat rate (Fig. 1C). The heat rate level increased almost equally with each injection, indicating a proportional relationship between the concentration of LPL and the reaction rate (Fig. 1D).

Next we compared the activity of LPL in plasma to that recorded with isolated human VLDL or with the synthetic lipid emulsion, Intralipid (with goat serum as a source of apoC-II), as substrate (**Fig. 2**). At the same initial triglyceride concentration (1 mM), the heat rate level was proportional to the concentration of LPL in all three systems, at least up to 400 pM (Fig. 2). The observed heat production rate was almost the same, suggesting that the total heat production was mainly due to hydrolysis of triglycerides. The calculated *P* values (unpaired, two-tailed distribution; pairwise comparison between slopes of the lines) were as follows: plasma/VLDL, $P = 0.006$; plasma/Intralipid, $P = 0.07$; VLDL/Intralipid, $P = 0.2$. The lowest detectable concentration of LPL differed only slightly between the substrate systems. It is generally accepted that a measurement is reliable when the determined parameter is at least ten times over the noise level. The noise level, calculated as the standard deviation of the heat rate level, was found to be 24.6 ± 2.1 nJ/s. Hence, for reliable determinations of LPL activity the change of heat rate must be over 250 nJ/s. Based on the slope of the relationship between heat rate and LPL concentration (Fig. 2, human plasma), it was possible to determine that 50 pM is the lowest concentration of LPL that can be reliably measured by the ITC instrument when the triglyceride concentration is 1 mM. The broad linearity range demonstrates that the ITC technique is suitable for quantitative measurements of LPL activity.

To investigate whether ITC could be used for measurement of LPL activity in human postheparin plasma, we injected small amounts of postheparin plasma from the syringe into the ITC cell containing triglyceride-rich normal human plasma. As with purified LPL, a linear relationship

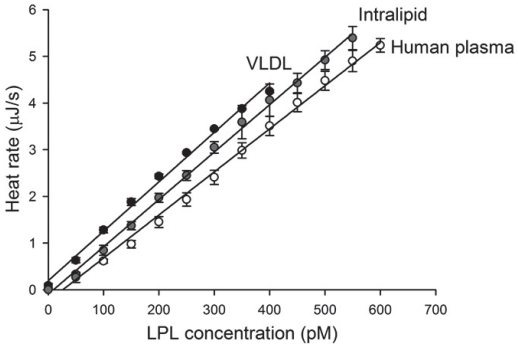

Fig. 2. ITC-based assays for determination of LPL activity using different substrate systems. Sequential injections of LPL into human plasma, substrate mixture containing human VLDL, or substrate mixture containing Intralipid. The initial triglyceride concentration was 1 mM in all cases. Each injection increased the LPL concentration by 50 pM. The slopes of the lines were as follows: human plasma 0.0092, Intralipid 0.0102, and VLDL 0.0106. The human plasma was diluted to contain 20 mM TRIS (pH 7.4). The substrate mixture used for VLDL contained 10 mg BSA/ml, 10 IU heparin/ml, 20 mM TRIS (pH 7.4), and 0.15 M NaCl. The substrate mixture used for Intralipid contained 63% inactivated serum from goat (as source for apoC-II) in addition to the mentioned constituents. The values are mean ± SD of three different measurements.

between the amount of postheparin plasma and the heat rate level was observed (**Fig. 3A**). Because postheparin plasma contains another triglyceride lipase in addition to LPL, named HL based on its tissue origin (26), we pretreated a sample of postheparin plasma with antibodies known to specifically inhibit HL (12, 24). The antibody concentration used in these experiments (1.5 mg/ml) had been shown to be sufficient to completely inhibit activity of HL (12, 24). After injection to the ITC cell, the slope of the heat rate versus plasma volume was only slightly decreased with plasma containing the antibodies compared with the original postheparin plasma diluted to the same extent with buffer. Analysis by *t*-test revealed that the differences between the slopes of the two lines (post-heparin plasma with and without the inhibitory antibody) were not significantly different ($P = 0.13$; paired, two-tailed distribution). To verify that the anti-HL IgG was able to fully inhibit HL, we made concentration curves with different amounts of IgG using ITC (Fig. 3C). We also used a specific HL assay with radiolabeled substrate to measure the remaining HL activity (Fig. 3D). These results demonstrate that the heat rate detected with injection of postheparin plasma to the ITC cell was almost fully due to LPL. Based on comparison of the activity of the purified LPL with that detected by injection of postheparin plasma (Fig. 2, human plasma and Fig. 3A), we estimated that the sample of postheparin plasma contained 7.3 pmol LPL per milliliter. This equals about 0.8 μg LPL/ml.

To demonstrate that the ITC assay for LPL activity could be adopted for general use, measurements were performed with a commercial preparation of human VLDL (Fig. 3B). The triglyceride concentration was lower (0.42 mM) in this preparation than in the previously used plasma samples (1 mM), and the recorded LPL activity was lower than that obtained in Fig. 3A (about one-fourth). The activity was, however, sufficiently high for a linear determination based on sequential injections of postheparin plasma to the ITC cell (Fig. 3B).

ITC can be used to record complete hydrolysis of plasma lipids by LPL

In the next experiments, ITC was used to monitor the kinetics for complete hydrolysis of available substrate lipids in plasma by LPL. For practical reasons, the amounts of LPL used were 40–100 times higher than those used for determination of initial rates. Examples of hydrolysis curves (run in triplicates) for two plasma samples, that differed in their initial triglyceride concentrations by a factor of 2.7, are shown in **Fig. 4A**. The areas under the curves correspond to the total heat production. The areas differed by a factor of 2.6, indicating a good correlation between total heat production and the initial triglyceride concentration of the plasma samples. The amounts of fatty acids released, as determined by the NEFA kit at the end of each reaction, were approximately 2-fold higher than the initial triglyceride concentrations in the plasma samples. This is in agreement with LPL being known to catalyze hydrolysis of the ester bonds at positions *sn*-1 and *sn*-3 of triglycerides (27) and that isomerization of acyl groups from the *sn*-2 position to the *sn*-1(3) positions is slow at pH 7.4. Thus, the actual substrate concentration can be considered to be equal to the concentration of hydrolyzable ester bonds, which is two times higher than the triglyceride concentration. Using results from several experiments, with plasma samples from different individuals, a linear correlation was found between the total heat production and the amounts of fatty acids released (Fig. 4B). The slope of this relationship was used for calculation of the apparent enthalpy ΔH, using the equation: $Q = [P]V\Delta H$, where Q is the total heat production, P is the concentration of released fatty acids, and V is the volume of the ITC cell (19). This calculation resulted in a ΔH value equal to 38.8 kJ/mol. To further analyze the curves for complete hydrolysis in Fig. 4A, the data were transformed into a plot of reaction rate versus remaining substrate concentration (Fig. 4C). This was obtained by subtracting the amount of hydrolyzed substrate at a chosen time point from the concentration of hydrolyzable ester bonds (the total area). This transformation enabled us to examine how the reaction rate depended on the substrate concentration, using data from a single hydrolysis curve. As can be seen, the reaction rate for the samples differed when the substrate concentration was high, but the rates were overlapping in the lower substrate range. This indicates that there might be detectable differences in the properties of plasma samples with regard to their ability to undergo hydrolysis by LPL.

ITC can be used to study the influence of added regulators on the LPL reaction in plasma

In the next set of experiments, we examined how the LPL activity in plasma was influenced by addition of apoC-II, apoC-III, apoA-V, ANGPTL3, or ANGPTL4. Experiments

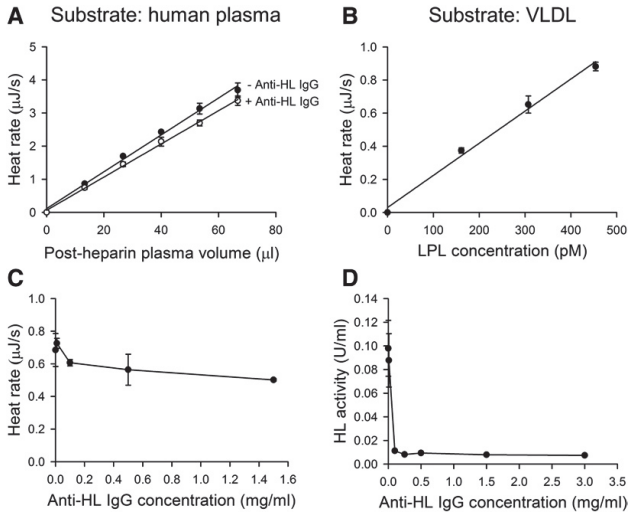

Fig. 3. LPL activity in postheparin plasma as measured by ITC. A: Five sequential injections of human postheparin plasma with antibodies to HL (+ Anti-HL IgG) or without (− Anti-HL IgG) were made to 1 ml of normal human plasma (the final concentration of triglyceride was 1 mM). The final concentration of anti-HL IgG was 1.5 mg/ml. B: Three sequential injections of human postheparin plasma were made into 1 ml of substrate mixture containing commercial VLDL (the final concentration of triglyceride and protein were 0.42 mM and 0.1 mg/ml, respectively), and 10 mg BSA/ml, 10 IU heparin/ml, 20 mM TRIS (pH 7.4), and 0.15 M NaCl. C: Single injections of postheparin plasma preincubated with IgG to HL were made to normal human plasma. The concentrations of anti-HL IgG during preincubations were as follows: 0 mg/ml; 0.01 mg/ml; 0.1 mg/ml; 0.5 mg/ml; and 1.5 mg/ml. D: Measurements of remaining HL activity after preincubation of human postheparin plasma with anti-HL antibodies using a radiolabeled triolein/gum arabic emulsion incubated at 1 M NaCl. Postheparin plasma was preincubated with antibodies to HL at the following concentrations: 0 mg/ml; 0.01 mg/ml; 0.1 mg/ml; 0.25 mg/ml; 0.5 mg/ml; 1.5 mg/ml; and 3.0 mg/ml. The values are mean ± SD of three determinations.

with apoC-II were carried out using Intralipid as substrate, because plasma normally contains sufficient amounts of apoC-II for full activation of LPL (28). The LPL concentration was held constant, while the apoC-II concentration was varied from 0 to 1,000 nM. ITC thermograms are presented in **Fig. 5A**. As expected, addition of apoC-II caused increased activity of LPL. To estimate the activation factor and the affinity of apoC-II for LPL, the heat rate values at 200 s were plotted against the apoC-II concentration (Fig. 5B). As can be seen, apoC-II activated LPL in a saturating fashion. For the analysis of the apoC-II activation data, we used a model proposed by Quinn, Shirai, and Jackson (29). The estimated maximal activation factor was 4.2 ± 1.2 and the K_d value was 22 ± 8 nM. The real time recordings demonstrated that LPL was unstable in the absence of apoC-II, or when the concentration of apoC-II was below 100 nM. Thus, in addition to activation, apoC-II also stabilized LPL. This is in accord with results from previous studies (30). The stabilizing effect of apoC-II is usually not recognized with other substrate systems used for measurements of LPL activity. The advantage with ITC is that the continuous monitoring of the reaction rate provides detailed information about the first minutes of the reaction.

For studies of the effects of apoC-III on the activity of LPL, experiments were conducted: *1*) under zero-order conditions when the LPL concentration was so low that the reaction rate was constant because the substrate was not significantly depleted (**Fig. 6A**); or *2*) under conditions with higher concentrations of LPL resulting in complete hydrolysis of the substrate (Fig. 6B). Under zero-order

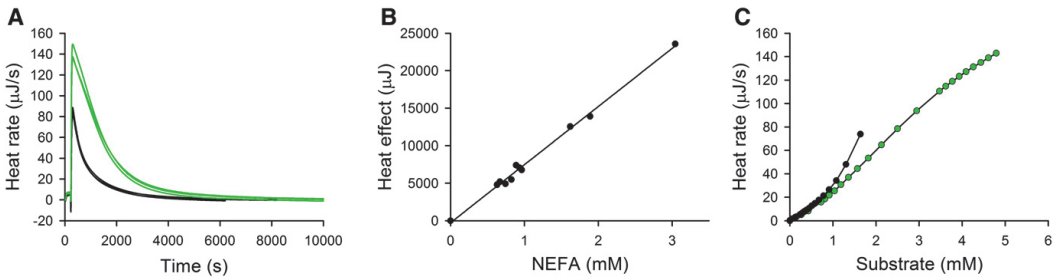

Fig. 4. Complete hydrolysis of lipids in human plasma by LPL as measured by ITC. A: Raw thermograms of total hydrolysis of human plasma lipids by LPL. Experiments were performed with plasma samples that contained 1 mM (black lines) or 2.7 mM (green lines) triglycerides. The concentration of added LPL was 22 nM. Three measurements for each plasma sample are presented. B: Relationship between the total heat production and the amounts of released fatty acids. Heat rates were obtained from full hydrolysis of 10 different human plasma samples on MicroCal Auto-iTC200. In these experiments, 200 μl of plasma was hydrolyzed by 8 nM LPL for 1 h. The surface areas of the curves for full hydrolysis were calculated in the MicroCal Origin and compared with the release of free fatty acids (final concentration minus initial concentration) measured using a NEFA kit. C: Transformation of the hydrolysis data presented in (A) into the relationship between heat rate and remaining substrate concentration (expressed as concentration of hydrolyzable ester bonds).

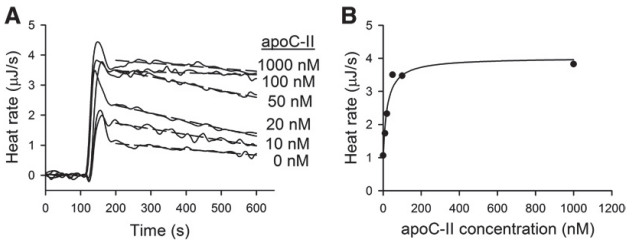

Fig. 5. Activation of LPL by apoC-II as studied by ITC. A: Raw ITC thermograms of hydrolysis of Intralipid by LPL in the absence or presence of apoC-II. The substrate mixture composition in the ITC cell was as follows: Intralipid corresponding to 1 mg triglyceride/ml, 10 mg/ml BSA, 10 IU/ml heparin, 0.15 M NaCl, and 10 mM TRIS (pH 7.4). Single injections of LPL were made in experiments that contained or did not contain human apoC-II. The concentration of LPL was 1.2 nM in all experiments. B: Hydrolysis heat rate as a function of apoC-II concentration. Heat rate points at 200 s were obtained from (A). The data were fitted using the following equation: $v = k_{cat} \cdot L \cdot [(\beta \cdot C + K_d)/(C + K_d)]$, where v is the reaction rate, k_{cat} is catalytic rate constant, L is the concentration of LPL, C is the concentration of apoC-II, β is the activation factor that indicates how much more active the LPL/apoC-II complex is compared with LPL alone, and K_d is the equilibrium dissociation constant (29).

conditions, a reduction of the reaction rate by apoC-III was observed at the start of the reaction and the reduction remained unchanged for more than 1 h (data not shown). This indicated that apoC-III affected the activity, rather than the stability of the enzyme. On sequential injections of LPL into plasma under zero-order conditions, without and with added apoC-III (15 or 50 μM), the presence of apoC-III clearly restricted the activity of LPL (Fig. 6A). Under conditions for complete lipolysis, addition of apoC-III caused an initial inhibition of lipolysis (Fig. 6B). With time, when much of the available substrate had been degraded, the heat rates without and with added apoC-III became similar. Transformation of the ITC data from Fig. 6B into plots of reaction rate versus calculated remaining substrate concentration illustrated that apoC-III inhibited LPL activity only when lipoproteins with high triglyceride content still remained (Fig. 6C). The added apoC-III (15 μM) corresponded approximately to a doubling of the concentration of apoC-III in normal human plasma (31).

It was interesting to test the effect of apoA-V in the ITC system. Evidence in vivo point to a stimulation of LPL activity by apoA-V, but the effect has been difficult to reproduce in vitro (32). Using ITC and zero-order conditions, low concentrations of apoA-V (10 nM) did not stimulate the activity of LPL (Fig. 6D). On the contrary, higher concentrations of apoA-V (100 nM) seemed to inhibit LPL activity. It should be noted that the normal concentration of apoA-V in human plasma is considerably lower, about 4 nM (33).

ANGPTL4 is considered to be a major regulator of LPL activity in vivo (9). The normal range for ANGPTL4 in plasma is between 0.04 and 3 nM (34). In experiments with ITC, suppression of LPL activity was seen by the presence of ANGPTL4 (the N-terminal fragment). A 10% drop of LPL activity was detected almost from the start of the experiment by the presence of added ANGPTL4 corresponding to an increase of the plasma concentration by only 10 nM. The decrease in activity persisted for the duration of the experiment (**Fig. 7A**). With higher concentrations of added ANGPTL4 (corresponding to 100 nM or 1,000 nM), the activity of LPL initially dropped more, and the heat rate continued to decrease after the first drop (Fig. 7A). These observations indicated that ANGPTL4 may affect LPL activity in plasma through two different mechanisms, one immediate that may involve inhibition of activity, but likely not irreversible inactivation. The other, seen at higher concentrations of ANGPTL4, is likely reflecting continuous irreversible inactivation of LPL. This occurred even though the enzyme was stable in plasma under the experimental conditions and the measurements were performed under the zero-order conditions, when the decrease of available substrates should not influence on the activity of LPL.

Finally we wanted to study the effects of the LPL-binding protein, GPIHBP1, shown by others to stabilize LPL against inactivation by ANGPTL4 (35). Also, the acidic N-terminal peptide of GPIHBP1 is known to bind and stabilize LPL (36, 37). When added to the ITC system under zero-order conditions, the peptide did not protect LPL from inactivation by ANGPTL4 (Fig. 7B). Studies with full-length ANGPTL3 demonstrated that it had similar effects on LPL as the N-terminal fragment of ANGPTL4, but ANGPTL3 appeared to be less potent than ANGPTL4 (Fig. 7C).

DISCUSSION

We have demonstrated that ITC can be used to obtain reliable and reproducible data illustrating the action of LPL on plasma lipoproteins. The advantage with ITC is the possibility to perform continuous measurements of LPL activity in plasma, using changes in heat rate for determination of the reaction rate. There is, in general, a linear relationship between the reaction rate and the heat rate (19). However, in the case of lipolysis in plasma, the heat rate includes heat effects of at least four different processes: hydrolysis of ester bonds, structural changes of the lipoproteins due to removal of lipids, neutralization of the released

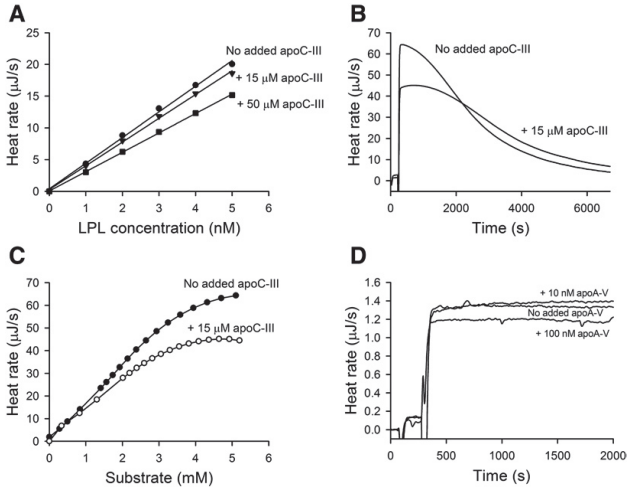

Fig. 6. Effect of apoC-III and apoA-V on LPL activity in plasma as studied by ITC. A: Effect of apoC-III on LPL activity under zero-order reaction conditions. Sequential injections of LPL were made into plasma samples in which the apoC-III concentration was increased by 15 μM, 50 μM, or not increased. The LPL concentration was increased by 1 nM per injection. The concentration of triglycerides in the ITC cell was 2 mM. B: Thermograms for complete hydrolysis by LPL in plasma when the concentration of apoC-III was not increased or was increased by 15 μM. The final concentration of LPL was 22 nM and the concentration of triglycerides was 2.7 mM. One representative curve from three different measurements is presented for each condition. C: Dependence of heat rate on substrate concentration as calculated from the curves presented in (B). D: LPL activity was measured in plasma in the absence or presence of added apoA-V corresponding to 10 nM or 100 nM. Single injections of LPL into the plasma were made. The concentrations of triglycerides and LPL in the ITC cell were 1 mM and 0.6 nM, respectively.

fatty acids by the buffer, and binding of the fatty acids to albumin. In spite of this complexity, several observations suggest that the heat rate is a suitable quantitative parameter for measurement of catalytic activity of LPL in plasma. The arguments are: *1*) that the heat produced by the action of LPL was proportional to the amount of fatty acids released; *2*) that LPL was stable in the ITC cell for a long period of time; *3*) that there was a linear dependency between the amount of LPL added to the ITC cell and the heat rate; *4*) that on complete hydrolysis of the plasma lipids, the total heat production was linearly dependent on the initial triglyceride concentration; and *5*) that the heat effect was nearly the same with the synthetic triglyceride emulsion Intralipid as with isolated VLDL or with triglyceride-rich plasma as substrate for LPL. Based on this, it is reasonable to assume that heat effects due to structural changes of the lipoproteins, or to other interactions established in the system, are negligible in comparison with those due to hydrolysis of ester bonds.

The ITC assay used for measurement of initial rates was sensitive enough to determine the levels of lipase activity in human postheparin plasma, but the activity present in preheparin human plasma was too low to be detected. The level of LPL activity in plasma before injection of heparin is only around 1% of that in postheparin plasma (12). In addition to LPL, heparin injection also releases endothelial lipase (EL) and HL from the vascular endothelium. Like LPL, these enzymes catalyze hydrolysis of triglycerides and phospholipids, and they may contribute to the ITC measurements. However, their substrate specificity is different from that of LPL. In the case of HL, we used inhibiting antibodies to demonstrate that HL did not significantly contribute to the lipase activity during the ITC assay. HL acts preferably on phospholipids in HDL and smaller VLDL, but it can also hydrolyze ester bonds in triglycerides (24, 38). Although it does not seem necessary in the ITC assay, we still advocate the use of inhibiting antibodies to HL for specific analyses of LPL activity in postheparin plasma. Slight variations in the substrate lipoproteins, or in the assay conditions, could otherwise allow HL to contribute to the hydrolysis. In the case of EL, it has been shown that TRLs are poor substrates (38). Furthermore, Badellino et al. (39) have shown that heparin administration increases the

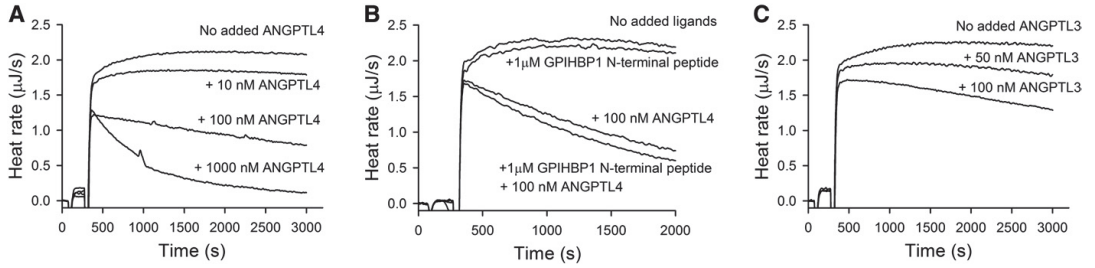

Fig. 7. Effects of ANGPTL4 or ANGPTL3 on LPL activity in plasma as studied by ITC. In (A) and (C), the LPL activity was measured in the absence or presence of different added concentrations of ANGPTL4 (A) or ANGPTL3 (C). In (B), the effect of ANGPTL4 on LPL was tested in the presence of the N-terminal peptide of GPIHBP1. In all experiments a single injection of LPL into the plasma sample was made. The concentrations of triglycerides and LPL in the ITC cell were 1 mM and 0.6 nM, respectively.

EL concentration in plasma only 3-fold. For comparison, LPL increases it more than 100-fold. In our ITC experiments, postheparin plasma was diluted 1.5 times with PBS or antibodies. Then 20 μl of this diluted sample was injected into the ITC cell, which contained 1 ml triglyceride-rich plasma. It follows that the concentration of EL was not significantly increased in the ITC cell after injection of postheparin plasma. Inhibiting antibodies to EL could be used to verify this conclusion.

The ITC assay is an alternative to assays that use radiolabeled (40) or fluorogenic substrates (41, 42), or directly measure released fatty acids by NEFA kits (43). Because plasma contains free fatty acids at high concentrations, [0.3–1 mM (44)], the NEFA kit is applicable only when significant amounts of fatty acids have been produced by the added LPL. In addition, most of the mentioned assays cannot be used for continuous registration of the lipolysis, and they are more laborious and time consuming than the ITC measurements. The use of plasma as substrate source is an advantage because plasma contains the natural substrate for LPL in the form of TRLs. The lipoproteins do not need to be isolated, meaning that the possible risk of damaging their native structure or changing their apolipoprotein composition can be avoided. For measurements of LPL activity, or functional studies of LPL and its controller proteins, it is important to use native substrates to be able to pick up functional effects that might be important in vivo. In addition, we demonstrate that commercially available human VLDL, or a synthetic emulsion of triglycerides like Intralipid, can be used as substrate in the ITC.

The broad linearity range, the high sensitivity, and the possibility to use an automatic setup are arguments for ITC for routine measurements of LPL activity. The continuous monitoring of the reaction rate allows accurate zero-order recordings of true initial rates. The stabilization of LPL by addition of apoC-II, as seen in the present study, is usually not detected in other substrate systems. Therefore the magnitude of the activation of LPL by apoC-II can easily be overestimated (30). ITC provided a method to carefully investigate possible effects of apoA-V on the catalytic activity of LPL. Animal experiments (45) and population studies in humans (46, 47) have demonstrated that apoA-V is an important regulator of triglyceride levels in plasma (48). apoA-V lowers triglyceride concentrations in vivo, but its effect has been difficult to reproduce in vitro using studies of LPL activity in different substrate systems (49). Our data from ITC adds to the notion that apoA-V does not directly stimulate the activity of LPL.

In addition to the usual determinations of the initial rates of hydrolysis, ITC allowed monitoring of the kinetics for complete lipolysis of the plasma samples by LPL. Transformation of a single lipolysis time course into a plot of heat rate versus remaining substrate concentration enabled us to follow how the reaction rate was changing during the degradation of TRLs to remnant particles. Comparison of plasma samples from two randomly selected volunteers demonstrated that individual differences could easily be picked up by ITC. This suggests that analyses of curves for total hydrolysis of plasma lipids by LPL can be used to search for possible causes for hypertriglyceridemia in individual subjects. We have not yet systematically explored this possibility.

The proteins apoC-III, ANGPTL3, and ANGPTL4 are known to suppress LPL activity in vivo and are all regarded as potential targets for drug development with the aim to lower triglyceride levels in plasma (6, 7). We found that addition of apoC-III to plasma could only moderately affect the activity of LPL. An increase of apoC-III by 50 μM reduced the activity of LPL by about 30%. These results are in line with the recent results of Gordts et al. (50), but are different from the data obtained with isolated chylomicrons as substrate for LPL where the inhibitory effect of apoC-III was stronger (51). In that case, complete inhibition was observed already by addition of apoC-III to a concentration of 10 μM. Our analyses of the ITC curves for complete hydrolysis of plasma lipids demonstrated that apoC-III affected LPL activity only when there were still TRLs in the system (at low degrees of lipolysis), but not later, when only triglyceride-poor lipoproteins remained (at high degrees of lipolysis). This can possibly be explained by expulsion of apoC-III from the surface of TRLs when their triglyceride content, or their size, is reduced. The distribution of apoC-III between plasma lipoproteins is known to be affected by the total triglyceride content in plasma, so that in plasma from subjects with elevated triglyceride levels, more apoC-III will be found with the TRLs and less with HDLs (52, 53). apoC-III was reported to displace LPL from triglyceride-rich emulsion particles (51). No direct interaction between LPL and apoC-III has so far been detected, meaning that the inhibition probably reflects competition between apoC-III and LPL for binding to the TRLs. Differences in the physical properties of the TRLs, as well as the levels of other plasma components, are likely to determine the binding of LPL to the TRLs, and thereby the lipolysis rate. HDLs are known to sequester apoC-III and other inhibiting apolipoproteins in an exchangeable fashion. ITC provides an easy method to study lipolysis directly in plasma, with all relevant components present, and to compare results from many different subjects.

Studies in recent years have identified some of the ANGPTLs as important regulators of lipid metabolism, and in particular as inhibitors/inactivators of LPL (9). There is as yet no consensus about by what mechanisms ANGPTL3 and ANGPTL4 suppress the activity of LPL. Results from several studies show that ANGPTL4 induces irreversible dissociation of active LPL dimers to inactive monomers (54–56). Shan et al. (57) have proposed that ANGPTL3 and ANGPTL4 inhibit LPL through different mechanisms and that only ANGPTL4 induces irreversible inactivation of LPL. Others have proposed that ANGPTL4 acts as a noncompetitive inhibitor by forming a reversible complex with LPL (58). Using ITC, addition of nanomolar concentrations of ANGPTL4 to plasma was required to see effects on LPL activity. This is at least one order of magnitude higher than the concentrations normally found in human plasma (34), and is in line with LPL being stable in plasma for the duration of the ITC experiments. We conclude that the concentrations of ANGPTL4 and ANGPTL3 in blood are too low to affect the activity of LPL. Our data confirm

the previous results of Nilsson et al. (59) that were obtained using other techniques to measure effects on LPL activity. In the ITC experiments, ANGPTL4 was more efficient in lowering of LPL activity compared with ANGPTL3, but the time courses for the inhibition of LPL were similar for the two proteins. In both cases, it was possible to distinguish an initial fast phase followed by a slow phase. This suggests that the ANGPTLs may use similar inhibition mechanisms in the plasma environment, including both inhibition of LPL activity and irreversible inactivation. It is likely that a reversible complex is first formed between LPL and the ANGPTL, and that this may cause inhibition of the catalytic activity against TRLs and lipoprotein-like substrates. The interaction may then lead to irreversible inactivation of LPL by formation of LPL monomers and dissociation of the ANGPTLs from LPL, as previously suggested (54). It is tempting to speculate that both mechanisms, inhibition and inactivation, may operate on LPL in vivo.

Development of drugs that reduce the inhibitory effect of apoC-III, ANGPTL3 (60), or ANGPTL4 (61) on LPL activity is currently ongoing, with the aim to reduce plasma triglyceride levels in patients at high risk for coronary heart disease. In addition, an apoC-II mimetic peptide was tested to lower plasma triglycerides in subjects with mild to moderate hypertriglyceridemia or in patients with severe hypertriglyceridemia due to deficiency of apoC-II (62, 63). ITC offers a convenient method to study the effects of new drug candidates in a plasma environment. With ITC, it is also possible to directly investigate the cause for the hypertriglyceridemia by direct incubation of plasma samples with LPL.

In the clinical laboratory, ITC can be used to find out whether hypertriglyceridemia in patients is caused by reduced amounts of active heparin-releasable LPL or by an unfavorable composition of the patient's plasma. In the first case, ITC would be an alternative to the standard radioisotope or fluorometric methods previously described. In the second case, ITC can be used to characterize plasma samples according to their properties as LPL substrates. This is a new way to analyze dysfunction of plasma lipoprotein metabolism, not easily attainable by other techniques.

In summary, ITC can be used for quantitative measurements of LPL activity in human postheparin plasma, for real-time recording of complete lipolysis of human plasma lipoproteins by LPL, and for investigations of the effects of some different control proteins on LPL activity in a plasma environment. We propose that studies of the action of LPL by ITC can be used for diagnostic purposes and for basic research, as well as tools in drug development.

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PUBLICATION III

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Apolipoprotein C-II mimetic peptide is an efficient activator of lipoprotein lipase in human plasma as studied by a calorimetric approach

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Apolipoprotein C-II mimetic peptide is an efficient activator of lipoprotein lipase in human plasma as studied by a calorimetric approach

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ABSTRACT

Elevated plasma triglyceride (TG) levels are associated with higher risk of atherosclerotic cardiovascular disease. One way to reduce plasma TG is to increase the activity of lipoprotein lipase (LPL), the rate limiting enzyme in plasma TG metabolism. An apolipoprotein (apo) C-II mimetic peptide (18A—CII—a) has been recently developed that stimulated LPL activity *in vitro* and decreased plasma TG concentration in animal models for hypertriglyceridemia. Since this peptide can serve as a new therapeutic approach for treatment of hypertriglyceridemia, we investigated how 18A—CII—a peptide influences LPL activity in human plasma. We used recently described isothermal titration calorimetry based approach to assess the peptide, which enables the analysis in nearly undiluted human plasma. The 18A—CII—a peptide was 3.5-fold more efficient in stimulating LPL activity than full-length apoC-II in plasma sample from normolipidemic individual. Furthermore, 18A—CII—a also increased LPL activity in hypertriglyceridemic plasma samples. Unlike apoC-II, high concentrations of the 18A—CII—a peptide did not inhibit LPL activity. The increase in LPL activity after addition of 18A—CII—a or apoC-II to plasma was due to the increase of the amount of available substrate for LPL. Measurements with isolated lipoproteins revealed that the relative activation effects of 18A—CII—a and apoC-II on LPL activity were greater in smaller size lipoprotein fractions, such as remnant lipoproteins, low-density lipoproteins and high-density lipoproteins. In summary, this report describes a novel mechanism of action for stimulation of LPL activity by apoC-II mimetic peptides.

1. Introduction

Epidemiological and genetic studies have clearly established that elevated triglycerides (TGs) in plasma are an independent risk factor of atherosclerotic cardiovascular disease (ASCVD) [1,2]. Increasing the activity of lipoprotein lipase (LPL), the main enzyme responsible for the hydrolysis of plasma TGs, is one possible approach to reduce the risk of ASCVD [3]. LPL requires apolipoprotein C-II (apoC-II) for its activity in plasma; homozygotes for apoC-II deficiency have severe hypertriglyceridemia, similarly to genetic LPL deficiency [4]. ApoC-II is a small protein that exchanges between triglyceride-rich lipoproteins (TRLs), such as chylomicrons (CMs) and very low-density lipoproteins (VLDLs), and high-density lipoproteins (HDLs). The mean apoC-II concentration in normolipidemic human plasma is approximately 4.5 μM [5]. Several studies

Abbreviations: apo, apolipoprotein; ANGPTL, angiopoietin-like protein; ASCVD, atherosclerotic cardiovascular disease; CETP, cholesteryl ester transfer protein; CM, chylomicron; HDL, high-density lipoprotein; ITC, isothermal titration calorimetry; LDL, low-density lipoprotein; LPL, lipoprotein lipase; NEFA, non-esterified fatty acid; SEC, size-exclusion chromatography; TG, triglyceride; TRL, triglyceride-rich lipoprotein; VLDL, very low-density lipoprotein.

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performed with isolated lipoproteins and artificial TG emulsions suggest that normal apoC-II levels in plasma are sufficient for full activation of LPL [5–7]. A recent study has revealed that an apoC-II mimetic peptide, named 18A–CII–a, efficiently decreases plasma TG concentration when given to apoE-knockout mice [8]. This was unexpected as apoC-II concentration has been shown to be positively correlated to TG levels in human plasma [9]. Furthermore, transgenic mice overexpressing human apoC-II have hypertriglyceridemia [10].

18A–CII–a is a bi-helical peptide that contains an 18-residue long amphipathic α-helix called 18A in its N-terminus, which binds to lipoproteins with a high affinity. 18A is linked via proline residue to another amphipathic α-helix containing residues 59–79 of human apoC-II that are needed for LPL activation. 18A–CII–a activated LPL similarly to full-length apoC-II when synthetic emulsion of TG was used as a substrate [8]. It also increased LPL activity in *ex vivo* plasma assays from patients with hypertriglyceridemia [8,11]; however, for practical reasons, plasma samples were at least 50-fold diluted in these assays. Dilution of the plasma may influence the effect of different ligands on LPL stability and activity. In addition to apoC-II, plasma contains numerous components that together with apoC-II influence LPL activity and/or stability. ApoC-I and apoC-III on TRLs and circulating angiopoietin-like proteins (ANGPTLs) 3, 4, 8 all inhibit LPL activity, whereas apoA-V increases LPL activity [12]. LPL activity is also stabilized by glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 and heparan sulfate proteoglycans [12–14].

We have recently developed an isothermal titration calorimetry (ITC) assay that allows the analysis of LPL activity, stability and ligand interactions in nearly undiluted human plasma [7]. This assay is based on the detection of the heat rate accompanying LPL hydrolysis of lipids. The ITC-based assay was successfully used to study the effects of apoC-III, apoA-V, ANGPTL3 and ANGPTL4 on LPL activity in undiluted human plasma [7]. In the present study, we applied our ITC-based approach to investigate the mechanism for how the 18A–CII–a peptide and apoC-II influence the activity of LPL in nearly undiluted human plasma.

2. Materials and methods

2.1. Reagents

LPL was purified from bovine milk and dialyzed to buffer containing 10 mM TRIS (pH 8.5, 4 °C) and 4 mM sodium deoxycholate [7]. Recombinant human apoC-II with a C-terminal His_6 tag was expressed in *E. coli* BL21 containing a pET29a plasmid, using an auto-induction protocol [15]. Following expression, cells were lysed in buffer containing 6 M GuHCl and purified with Ni-affinity (HisTrap HP), anion-exchange (HiTrap Q FF) and size-exclusion chromatography (SEC) (Superdex 200 10/300 GL) following standard protocols (GE Healthcare Life Sciences) using the indicated buffers containing 6 M urea. The purified protein was dialyzed against 10 mM ammonium bicarbonate and lyophilized. The 18A–CII–a peptide and an inactive analog called 18A–CII–i were synthesized as previously described [8]. Four residues critical for LPL activation were changed in the 18A–CII–i peptide to alanine [8]. Peptides and apoC-II were dissolved in 35 mM $NaHCO_3$, pH 8.5 buffer, kept at 4 °C and used within one week. Samples of non-fasting human EDTA-plasma were purchased from the Tallinn Blood Centrum. Normolipidemic plasma samples were aliquoted, stored at −80 °C and were used immediately after thawing. Hypertriglyceridemic plasma samples were only used fresh.

2.2. Human plasma separation by SEC

Human plasma was separated using Superose 6 10/300 GL (GE Healthcare Life Sciences) column. 500 μl of nonfasting human plasma (TG = 111 mg/dl) was injected to the column. Elution was performed in a 20 mM TRIS, 0.15 M NaCl, 1 mM EDTA, pH 7.4 buffer. Fractions of 500 μl were collected. TGs and cholesterol were measured in each fraction, using Triglyceride Colorimetric assay kit (Cayman, USA) and Cholesterol Fluorometric Assay Kit (Cayman, USA).

2.3. ITC measurements

Experiments were performed on a Nano ITC model 5300 (TA Instruments, USA) or MicroCal PEAQ-ITC (Malvern Panalytical) at 25 °C [7]. Undiluted human plasma samples were mixed with 20 mM TRIS pH 7.4 buffer and with 18A-CII peptides, apoC-II or 35 mM $NaHCO_3$, pH 8.5 buffer. This resulted in only a 1.2-fold dilution of the plasma samples. Next, plasma samples were loaded to the ITC sample cell. Syringe was filled with purified LPL. To determine initial reaction rates (Fig. 1 and Fig. 3B), five sequential injections of 0.2 nM LPL were made into the ITC cell. The resulting slope of the relationship between heat rate and LPL concentration represented specific activity of LPL. Relative activity of LPL (%) in Fig. 1 was calculated as a ratio of the slope of the experiment where 18A-CII peptides or apoC-II were added to the slope of the experiment where no ligands were added. Sample cells were washed after each experiment [7].

2.4. Non-esterified fatty acid (NEFA) assay for measurement of LPL activity

LPL activity was determined by measuring the generation of NEFAs after lipolysis of human lipoproteins [8] (Fig. 3C) which were normalized to contain 1.7 μg of TGs in incubation mixtures, except HDL, which contained 1.3 μg of TGs. In the case of HDL this was the highest achievable amount. 10 μM 18A–CII–a or apoC-II, 7 nM LPL, 1% BSA, and PBS were added to yield a final reaction volume of 50 μl. Reaction mixtures were incubated for 30 min on ice and then for 1 h at 37 °C. NEFAs were determined using NEFA-HR (2) (FUJIFILM Wako Diagnostics, USA) kit.

3. Results

3.1. Effects of 18A-CII peptides and apoC-II on LPL activity in human plasma

The effects of 18A–CII–a, its inactive analog 18A–CII–i, and full-length apoC-II on LPL activity were first studied by ITC in undiluted human plasma obtained from a subject with normal TG level. The experiments were performed under conditions in which the concentration of LPL was sufficiently low (0.2–1 nM) that consumption of substrate did not influence the reaction rate, meaning that initial rates were determined under zero-order conditions. As can be seen in Fig. 1A, the peptides and apoC-II had different effects on LPL activity. Addition of 18A–CII–a to human plasma increased LPL activity in a concentration-dependent manner, reaching a 77% increase at 50 μM, the highest tested concentration (Fig. 1A). In contrast, apoC-II increased LPL activity up until 20 μM of apoC-II was added, reaching only a maximum increase level of 20%. Higher concentrations led to an inhibition of LPL activity; 14% lower activity compared to baseline was detected when 50 μM of apoC-II was added (Fig. 1A). 18A–CII–i decreased LPL activity in a concentration-dependent manner. The maximum inhibition of 32% occurred when 50 μM

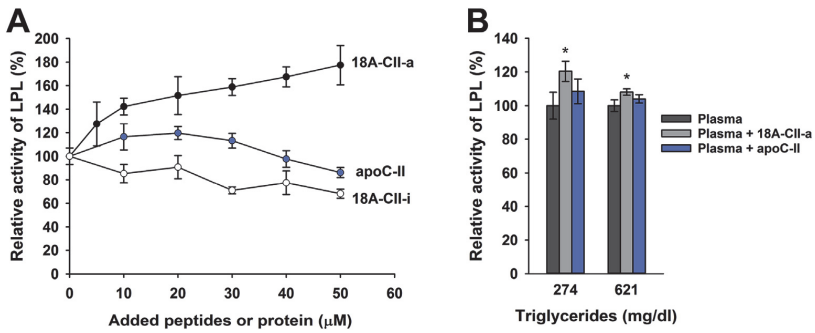

Fig. 1. Effects of 18A-CII peptides and apoC-II on LPL activity in normolipidemic (A) and hypertriglyceridemic (B) human plasma samples as measured by ITC. TG concentration of plasma sample in panel A was 111 mg/dl. In panel B, 10 μM 18A–CII–a, 10 μM apoC-II or buffer was added to pooled hypertriglyceridemic plasma samples with an average of 274 or 621 mg/dl TG concentration. The values are mean ± SD of three different measurements. In panel B, data were compared by two-tailed *t*-test; *P < 0.05 compared with experiments where no ligands were added.

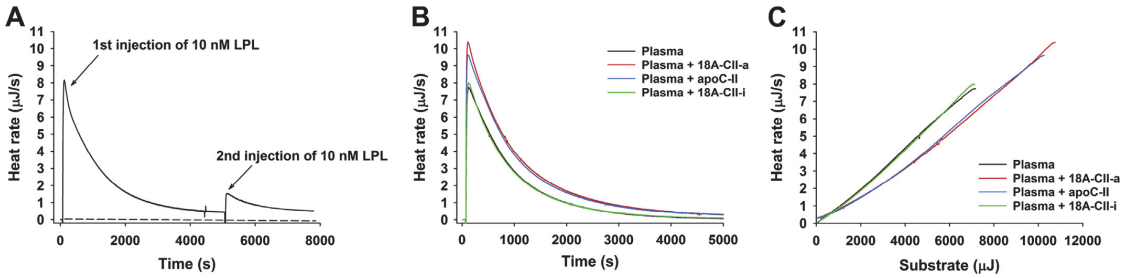

Fig. 2. Complete hydrolysis of available substrate in human plasma by LPL as measured by ITC. (**A**) Two injections of LPL were made into ITC cell containing human plasma. (**B**) One injection of 10 nM LPL was made into ITC cell containing human plasma with no added ligands or with added 10 μM 18A–CII–a, 10 μM apoC-II or 10 μM 18A–CII–i. (**C**) Data presented in panel B was transformed into the relationship between heat rate and remaining substrate concentration (area). TG concentration of the plasma samples were 62 mg/dl. One representative curve from three different measurements is presented for each condition.

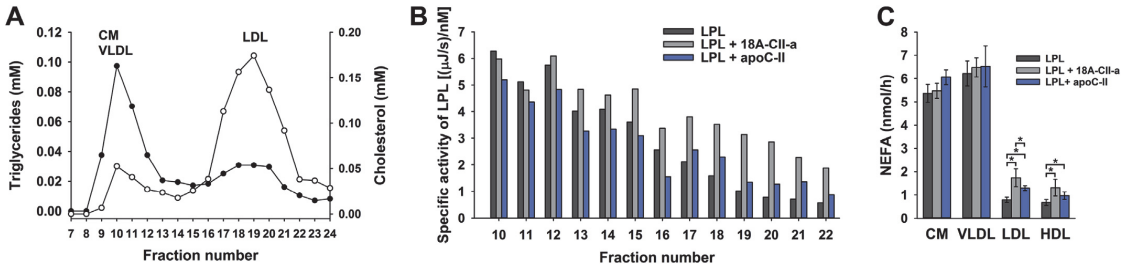

Fig. 3. Effects of 18A-CII-a and apoC-II on LPL activity in human plasma fractions. (A) Human plasma separation using SEC. TGs (●) and cholesterol (○) were measured in each fraction. **(B)** LPL activity determination by ITC in SEC fractions. The fractions were concentrated 5-fold and mixed with 10 μM 18A–CII–a, 10 μM apoC-II or buffer and 10 mg/ml BSA and 10 IU/ml heparin. **(C)** Effects of 18A–CII–a and apoC-II on LPL activity in lipoproteins isolated by density gradient ultracentrifugation as measured by NEFA assay. The values are mean ± SD of four measurements. Data were analyzed by Mann-Whitney test, *P < 0.05.

of 18A–CII–i was added (Fig. 1A). The effects of 18A–CII–a and apoC-II were also tested in pooled hypertriglyceridemic plasma samples (Fig. 1B). Addition of 10 μM 18A–CII–a peptide to pooled plasma samples with a mean TG level of 274 mg/dl (n = 6) or 621 mg/dl (n = 3) resulted in a 20% or 8% increase in LPL activity, respectively. In contrast, increasing apoC-II concentration by 10 μM in these plasma samples did not cause a statistically significant increase in LPL activity.

3.2. Effects of 18A-CII peptides and apoC-II on complete hydrolysis of available substrate in human plasma by LPL

Next, using ITC, we investigated how the addition of 18A-CII peptides or apoC-II influenced the kinetics of complete hydrolysis of available substrate lipids in plasma by LPL. To carry out these measurements within a traceable time period, LPL concentration was increased up to 10 nM, which was 50-fold higher than in the

initial rate experiments presented in Fig. 1. In the first set of experiments, LPL was injected into plasma that did not contain the test peptides or additional apoC-II. After 5000 s, the heat rate level was close to the initial level, suggesting that the reaction catalyzed by LPL was almost complete (Fig. 2A). To be confident that this was not influenced by the inactivation of LPL, a second injection of LPL was made. This resulted only in a relatively small increase of the heat rate, confirming that complete hydrolysis of the available substrate was nearly achieved during the first injection.

The complete hydrolysis experiments were then performed after addition of 10 μM 18A—CII—a, 10 μM 18A—CII—i or 10 μM apoC-II (Fig. 2B). Areas under the complete hydrolysis curves of plasma samples with added 18A—CII—a (area = 10408 ± 953 μJ, mean of 3 measurements ± SD) or apoC-II (area = 9892 ± 1125 μJ) were ~1.5-fold larger than for plasma samples without added ligands (area = 7109 ± 292 μJ). When 18A—CII—i was added there was almost no effect on the area of the complete hydrolysis curve (area = 6913 ± 851 μJ) compared to the reference curve (Fig. 2B). The area under a corresponding thermogram is equal to the total heat production, which is proportional to the hydrolyzed TG concentration of the plasma sample [7]. Thus, these experiments indicated that the addition of 18A—CII—a or apoC-II to human plasma increased the amount of available substrate for LPL.

To further analyze the complete hydrolysis results, the data presented in Fig. 2B were transformed into a relationship between heat rate (reaction rate) and remaining substrate concentration (area) (Fig. 2C). This was obtained by subtracting the hydrolyzed substrate concentration at a chosen time point (area up to this point) from the total substrate concentration (the total area). These relationships revealed how the addition of the peptides or apoC-II influenced reaction rate dependency on substrate concentration. Although there was more available substrate after the addition of 18A—CII—a or apoC-II, the "new available substrate" was not hydrolyzed as efficiently by LPL as the substrate that was first available without the addition of 18A—CII—a or apoC-II (Fig. 2C). The kinetics of lipid hydrolysis by LPL was not changed after addition of 18A—CII—i compared to the experiment where no ligands were added.

3.3. Effects of 18A—CII—a and apoC-II on LPL activity in human plasma fractions

To investigate which lipoprotein fractions LPL activity was increased by the 18A—CII—a peptide, human plasma was separated by SEC (Fig. 3A). The effects of 18A—CII—a and apoC-II were investigated on each plasma fraction separately using ITC (Fig. 3B). As expected, the specific activity of LPL was the highest in the fractions 10—13 that contained TRLs. Interestingly, the addition of 18A—CII—a to fraction 10 or 11 did not increase LPL activity. However, 18A—CII—a modestly increased LPL activity in fractions 12 and 13, containing smaller size TRLs. Addition of extra apoC-II to these fractions even reduced the activity of LPL. Specific activity of LPL decreased as the following fractions containing smaller size lipoproteins were used as a substrate. At the same time, the relative effect of 18A—CII—a on LPL activity started to increase with the following fractions containing remnant lipoproteins. In Table 1, LPL activities from Fig. 3B are presented as a ratio of the specific activity of LPL with added 18A—CII—a or apoC-II to the specific activity of LPL without the added peptide or protein. The relative effect of 18A—CII—a on LPL activity was the highest with fraction number 20, which contains low-density lipoproteins (LDLs) and likely smaller size remnant lipoprotein particles (Table 1 and Fig. 3B). Addition of the peptide to this fraction caused a 3.7-fold increase in LPL activity. Effect of apoC-II in this fraction calculated as a fold change in LPL activity was more than 2 times lower (Table 1).

Table 1
Fold change in LPL activity after addition of 18A-CII-a or apoC-II to human plasma fractions. Fold change was calculated from data presented in Fig. 3B as a ratio of the specific activity of LPL with added 18A—CII—a or apoC-II to the specific activity of LPL without the added ligands.

Fraction number	Fold change in LPL activity	
	18A—CII—a	apoC-II
10	0.95	0.83
11	0.94	0.85
12	1.06	0.84
13	1.2	0.82
14	1.13	0.82
15	1.35	0.86
16	1.32	0.6
17	1.79	1.21
18	2.22	1.45
19	3.1	1.34
20	3.67	1.64
21	3.23	1.93
22	3.26	1.51

Next, the effects of 18A—CII—a and apoC-II on LPL activity were examined in CM, VLDL, LDL and HDL fractions isolated from human plasma by density gradient ultracentrifugation, using a NEFA assay (Fig. 3C). Although the addition of 18A—CII—a or apoC-II to CM or VLDL samples tended to somewhat increase LPL activity, these effects were not statistically significant. Both 18A—CII—a and apoC-II had their largest relative effects on LPL activity in LDL and HDL fractions.

4. Discussion

The novel ITC assay of LPL that we developed enabled us to more carefully investigate drug candidate properties of the 18A—CII—a peptide, whose TG-lowering effect has been shown in various mouse models for hypertriglyceridemia [8,16,17] and in apoC—II—knockout zebrafish [18]. The main advantage of the ITC assay is that it makes it possible to perform experiments in human plasma samples with only minimal dilution, thus insuring that the concentration and proportions of lipoproteins and LPL regulators (activators and inhibitors), as well as other plasma components that can influence the LPL activity remained physiological. Furthermore, the results of measurements in undiluted plasma also take into account the possible macromolecular "crowding effect" of plasma on LPL activity and interactions [19].

Although addition of both apoC-II and 18A—CII—a peptide to plasma increased LPL activity, 18A—CII—a peptide was a more potent activator of LPL than apoC-II. The maximum increase in LPL activity after addition of 18A—CII—a to normolipidemic plasma sample was 3.5-fold greater than in the case of extra apoC-II. Unlike apoC-II, high concentrations of 18A—CII—a did not inhibit LPL activity. The inhibitory properties of apoC-II can possibly be attributed to its N-terminal domain, since this region is different in 18A—CII—a and apoC-II. Although 18A—CII—a increased LPL activity in all tested concentrations in both normolipidemic and hypertriglyceridemic plasma samples, the effect was larger in the samples obtained from normolipidemic individuals.

The observation that the addition of apoC-II or 18A—CII—a peptide to normolipidemic human plasma increased LPL activity was unexpected based on clinical reports of apoC-II deficiency. In general, heterozygotes for apoC-II deficiency have normal TG levels in plasma, suggesting that their plasma contains sufficient amounts of apoC-II for full LPL activation [5]. Our results demonstrate, however, that at least in some cases, LPL is not saturated by apoC-II. This is in line with a recent study that showed that the

downregulation of a long non-coding RNA, termed liver-specific TG regulator (lncLSTR), increased plasma apoC-II levels and LPL activity in mice, which resulted in decreased plasma TG levels [20]. Also, it was recently demonstrated that endogenous apoC-II levels are insufficient in mice to rapidly clear TGs after intravenous injection of TG-rich emulsion. Injection of apoC-II mimetic peptide to these mice helped to overcome the insufficiency of apoC-II and TG-rich emulsion was rapidly cleared [17].

The increase in LPL activity after addition of 18A–CII–a or apoC-II to plasma was due to the increase of the amount of available substrate for LPL. This indicates that part of the substrate in plasma was not available for LPL without addition of 18A–CII–a or extra apoC-II. This also suggests that LPL was not saturated with the substrate, meaning that part of LPL was not active in plasma. It is likely that "inactive" LPL was bound to lipoproteins but was not hydrolyzing TGs. After addition of 18A–CII–a or apoC-II, this part of LPL became activated. It has been shown that while LPL prefers TRLs as a substrate, it can distribute between all classes of lipoproteins in plasma [21,22]. It is possible that some of the particles which became available for LPL after addition of 18A–CII–a simply did not contain enough apoC-II. While the relative effects of 18A–CII–a and apoC-II on LPL activity were greater in smaller size lipoproteins, such as remnant lipoproteins, LDLs and HDLs, 18A–CII–a also slightly enhanced LPL activity in smaller size TRL fractions. Thus, it is likely that fraction of TGs in almost all lipoprotein classes was not available for LPL and became available only after addition of the peptide. Overall, our data suggest that 18A–CII–a helps to hydrolyze smaller TRLs and makes otherwise poorly hydrolysable remnant lipoproteins, LDLs and HDLs more available for LPL.

The relatively small stimulating effect of 18A–CII–a on the hydrolysis of isolated TRLs was also unexpected as it was previously shown that 18A–CII–a lowers VLDL-TG concentration in apoE-knockout mice [8] and also in mice with a defective truncated form of apoC-II [16]. This suggests that, *in vivo*, there may be additional mechanisms responsible for the TG lowering effect of the peptide. At least in humans, TG rapidly equilibrates *in vivo* between lipoproteins by cholesteryl ester transfer protein (CETP) and thus a significant fraction of TG hydrolysis can possibly occur on lipoprotein particles besides TRLs. The main focus on CETP has been its role in equilibrating cholesteryl esters between lipoproteins, but in terms of energy metabolism it may be that one of its main roles is the transfer of TG from TRL to other lipoproteins to enhance overall lipolysis. From an energy delivery standpoint, the re-delivery of any unhydrolyzed TG to the liver by remnant uptake defeats the main purpose for VLDL secretion. It is important to note, however, while the relative effects of 18A–CII–a and apoC-II were larger on smaller size lipoproteins, much more TGs are present in TRLs than in LDLs and HDLs. Thus, the small relative effect of 18A–CII–a on TRL degradation may have larger impact, than the effect on smaller particles, on total reduction of TGs *in vivo*. Finally, high levels of TGs in remnant lipoproteins and in LDLs are associated with increased cardiovascular mortality [2,23]. In fact, it was recently shown in ARIC, a large epidemiologic study of heart disease, that the TG content of LDL was a better predictor of ASCVD risk than LDL-Cholesterol [24]; thus, the lowering of TG on remnants and LDL could be beneficial for reducing ASCVD risk. At the same time, further investigations are needed to ascertain whether the increased lipolysis of HDL by LPL has any impact on ASCVD risk.

In conclusion, our results demonstrate that apoC-II mimetic 18A–CII–a has different properties in promoting lipolysis than the human endogenous apoC-II protein. The 18A–CII–a peptide is significantly more efficient in stimulating LPL activity in human plasma and it does not inhibit LPL activity at higher concentrations unlike apoC-II. Thus, while increasing the concentration of apoC-II in individuals with common forms of hypertriglyceridemia would likely not be beneficial, based on our findings treating such patients with 18A–CII–a could be an effective way to lower TG and thus supports future development of this peptide.

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Robert Uibu, Bachelor's Degree, 2017, (sup) Aivar Lõokene, Mart Reimund. Optimization of apolipoprotein C2 expression and functional studies in human blood plasma. Tallinn University of Technology, Department of Chemistry and Biotechnology.

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2014	Biochemistry (Bachelor's students), supervision of practical works

Publications

Villo, L., Risti, R., Reimund, M., Kukk, K., Samel, N., Lõokene, A. (2019) Calorimetric approach for comparison of angiopoietin-like protein 4 with other pancreatic lipase inhibitors. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids.* In press, DOI: 10.1016/j.bbalip.2019.158553

Reimund, M., Wolska, A., Risti, R., Wilson, S., Sviridov, D., Remaley, A. T., Lõokene, A. (2019) Apolipoprotein C-II mimetic peptide is an efficient activator of lipoprotein lipase in human plasma as studied by a calorimetric approach. *Biochemical and Biophysical Research Communications.* 519 (1), 67–72.

Reimund, M., Kovrov, O., Olivecrona, G., Lõokene, A. (2017) Lipoprotein lipase activity and interactions studied in human plasma by isothermal titration calorimetry. *Journal of Lipid Research*. 58 (1), 279−288.

Kaabel, S., Adamson, J., Topić, F., Kiesilä, A., Kalenius, E., Öeren, M., Reimund, M., Prigorchenko, E., Lõokene, A., Reich, H. J., Rissanen, K., Aav, R. (2017) Chiral hemicucurbit[8]uril as an anion receptor: selectivity to size, shape and charge distribution. *Chemical Science*. 8 (3), 2184−2190.

Reimund, M., Larsson, M., Kovrov, O., Kasvandik, S., Olivecrona, G., Lõokene, A. (2015) Evidence for Two Distinct Binding Sites for Lipoprotein Lipase on Glycosylphosphatidylinositol-anchored High Density Lipoprotein-binding Protein 1 (GPIHBP1). *Journal of Biological Chemistry*. 290 (22), 13919−13934.

Industrial property

Invention: Method for calorimetric determination of the lipoprotein lipase activity in human plasma environment; Owner: Tallinn University of Technology; Authors: A. Lõokene, M. Reimund, O. Kovrov, G. Olivecrona; Priority number: US62/350,747; Priority date: 16.06.2016; International application number: PCT/EP2017/064850

Special courses

2013 EMBO Practical Course "Modern biophysical methods for protein-ligand interactions" Oulu, Finland

2013 American Society for Mass Spectrometry Short Course „Protein Structural Analysis by Mass Spectrometry: Hydrogen Exchange & Covalent Labeling" Minneapolis, MN, USA

2012 Tartu Proteomics Summer School, Practical course „Quantitative proteomics" University of Tartu, Institute of Technology, Estonia

Conference presentations (poster)

2018 "ApoC-II mimetic peptide increases the activity of LPL on triglyceride-poor/cholesterol-rich lipoproteins in human plasma", *3rd European MicroCal User Meeting*, London, England.

2018 "ApoC-II mimetic peptide increases the activity of LPL on triglyceride-poor/cholesterol-rich lipoproteins in human plasma", *41st European Lipoprotein Club meeting,* Tutzing, Germany.

2018 "ApoC-II mimetic peptide increases the activity of LPL on triglyceride-poor/cholesterol-rich lipoproteins in human plasma", *Gordon Research Conference and Seminar "Lipoprotein Metabolism"*, Waterville Valley, NH, USA.

2017 "Apolipoprotein C-II mimetic peptide increases the activity of lipoprotein lipase on remnant lipoproteins in human plasma", *XX Lipid Meeting Leipzig*, Leipzig, Germany.

2016 "Isothermal titration calorimetry: a tool for investigation of lipoprotein lipase activity and interactions in human plasma", *Gordon Research Conference and Seminar "Lipoprotein Metabolism"*, Waterville Valley, NH, USA.

2014 "Evidence for Two Distinct Binding Sites for Lipoprotein Lipase on Glycosylphosphatidylinositol-anchored High Density Lipoprotein-binding Protein 1", *Gordon Research Conference and Seminar "Lipoprotein Metabolism"*, Waterville Valley, NH, USA.

ELULOOKIRJELDUS

Isikuandmed

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Hariduskäik

2012–…	Tallinna Tehnikaülikool, keemia ja geenitehnoloogia (keemia), PhD
2010–2012	Tallinna Tehnikaülikool, rakekenduskeemia ja biotehnoloogia (biokeemia), MSc (cum laude)
2007–2010	Tallinna Tehnikaülikool, rakekenduskeemia ja biotehnoloogia, BSc
2004–2007	Rapla Vesiroosi Gümnaasium

Keelteoskus

Eesti (emakeel), inglise (kõrgtase)

Teenistuskäik

2017–…	Tallinna Tehnikaülikool, keemia ja biotehnoloogia instituut, nooremteadur
2016	Tallinna Tehnikaülikool, keemianstituut, nooremteadur

Juhendatud väitekirjad

Annamaria Rahumeel, magistrikraad, 2018, (juh) Aivar Lõokene, Mart Reimund. Lipoproteiinlipaasi stabiilsuse ja aktiivsuse regulatsioonimehhanismid füsioloogilistes tingimustes. Tallinna Tehnikaülikool, keemia ja biotehnoloogia instituut.

Robert Risti, magistrikraad, 2017, (juh) Mart Reimund, Aivar Lõokene. Apolipoproteiin C-II analoogpeptiidi toimemehhanismi uurimine inimese vereplasmas isotermilise tiitrimis-kalorimeetria meetodil. Tallinna Tehnikaülikool, keemia ja biotehnoloogia instituut.

Robert Uibu, bakalaureusekraad, 2017, (juh) Aivar Lõokene, Mart Reimund. Apolipoproteiin C2 ekspressiooni optimeerimine ja toimemehhanismi uurimine inimese vereplasmas. Tallinna Tehnikaülikool, keemia ja biotehnoloogia instituut.

Liina Roosileht, magistrikraad, 2016, (juh) Aivar Lõokene, Mart Reimund. Inimese pankrease lipaasi ja angiopoietiini-sarnase valgu 4 vahelise kompleksi moodustumise uurimine. Tallinna Tehnikaülikool, keemiainstituut.

Annamaria Rahumeel, bakalaureusekraad, 2016, (juh) Mart Reimund. Angiopoietiini-sarnase valk 4 ja lipoproteiinlipaasi vahelise interaktsiooni uurimine inimese vereplasmas. Tallinna Tehnikaülikool, keemiainstituut.

Jette Rindesalu, magistrikraad, 2015, (juh) Mart Reimund, Aivar Lõokene. GPIHBP1 N-terminaalse domeeni spetsiifilisuse uurimine interaktsioonis lipoproteiinlipaasiga. Tallinna Tehnikaülikool, keemiainstituut.

Jette Rindesalu, bakalaureusekraad, 2013, (juh) Mart Reimund. Lipoproteiinlipaasi struktuuri uurimine keemilise ristsidumise meetodil. Tallinna Tehnikaülikool, keemiainstituut.

Õppetöö

2015–2018	Interaktoomika (magistritudengid), praktiliste tööde juhendamine
2014	Biokeemia (bakalaureusetudengid), praktiliste tööde juhendamine

Publikatsioonid

Villo, L., Risti, R., Reimund, M., Kukk, K., Samel, N., Lõokene, A. (2019) Calorimetric approach for comparison of angiopoietin-like protein 4 with other pancreatic lipase inhibitors. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids.* In press, DOI: 10.1016/j.bbalip.2019.158553

Reimund, M., Wolska, A., Risti, R., Wilson, S., Sviridov, D., Remaley, A. T., Lõokene, A. (2019) Apolipoprotein C-II mimetic peptide is an efficient activator of lipoprotein lipase in human plasma as studied by a calorimetric approach. *Biochemical and Biophysical Research Communications.* 519 (1), 67–72.

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Kaabel, S., Adamson, J., Topić, F., Kiesilä, A., Kalenius, E., Öeren, M., Reimund, M., Prigorchenko, E., Lõokene, A., Reich, H. J., Rissanen, K., Aav, R. (2017) Chiral hemicucurbit[8]uril as an anion receptor: selectivity to size, shape and charge distribution. *Chemical Science*. 8 (3), 2184−2190.

Reimund, M., Larsson, M., Kovrov, O., Kasvandik, S., Olivecrona, G., Lõokene, A. (2015) Evidence for Two Distinct Binding Sites for Lipoprotein Lipase on Glycosylphosphatidylinositol-anchored High Density Lipoprotein-binding Protein 1 (GPIHBP1). *Journal of Biological Chemistry*. 290 (22), 13919−13934.

Tööstusomand

Patentne leiutis: Method for calorimetric determination of the lipoprotein lipase activity in human plasma environment; Omanik: Tallinna Tehnikaülikool; Autorid: A. Lõokene, M. Reimund, O. Kovrov, G. Olivecrona; Prioriteedi number: US62/350,747; Prioriteedi kuupäev: 16.06.2016; Rahvusvaheline taotluse number: PCT/EP2017/06485

Täiendusõpe

2013 EMBO Practical Course "Modern biophysical methods for protein-ligand interactions" Oulu, Soome.

2013 American Society for Mass Spectrometry Short Course „Protein Structural Analysis by Mass Spectrometry: Hydrogen Exchange & Covalent Labeling" Minneapolis, MN, Ameerika Ühendriigid.

2012 Tartu Proteomics Summer School, Practical course „Quantitative proteomics" Tartu Ülikool, tehnoloogiainstituut, Eesti.

Konverentside ettekanded (poster)

2018 "ApoC-II mimetic peptide increases the activity of LPL on triglyceride-poor/cholesterol-rich lipoproteins in human plasma", *3rd European MicroCal User Meeting*, London, Inglismaa.

2018 "ApoC-II mimetic peptide increases the activity of LPL on triglyceride-poor/cholesterol-rich lipoproteins in human plasma", *41st European Lipoprotein Club meeting,* Tutzing, Saksamaa.

2018 "ApoC-II mimetic peptide increases the activity of LPL on triglyceride-poor/cholesterol-rich lipoproteins in human plasma", *Gordon Research Conference and Seminar "Lipoprotein Metabolism"*, Waterville Valley, NH, Ameerika Ühendriigid.

2017 "Apolipoprotein C-II mimetic peptide increases the activity of lipoprotein lipase on remnant lipoproteins in human plasma", *XX Lipid Meeting Leipzig*, Leipzig, Saksamaa.

2016 "Isothermal titration calorimetry: a tool for investigation of lipoprotein lipase activity and interactions in human plasma", *Gordon Research Conference and Seminar "Lipoprotein Metabolism"*, Waterville Valley, NH, Ameerika Ühendriigid.

2014 "Evidence for Two Distinct Binding Sites for Lipoprotein Lipase on Glycosylphosphatidylinositol-anchored High Density Lipoprotein-binding Protein 1", *Gordon Research Conference and Seminar "Lipoprotein Metabolism"*, Waterville Valley, NH, Ameerika Ühendriigid.