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Hepatic Lipase Regulates LipoProtein Trafficking in Hepatocytes

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HEPATIC LIPASE REGULATES LIPOPROTEIN TRAFFICKING IN HEPATOCYTES

by

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Abstract

The production of very low density lipoprotein and high density lipoprotein particles by the liver is a tightly regulated process, which begins with synthesis and assembly of core protein components in the rough endoplasmic reticulum. Factors influencing the production and metabolism of these particles are of immediate medical relevance, as their malfunction or hyperactivity can lead to an assortment of disease states. Hepatic lipase is a secreted liver enzyme, with many previously described roles in the metabolism and clearance of both high and low density lipoproteins. Increased production and assembly of this enzyme is an indicator of metabolic dysfunction, while its absence or insufficiency leads to pre-mature atherosclerosis and death. The present study shows that this enzyme's role in lipoprotein metabolism is not confined to the degradation and clearance of these particles after they have been secreted. Experiments using co-immunoprecipitation targeted at hepatic lipase demonstrate that this protein interacts with ApoA1 and ApoB100, the core protein components of HDL and VLDL respectively, at the ER level in hepatocytes, as part of an enormous multi-subunit protein complex. This interaction with ApoA1 leads to decreased competence of hepatocytes to secrete HDL, which confers a pro-atherogenic phenotype. Analysis of ER to Golgi VLDL transport vesicles, produced with a cell-free *in vitro* budding assay, has revealed that hepatic lipase is co-secreted between these compartments with immature VLDL particles. Further analysis of cytosol isolated from hepatocytes demonstrates an interaction between hepatic lipase and the LDL-receptor related protein in a post-Golgi vesicle; the significance of which will be investigated in future studies.

Acknowledgements

Special thanks to Dr. Shadab Siddiqi for believing in me, and for giving me the opportunity to work in his lab and on this project, as well as for providing continued guidance and mentorship throughout this process and in helping direct my future. Thanks to Shaila Siddiqi for sample preparation, and for taking the time to teach me all of the methods hands on, as well as for being there to help on countless occasions with presentations and experiments.

Big thanks to all of my lab mates; Phil, Bhavesh, Faisal, Abigail, and Dr. Olga. Without your help and support day in and day out none of this would be possible. Additional thanks to Jeffrey Hatcher for assistance putting together the illustration and for continued mentorship.

Thank you Dr. Robert Borgon and Dr. Seth Elsheimer for taking the time to be on my committee. Additionally, both of your courses were instrumental in inspiring me to pursue research and a career in science, which I am grateful for on a level I cannot put into words.

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Chapter 1: Literature Review and Aims

Introduction

The aims of this study are twofold; identify the role played by hepatic lipase in ApoA1 trafficking, particularly at the level of the endoplasmic reticulum (ER), and also to determine the relationship between VLDL secretion and hepatic lipase secretion. This information will aid in the development of drugs aimed at increasing the ability of hepatocytes to secrete ApoA1/HDL. Increased ApoA1/HDL serves to increase reverse cholesterol transport and to prevent LDL oxidation, both of which in turn will decrease the risk for developing atherosclerosis. The process of ApoA1 retention by pre-folded hepatic lipase can be targeted at the level of transcription by anti-sense oligonucleotides, which target siRNA encoding the hepatic lipase gene, or at the post-translational level by introducing some factor that disrupts the association between these two proteins.

VLDL trafficking between the ER and cis-Golgi is a tightly regulated process, the rate of which ultimately limits the secretion of VLDL. Enhance VLDL secretion is an independent risk factor for developing atherosclerosis, which is exacerbated in different forms of metabolic disturbances, such as insulin resistance. Thus, identification of factors that diminish or enhance the rate at which ER to Golgi movement occurs are of utmost importance to developing treatments directed at modulating VLDL secretion. This study aims to lay ground work for future studies aimed at elucidating the means by which hepatic lipase alters VLDL trafficking and secretion in hepatocytes.

Early Observations

Human plasma typically contains little to no lipolytic activity. Early observations by Hahn in 1943 demonstrated that addition of heparin to lipemic human serum would rapidly induce lipid hydrolysis, which led to the eventual discovery of lipoprotein lipase [1]. While this was an exciting development in lipid metabolism, it was well known that lipoprotein lipase activity alone could not account for 100% of the lipolysis induced in human serum upon heparin injection. Some twenty years after the description of lipoprotein lipase, Sinderman et al, described the presence of heparin inducible lipase of hepatic origin [2]. A clear distinction between the two proteins was drawn by need for ApoCII as a cofactor. Lipoprotein lipase demonstrated no lipolytic activity in 1 molar NaCl, due to the fact that these conditions disrupt interaction with ApoCII, while hepatic lipase retained full activity [3, 4]. Clinically, this is used to assess risk for cardiovascular disease. NaCl sensitive lipolytic activity is subtracted from total lipolytic activity to indirectly measure hepatic lipase activity, which is directly related to cardiovascular disease [5].

Overview

Hepatic lipase is a lipolytic enzyme produced in the liver, adrenal cortex, and macrophages [6, 7]. The production of this enzyme by steroidogenic organs such as the ovaries and testis, in addition to those previously listed, is still a subject of debate. The mature enzyme is found in two inactive pools within the body; anchored to heparan sulfate proteoglycans (HSPG)

of hepatocytes and endothelial cells, as well as bound to ApoA1 and ApoA2 containing HDL, which are responsible for its release from the surface of hepatic sinusoids [8, 9].

The enzyme shows dual functionality as an A1 phospholipase as well as triglyceride lipase. It has been demonstrated that the enrichment of HDL particles with triglycerides (HDL_2) as well as anionic lipids regulates the lipolysis of VLDL triglycerides by hepatic lipase [8]. This enzyme is also responsible for the maturation of HDL_2 to type three high density lipoproteins, and for the transformation of intermediate density lipoproteins (IDL) to low density lipoproteins (LDL). The initial discovery of this function originates from clinical cases, in which patients with a *LIPC* loss of function mutation presented with elevated concentrations HDL_2 as well as elevated IDL [10]. Both of the previously mentioned functions represent the latter steps in the degradation processes of the respective lipoprotein particles. Thus, the catalytic activity of hepatic lipase plays a large role in determining lipoprotein subtype profile, which used clinically to assess cardiovascular disease risk.

In cases of insulin resistance, the over activity and expression of hepatic lipase has been observed, and implicated in the formation of small dense LDL, which are known to promote atherosclerosis [11, 12]. Another feature of insulin resistance is an increase in the secretion of VLDL particles by the liver [13]. While the two factors independently increase risk for atherosclerosis, no study to date has described how the two pathophysiological states relate to each other, or if hepatic lipase expression has an impact on the trafficking or secretion of VLDL from hepatocytes in the insulin resistant state.

In addition to the lipolytic function for which hepatic lipase was named, a likewise important ligand binding function was later described. This function aids in the uptake of whole low density lipoprotein particles, in addition to the selective uptake of cholesterol esters from high density lipoproteins [14]. The protein's role in HDL-c uptake mediates a reduction in levels of HDL-c, which is observed to have an inverse relationship with blood triglycerides in the diabetic dyslipidemia [11]. Lowered serum HDL-c levels and elevated triglycerides have been implicated as a risk for cardiovascular disease [15]. Further studies revealed that the uptake of these whole LDL particles is due to a direct interaction between hepatic lipase and ApoB100, which is the core protein component of LDL [16]. The ligand binding or "bridging" function of hepatic lipase is dependent on the presence of heparan sulfate proteoglycans, and is further mediated by LDL receptor related protein (LRP), or the LDL receptor as a separate pathway [17-19].

This process is also sensitive to the protein composition of lipoprotein particles, which changes throughout their maturation. For cells up taking these triglyceride rich remnant particles by LRP mediated endocytosis, ApoE is a required cofactor, in addition to the cell surface proteoglycans [17]. In the fasted state, the majority of ApoE is confined to HDL particles. The transfer of ApoE to LDL occurs in the post-prandial hyperlipidemic state when triglyceride rich VLDL is being produced by the liver. While ApoE is a necessary cofactor for the endocytosis of LDL particles and HDL cholesterol ester uptake, its presence on HDL inactivates the catalytic activity of hepatic lipase by reducing the ability of HDL particles to remove the enzyme from cell surface HSPG. Simultaneous to the exchange of proteins in this lipemic state, cholesterol ester transfer protein mediates the exchange of cholesterol esters on HDL particles for triglycerides present on VLDL or IDL particles. Collectively, these processes mediate triglyceride

enrichment of HDL particles, which can be used later as an energy substrate; along with the cholesterol enrichment of ApoB containing particles (LDL), which are then taken up by and degraded in the liver with the aid of hepatic lipase's ligand binding function.

It has been observed that the expression of scavenger receptor-B1 and hepatic lipase are regulated similarly in the liver as well as in steroidogenic organs, and that their expression is inversely related to cellular cholesterol content [20]. These two proteins act in concert to facilitate cellular cholesterol ester uptake in perfused rat livers [21]. Studies with hepatic lipase/ApoA1 double knockout mice have shown that the female knockout mice produce a lesser amount of progesterone when compared to non-transgenic littermates, and that this is due to a lack of available substrate in steroidogenic tissues [22]. Taken together, these studies demonstrate the current understanding of role this enzyme plays in the sequestration of cholesterol by different cell types, along with the role played by SR-B1 in assisting with this process.

Factors which mediate the maturation of hepatic lipase in the endoplasmic reticulum (ER) have been well studied. Doolittle et al, have shown in Chinese hamster ovary cells that the rate limiting step in the enzyme's secretion is proper folding at the ER level, and that this process entails the formation of a myriad of complexes of differing composition, some of which include the chaperones calnexin and lipase maturation factor 1 [23, 24]. Maturation of hepatic lipase takes hours, as opposed to minutes in the case of its close genetic cousin lipoprotein lipase, and as much as half of the initially translated enzyme is degraded by a proteasome mediated pathway, which has yet to be described. In addition to this, the mechanisms directly regulating

this enzyme's secretion, and the components with which it secretes from the ER in hepatocytes has not been described.

Transgenic studies

While the ligand binding function of hepatic lipase has been deemed "anti-atherogenic" by some transgenic studies due to its role in facilitating LDL uptake [25, 26], other studies using tissue specific expression in macrophages showed that this same role increased the formation of atherosclerotic plaques [27]. When compared to catalytically inactive variants of hepatic lipase, Gonzalez-Navarro, H. et al, were able to demonstrate that the ligand binding function decreased the formation of aortic leasions in ApoE knockout mice, which are used as an animal model for atherosclerosis. Despite a similar reduction in ApoB containing lipoprotiens between groups expressing the catalytically active and catalytically inactive varriants of the enzyme, aortic lesion formation was actually increased in mice expressing the catalytically active form of hepatic lipase, when compared to the control group expressing neither varriant. These studies show that the bridging function of hepatic lipase is essential to the clearance of ApoB100 containing lipoprotien remnants by the liver and that this can occur independent of its catalytic function. However, the same ligand binding function may also aid in the uptake of small dense LDL particles by artery dwelling macrophages, and contribute to the pathogenesis of atherosclerosis.

In a recent study performed by Escola-Gil et al., it was shown that mice lacking hepatic lipase or endothelial lipase demonstrate an increase in macrophage to feces reverse cholesterol transport, as well as HDL anti-oxidant properties [28]. RCT and prevention of LDL oxidation are

two of the primary reasons why HDL is considered “good cholesterol”. Both processes directly inhibit foam cell formation and growth, which describes the process by which macrophages accumulate cholesterol within fatty plaques and occlude the lumen of arteries [29]. The oxidation of LDL molecules leads to their recognition by the CD36 and SR-A1 receptors present on macrophages. As the accumulation of cholesterol by these cells proceeds, changes in gene expression occur in an effort to restore homeostasis. Cholesterol efflux mediated by ApoA1 containing HDL is increased to restore balance. The aforementioned study showed that when macrophages containing tritium labeled cholesterol were injected into mice lacking either lipase, they were able to excrete more of the radioactive tritium through their feces as bile when compared to the wild type control. Furthermore, HDL particles isolated from either group of knockout mice showed an increased ability to protect LDL molecules from oxidation *in vitro.* This characteristic was attributed to increased levels of ApoA1 in HDL particles from both knockout groups. These observations indicate that hepatic lipase may exert a negative impact on the ability of macrophages to secrete HDL, and that in the absence of hepatic lipase, LDL particles may less prone to oxidation.

Mice lacking hepatic lipase have also demonstrated protection against hepatic steatosis as well as obesity in response to a high fat diet [30]. Taken with the observation that impaired secretion of VLDL leads to the development of hepatic steatosis [31], this indicates that hepatic lipase may play some role in down regulating VLDL secretion, or that its absence can be compensated for in mice. Furthermore, recent studies using metabolic profiling as well as *in vitro* and *in vivo* assessments of gene expression have demonstrated that VLDL triglyceride hydrolysis by hepatic lipase selectively activates the nuclear receptor PPARδ [32]. This receptor is known

to induce production of adipocyte differentiation related protein, which is associated with lipid droplet formation as well as adipocyte proliferation and enlargement. This is paralleled by observations from the previously mentioned study in hepatic lipase knock-out mice, which demonstrated reduced macrophage infiltration of peritoneal fat. Based on this observation it was proposed that free fatty acids produced by hepatic lipase activity in these fat dwelling macrophages contributes to worsening inflammation and increased substrate availability for triglyceride storage by adipocytes.

While many studies have implicated the significance of the catalytic and "bridging" functions of hepatic lipase in lipoprotein metabolism outside of the cell, recent studies have called into question the role played by this protein inside of the cell. Yao's group has demonstrated that hepatocytes from LIPC null mice (lacking hepatic lipase) transfected with human hepatic lipase secrete less ApoA1 when compared to control [33]. This study also analyzed the impact of transfection with mouse hepatic lipase, which lacks the C-terminal heparin binding domain. A similar decrease in ApoA1 secretion was observed in this group, and an increase in unfolded hepatic lipase residing in the ER, relative to human hepatic lipase transfection, was also observed. This indicates that the C-terminal heparin binding domain, along with the N-terminal heparin binding domain present in both species, may play a role in the proper folding and secretion of this enzyme.

The study proposed that the observed impact of this enzyme on ApoA1 secretion may occur as a result of direct interaction between ApoA1 and hepatic lipase, or alternatively by changes in gene expression due to the activity of hepatic lipase. The observation that the selective hydrolysis of VLDL triglyceride by hepatic lipase leads to PPARδ activation indicates

that changes in gene expression are not entirely out of the question. Furthermore, recent *in vitro* studies have suggested that hepatic lipase becomes active in the ER, leading to decreased availability of triglycerides, which are necessary for secretion of VLDL [34]. However, aforementioned studies into the maturation of hepatic lipase have indicate that the protein is rapidly secreted from the ER upon dimerization, which is also when the enzyme gains catalytic activity [24]. The rate limiting step in the secretion of VLDL from the liver is movement from the ER to the cis-Golgi, in a specialized VLDL transport vesicle, or VTV [35].

Aims

Aim 1: The primary aim of the present study is to identify whether or not any interaction between hepatic lipase and ApoA1 exists at the ER level. The majority of ApoA1 found in circulation originates from the liver, and thus any factor affecting the proper secretion of this crucial, anti-atherogenic lipoprotein from hepatocytes warrants investigation. This was accomplished using co-immunoprecipitation, silver staining and western blot analysis.

Aim 2: The secondary aim of this study is to assess the relationship between hepatic lipase and VLDL movement from the ER to the cis-Golgi. ApoB100 is the core component of VLDL molecules, and our lab has extensively studied the mechanisms related to the secretion of these pro-atherogenic particles. This was analyzed with an *in vitro* budding assay, TCA precipitation of budding fractions, co-immunoprecipitation, silver staining, and western blot analysis.

Chapter 2: Materials and Methods

SDS-PAGE and western blot analysis

Protein concentration was determined by the Bradford method [36]. Proteins were separated by SDS-PAGE and then transferred to nitrocellulose membranes (Bio-Rad). Western blot protein analysis was accomplished with ECL western blot reagent (GE Healthcare), and autoradiography film (MIDSCI, St. Louis, MO). Secondary antibodies were conjugated to horseradish peroxidase (Santa Cruz Biotechnology), with luminol as the light producing substrate in ECL.

Co-immunoprecipitation

Samples containing 500 μg protein were incubated with rabbit anti-hepatic lipase antibodies for 4 hours at 4°C while rotating (Santa Cruz Biotechnology). ER samples were incubated in 2% triton-X for 10 minutes at 4°C to solubilize membranes. After 4 hours incubation, secondary antibodies conjugated to agarose beads were added (Santa Cruz Biotechnology). After the addition of secondary antibodies targeting the FC region of rabbit antibodies, the samples were incubated at 4°C over night while rotating.

The formed immune complexes were washed 10 times with cold PBS. Samples were subsequently boiled and vortexed to release their contents. The supernatant was then separated by SDS-PAGE. The same procedure was performed simultaneously with anti-IgG primary antibodies as a control.

Preparation of Hepatic Cytosol

Hepatocytes were washed thoroughly in Krebs buffer, and then cytosol buffer (25 mM HEPES, 125mM KCl, 2.5mM $MgCl_2$, .5 mM DTT, protease inhibitors, pH 7.2). Cells were homogenized using a Parr bomb at 1100 psi for 40 minutes. Samples were then centrifuged at 40,000 RPM for 95 minutes (Beckman rotor, 70.1, Ti). The supernatant was collected at cytosol.

Samples were then dialyzed with fresh cytosol buffer, concentrated with a Centricon filter, and ultrafiltration membrane with a 10 kDa cutoff (Millipore, Billerica, MA). Final concentration of cytosol samples was determined to be 19 mg/mL by Bradford method.

Preparation of ER samples

Hepatocytes were homogenized in .25M sucrose in 10 mM HEPES, 50 mM EDTA, and protease inhibitor, using a Parr bomb at 1100 psi for 40 minutes. ER, cis-Golgi, and trans-Golgi fractions were isolated by step gradient ultracentrifugation [35]. This step was repeated to ensure purity.

In Vitro ER Budding

VTV generation was induced by incubating intact rat liver ER membranes (500μg protein) with rat liver cytosol (1mg protein) and an ATP generating system 5 mM Mg^{2+}, 0.5 mM Ca^{2+}, 5 mM DTT, 1 mM GTP, 1 mM E600. Final volume was adjusted to 500 μl with transport buffer, (30 mM HEPES, 250 mM sucrose, 2.5 mM MgOAc, 30 mM KCl; pH 7.2) [36].

Termination of the reaction was achieved by placing the tubes on ice. The mixture was overlaid on a continuous sucrose gradient, .2 molar to 2.1 molar, and centrifuged with a Beckman rotor SW41 at 25,900 rpm for 2 h at 4 °C [36]. 500 µl aliquots were separated from the continuous gradient and stored at -20°C until needed.

TCA Precipitation

150 µl of each 500 µl fraction from the sucrose gradient used to resolve the VTV budding assay were incubated with 150 µl of 30% trichloroacetic acid (TCA) at 4°C for 30 minutes, after thorough vortexing. Samples were then pelleted by 15 minute centrifugation at 13400 RPM in a bench top centrifuge. The supernatant was discarded. Cold (-20°C) acetone was added to the pellet, vortexed thoroughly, and incubated at 4°C for 30 minutes. Samples were then pelleted by 15 minute centrifugation at 13400 RPM in a bench top centrifuge. The supernatant was discarded. 15 µl of .5 normal NaOH was added to the pellet and incubated at room temperature for 10 minutes to neutralize the excess trichloroacetic acid. Samples were stored at -20°C until use.

Silver Staining

After immunoprecipitation was performed as described previously, samples were resolved by 4%-20% SDS-PAGE. To prevent diffusion of proteins, the gel was incubated in 200 ml fixing buffer (10% accetic acid v/v, 30% ethanol v/v in d.i. H_20) while rocking at room temperature for 1 hour. To complete fixing, the gel was transferred to incubation buffer (.83 M sodium acetate, 30% ethanol v/v, 8 mM sodium thiosulfate, .5% v/v glutaraldehyde) and left

rocking over night at room temperature. The gel was washed three times in d.i. water to remove excess fixing agents. Silvering was accomplished by incubating the gel in silvering agent (0.02% v/v formaldehyde, 12 mM silver nitrate) for 20 minutes while rocking at room temperature in the absence of light. Developing of the silver stained bands was accomplished by repetitive incubation/washing with developing buffer (236 mM sodium carbonate, .01% v/v formaldehyde), until bands were adequately visible. Termination of the reaction was accomplished with rinsing and incubation in 1% m/v glycine.

Chapter 3: Results and Discussion

To identify protein complexes that impact the secretion of lipoproteins in the endoplasmic reticulum of rat hepatocytes, immunoprecipitation was performed using primary antibodies against hepatic lipase, and resolved by SDS-PAGE. Based on the presence of a bands with an apparent molecular mass at 100kDa and 250kDa in the non-boiled sample, and bands at approximately 28kDa and 53kDa in the boiled sample of our silver staining procedure, seen in **Figure 1**, further immunoprecipitation and western blot analysis was performed to determine definitively if hepatic lipase forms complexes with ApoA1, ApoB100, or other lipoprotein components at the ER level. Analysis of cytosolic samples as well as VTV budding samples was performed later in order to determine the paradigm responsible for hepatic lipase secretion.

Silver Staining

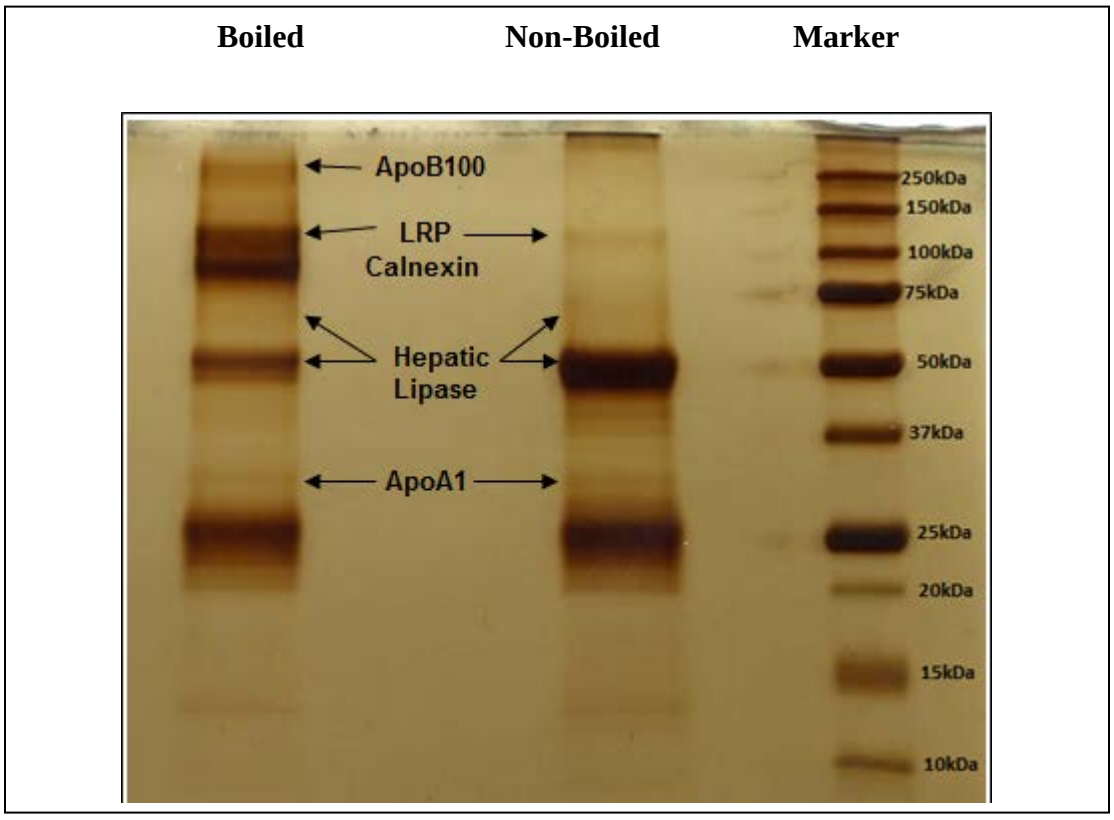


Figure 1 Silver staining was performed on ER co-immunoprecipitation samples, with primary anti-hepatic lipase antibodies. Boiling was performed for 5 minutes at 100°C prior to SDS-PAGE for indicated samples. Samples were resolved by 4% to 20% gradient SDS-PAGE.

The lane containing boiled samples is representative of separated components belonging to various ER complexes in which hepatic lipase is involved. The non-boiled sample was not able to resolve the complex, due to its excessive mass. The IgG heavy chain and light chain are observed at 50kDa and 25kDa, respectively, are present in both samples.

ApoA1

To confirm the identity of the 28 kDa band in the boiled samples of Figure 1 as ApoA1, immunoprecipitation of ER samples was performed in the same fashion, resolved by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by western blot.

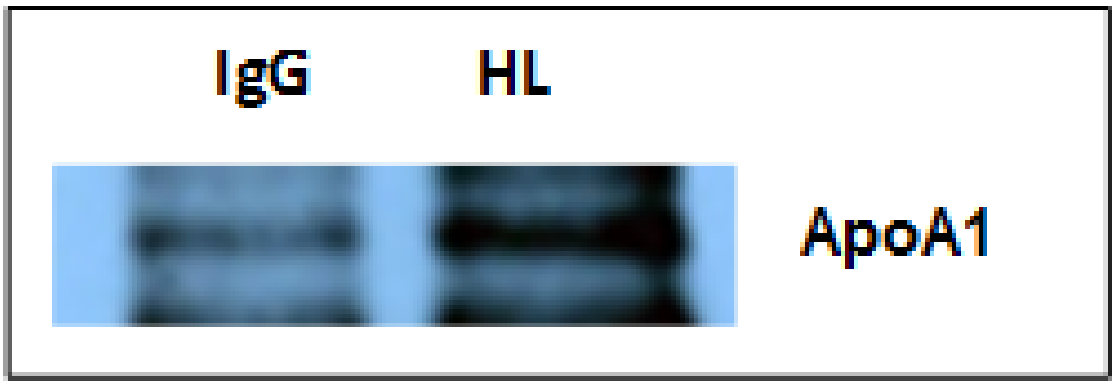
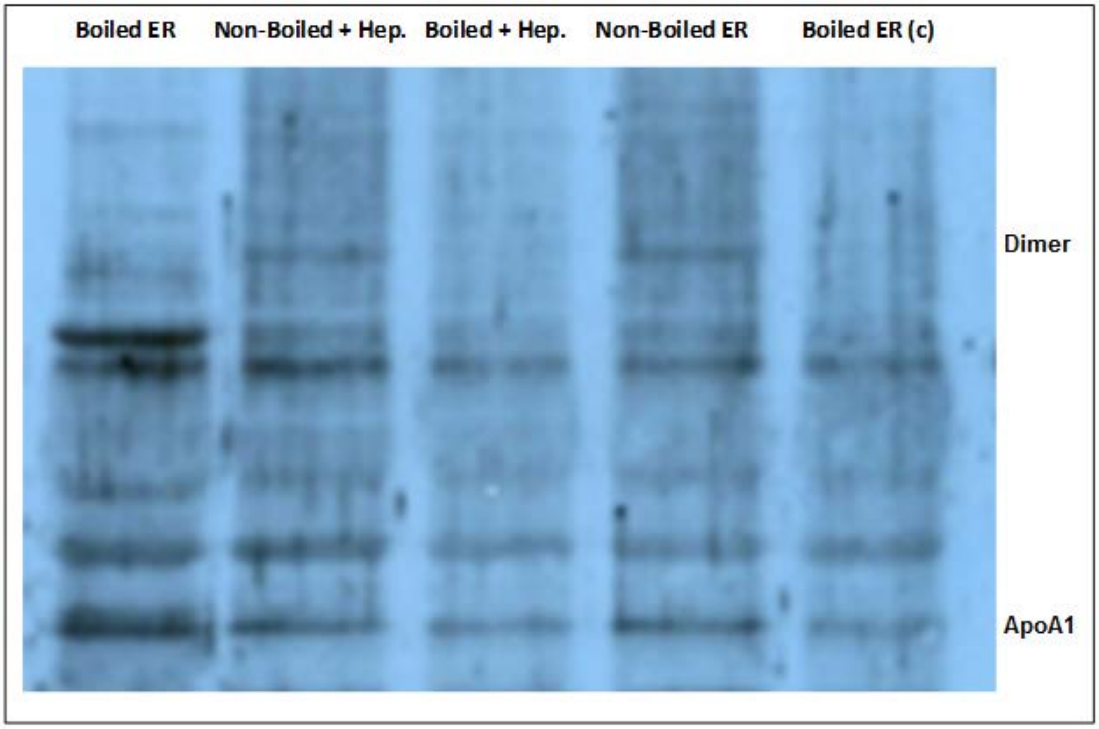


Figure 2 Western blot of ApoA1 from anti-hepatic lipase immunoprecipitation samples, from rat hepatic cytosol and ER. ER control was solubilized in 2% triton-X detergent prior to SDS-PAGE. HL denotes experimental observation using primary anti-hepatic lipase. IgG denotes control using primary anti-IgG. Samples were resolved on 12% SDS-PAGE.

To verify complex formation, analysis of boiled and non-boiled ER samples was performed with and without the presence of heparin. Heparin at a concentration of 100 μg/μl has been shown to block the association of hepatic lipase with LRP [19]. Based on this, incubation of ER samples with this concentration of heparin was used to investigate complex formation while blocking the N and C terminal heparin binding domains of hepatic lipase. Previous studies have demonstrated that mice expressing hepatic lipase deficient in the heparin binding domains present with low concentrations of ApoA1 HDL [37]. Thus, blocking these domains with heparin

was employed in this study to analyze the how this affects the interaction of hepatic lipase with ApoA1, which we have hypothesized mediates previously observed reductions in ApoA1 secretion.



a)

b)

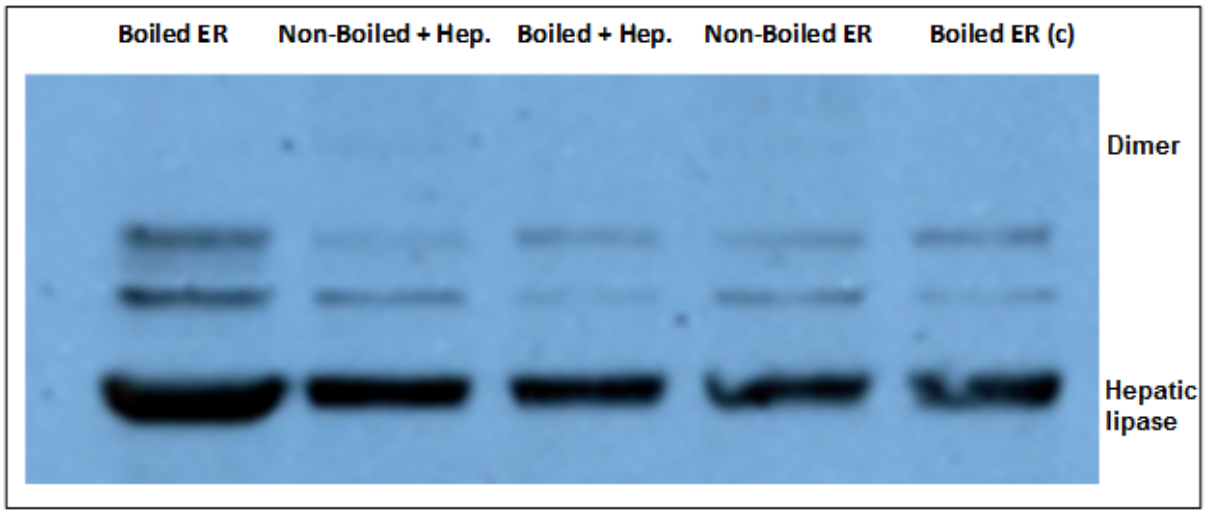


Figure 3 Western blot of **a)** ApoA1 **b)** hepatic lipase, from hepatic ER samples resolved by 4-20% gradient SDS-PAGE. All lanes contain 42µg total protein. Membranes were solubilized in

2% triton-X detergent. Boiled ER was kept at 4°C and boiled for 5 minutes at 100°C prior to SDS-PAGE. All heparin and non-heparin samples were incubated at 4°C for one hour after heparin was added to the positive samples, and subsequently incubated at 37°C for one hour. Samples were either boiled for 5 minutes prior to SDS-PAGE indicated by “boiled”, or loaded after incubation at 37°C, as indicated by “non-boiled”. Boiled ER (c) was incubated at described for heparin/non-heparin containing samples and boiled as described prior to SDS-PAGE to control for artifacts created by the incubation process.

ApoB100

In order to confirm the suspected identity of the 250kDa band as ApoB100 in the boiled sample of our silver staining experiment Figure 1, western blot analysis was performed on ER immunoprecipitation samples. Further analysis with immunoprecipitation experiments were performed to determine if interaction between these proteins occurs within vesicles present in the cytosol bound for fusion with the Golgi, or cell membrane.

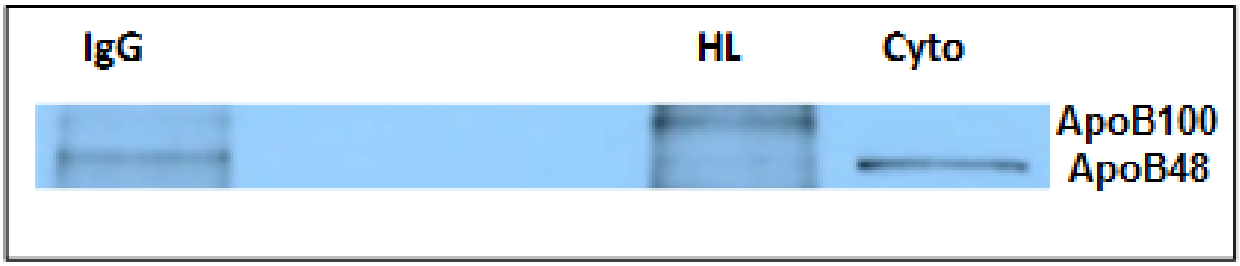


Figure 4 Western blot for ApoB100 originating from anti-hepatic lipase co-immunoprecipitation samples from rat hepatic cytosol. IgG denotes the control using primary anti-IgG antibodies. The middle lane labeled HL denotes the experimental observation with primary anti-hepatic lipase antibodies. To the right is a cytosolic sample added as a control, containing 40 μg total protein. Samples were resolved on 12% SDS-PAGE. Bands at 250kDa represent the truncated ApoB48 isoform, while the band at a greater molecular mass denotes the full length 515kDa ApoB100 band. Both transcripts are produced in rat hepatocytes [38].

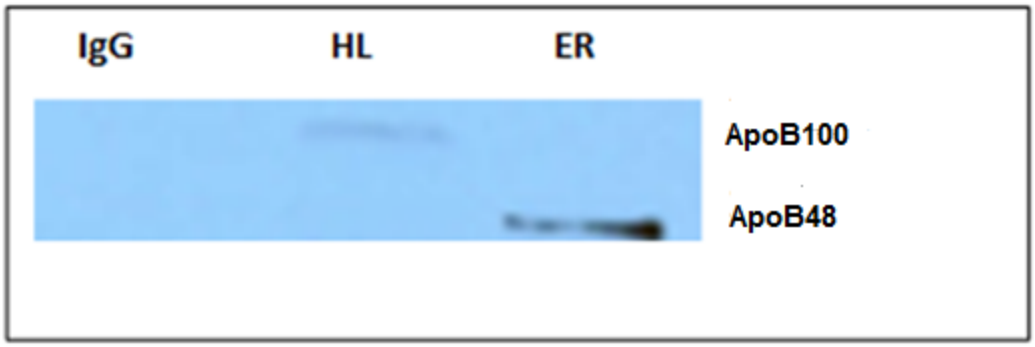
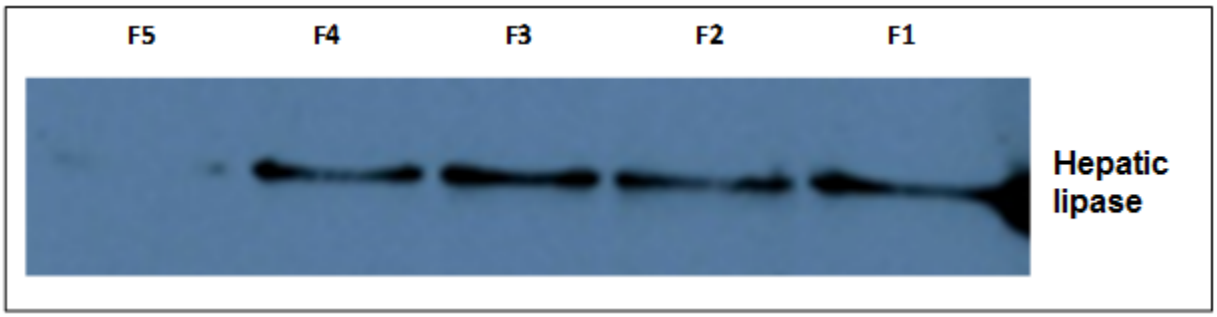


Figure 5 Western blot of ApoB100 from anti-hepatic lipase immunoprecipitation samples, from rat hepatic ER. IgG denotes control using primary anti-IgG. The middle lane labeled HL denotes the experimental observation with primary anti-hepatic lipase antibodies. ER control was solubilized in 2% triton-X detergent prior to immuno-precipitation. Samples were resolved on 12% SDS-PAGE. Bands at 250kDa represent the truncated ApoB48 isoform, while the band at a greater molecular mass denotes the full length 515kDa ApoB100 band. Both transcripts are produced in rat hepatocytes [38].

Hepatic Lipase is Present in VTVs

TCA precipitation was performed on VTV budding fractions in order to determine what paradigm mediates the secretion of hepatic lipase from the endoplasmic reticulum. The COPII complex mediates the selective movement of nascent proteins from the endoplasmic reticulum to the cis-Golgi [39]. This COPII coat induces the formation of protein transport vesicles, or PTVs, which deliver nascent protein cargo to the cis-Golgi. Our lab has previously demonstrated that ApoB100, the core component of VLDL particles, is transported between these cellular compartments in a specialized VLDL transport vesicle, or the VTV. This is vesicle biochemically distinct from the PTV in terms of protein composition, size, and density [35]. This process is of particular significance, because it is the rate limiting step in VLDL secretion. While the process of VTV formation is dependent on components of the COPII complex, there are distinct differences in the requirements for budding of each vesicle.

a)



b)

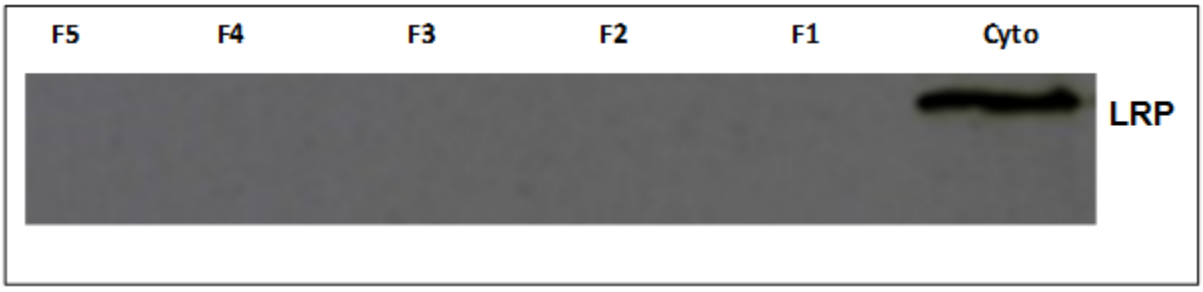


Figure 6 Western blots of **a)** hepatic lipase and **b)** LRP, from the four low density fractions of VTV budding assay continuous sucrose gradient, F1 being the lowest density. Samples were resolved on 12% SDS-PAGE after TCA precipitation. A cytosol samples was included as control for the efficacy of LRP identification.

LDL Receptor Related Protein

Figure 7 Western blot of LRP originating from anti-hepatic lipase co-immunoprecipitation samples from rat hepatic cytosol. IgG denotes the control using primary anti-IgG antibodies. The middle lane labeled HL denotes the experimental observation with primary anti-hepatic lipase antibodies. To the right is a cytosol sample added as a control, containing 40 μg total protein. Samples were resolved on 12% SDS-PAGE.

ApoAIV

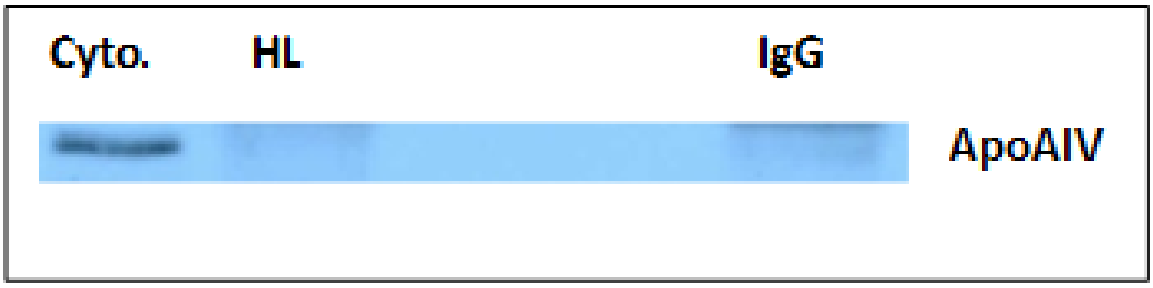


Figure 8 Western blot of ApoAIV originating from anti-hepatic lipase co-immunoprecipitation samples from rat hepatic cytosol. IgG denotes the control using primary anti-IgG antibodies. The middle lane labeled HL denotes the experimental observation with primary anti-hepatic lipase antibodies. To the right is a cytosol sample added as a control, containing 40 μg total protein. Samples were resolved on 12% SDS-PAGE.

Illustration

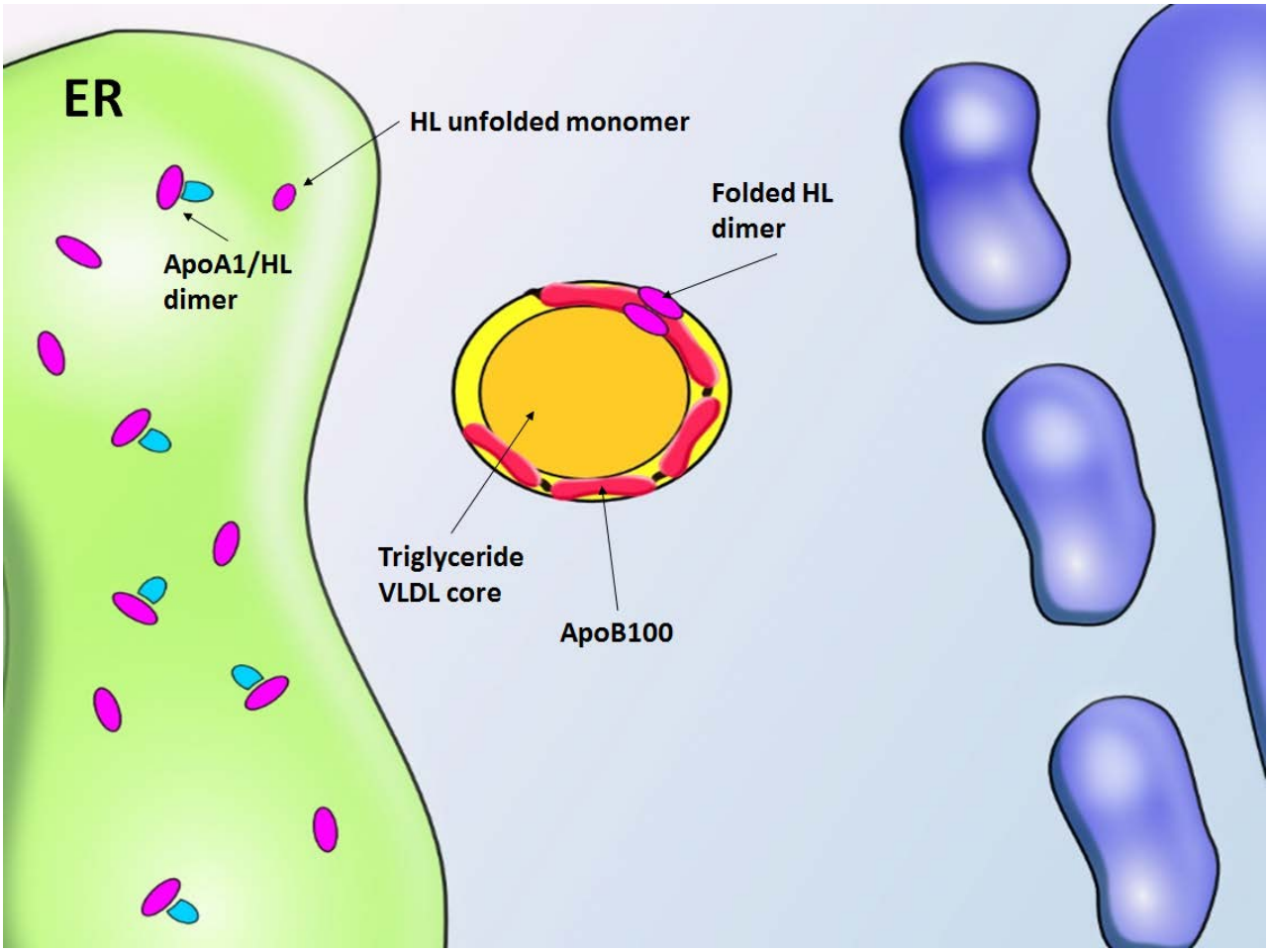


Figure 9 Schematic illustration of significant discoveries established in the present study. In the ER, hepatic lipase, which is present in form of inactive, unfolded monomers, forms unproductive dimers with nascent ApoA1 molecules. This effectively decreases the rate at which hepatocytes are able to secrete ApoA1/HDL, thus causing an unfavorable lipoprotein profile that may contribute to the development of atherosclerosis.

Discussion

The intentions of the present study were to investigate hepatic lipase complex formation along the secretory pathway in hepatocytes; with special attention to interactions with nascent high and very low density lipoproteins. Additionally, the study aimed to determine what secretory pathway is responsible for the secretion of properly folded, active, hepatic lipase dimers. To determine significant interactions at the ER and cytosol level, co-immunoprecipitation was used in conjunction with silver staining and western blotting. An *in vitro* budding assay was performed to produce and isolate ER to Golgi transport vesicles, which were then analyzed with TCA precipitation and subsequent western blotting. Our data supports a model by which inactive hepatic lipase monomers interact with ApoA1 at the ER level, and with ApoB100 in the VTVs as an active dimer **Figure 9**.

The band at 53kDa in **Figure 1** represents hepatic lipase, along with the IgG heavy chain at 50kDa. A band is also observable at 28kDa in the boiled as well as the non-boiled samples, representing ApoA1. The interaction of these proteins in the ER was confirmed by immunoprecipitation experiments, **Figure 2**. While non-specific binding was observed in our IgG control, the signal was significantly more pronounced in our experimental observation using anti-HL primary antibodies. Based on the fact that a band is observable at this mass with and without boiling in our silver staining experiment indicates that this is a weak interaction, and may be unstable under harsh conditions. Namely, the highly reducing environment created by buffers used in SDS-PAGE, as well as due to vortexing used to solubilize proteins prior to SDS-PAGE.

To verify the existence of a hetero-dimeric complex of ApoA1 (28 kDa) and hepatic lipase (53kDa), further western blot analysis was performed on boiled and non-boiled ER samples, both with and without heparin. Based on the presence of bands at approximately 81 kDa in both non-boiled samples with immunoreactivity to ApoA1 **Figure 3a)** as well as to hepatic lipase **Figure 3b)**, we report that these two proteins interact in the ER of hepatocytes. This interaction may explain observations of decreased ApoA1 secretion due to hepatic lipase expression, which would confer a pro-atherogenic phenotype *in vivo*. Interestingly, the inclusion of heparin did not produce marked changes in the association of these two proteins. This may be attributed to insufficient binding of heparin to the appropriate domains of hepatic lipase, or to a lack of appreciable impact of this association on ApoA1 binding at this level.

It has been demonstrated previously that hepatic lipase and ApoB100 interact in the extracellular fluid *in vivo* [16]. In the present study we have demonstrated a specific interaction between ApoB100 in the hepatic ER **Figure 5**, as well as in hepatic cytosol **Figure 4**. The faint band observed at 515 kDa in both ER and cytosol derived immunoprecipitation samples indicates specific binding between ApoB100 and hepatic lipase, but not hepatic lipase and ApoB48. While both variants are present in the liver of rodents, only the full length ApoB100 is produced in human hepatocytes [38].

The production of VLDL particles depends on the availability of triglycerides, and in their absence, ApoB molecules are often degraded [40, 41]. This elevated lipid content causes the VTVs budding from the endoplasmic reticulum to have a low density, which is why they are present in the lowest density fractions of the continuous sucrose gradient used to resolve our budding assay. As previously reported by our lab, the VLDL transport vesicle, which transports

VLDL particles from the ER to the cis-Golgi, contains both ApoB100 and ApoAIV, but not ApoA1 [42]. Our present study demonstrates the presence of hepatic lipase in the VTV **Figure 6 a)**, which had not been identified in this vesicle in previous proteomic analyses of the VTV conducted by our lab. The identification of hepatic lipase in this transport vesicle explains its presence in cytosolic samples in association with ApoB100, as VTVs are present in the cytosol when moving between the ER and cis-Golgi.

This data suggests that an interaction between hepatic lipase and ApoB100 may mediate the movement of hepatic lipase from the ER to the cis-Golgi. Further studies should be conducted in cell culture to identify what impact this protein has on VLDL maturation, and to determine if changes in its expression can alter the rate or volume of VLDL secretion. Based on previous reports and what our data has indicated, hepatic lipase gains catalytic activity upon dimerization, at which point it is rapidly secreted from the ER; in the VTV [24]. Thus, this could represent a significant interaction involving the VLDL molecule within the VTV. Further studies should be conducted to determine definitively if hepatic lipase binding significantly alters VLDL transport to the cis-Golgi.

Calnexin is a 90kDa integral membrane chaperone that assists in the proper folding of proteins in the endoplasmic reticulum, and it is known to be specifically involved in hepatic lipase maturation [24]. Thus, it can be inferred that this protein is responsible for the band at 90kDa in the boiled sample of **Figure 1**. The band at 110kDa in the boiled sample may represent LRP, due to their previously described association in mediating LDL particle uptake. However, due to differences in local environments in the ER and the extracellular fluid, namely the lack of

mature glycosaminoglycans, further analysis was performed with immunoprecipitation to assess this possibility.

Figure 7 demonstrates a specific interaction between hepatic lipase and LRP in rat hepatic cytosol. Hepatic lipase is known to bind with LRP in the presence of heparan sulfate proteoglycans, which form at the Golgi level [19]. The vesicles in which these proteins are transported are present in the cytosol, as they transport their cargo between sub-cellular compartments, which is why they are present in discrete amounts. Based on the absence of LRP in VTVs **Figure 6 b)**, which contain hepatic lipase, and that fact that heparan sulfate proteoglycans mature and are secreted from the Golgi, this interaction between LRP most probably occurs in post-Golgi vesicles, bound for delivery to the cell membrane or for proteosomal degradation. Further studies should investigate this interaction in the trans-Golgi network, and post-Golgi vesicles. Because interaction between these proteins could not be confirmed by western blot analysis, the identity of the 110 kDa band in our silver staining procedure remains inconclusive, and warrants further investigation.

Based on the presence of equal non-specific binding in samples using hepatic lipase and IgG primary antibodies **Figure 8**, this data shows no specific interaction between ApoA4 and hepatic lipase in rat hepatic cytosol. Previous reports from *in vivo* observations have demonstrated a lack of physiological impact of exogenous HL administration on serum ApoAIV levels [43]. Hepatic lipase is known to interact with ApoA1 and ApoB100 containing particles via its bridging function, but not to ApoAIV containing particles. Taken with our data demonstrating interactions between hepatic lipase, ApoB100, LRP, and ApoA1, it can be stated with confidence that undescribed intricacies to the function of hepatic lipase exist, and that they

are mediated by direct interaction with lipoproteins and lipoprotein receptors along the secretory pathway.

Conclusion and Future Directions

The identification of a direct interaction between hepatic lipase and ApoA1 at the ER level, as well as the existence of an 81 kDa complex with immunoreactivity to both proteins in this same compartment, strongly suggests that direct binding of hepatic lipase to ApoA1 plays a role in diminishing the ability of hepatocytes to secrete HDL. Furthermore, the identification of direct interaction between hepatic lipase and ApoB100 in both the ER and in the VTV suggests that this protein's secretion is co-regulated with VLDL secretion. This interaction may also mediate degradation by a yet described pathway involving LRP at the Golgi level.

Based on the presented data and its interpretation, future studies should be aimed at determining the impact of varying levels of hepatic lipase expression on VTV formation, as well as its impact on the mechanisms responsible for movement of HDL particles from the endoplasmic reticulum, which is not well characterized in and of itself. The point along the secretory pathway at which lipidation of VLDL particles is complete is still a subject of debate. Taken with the fact that movement between the ER and cis-Golgi in the VTV is the rate limiting step in VLDL secretion, the discovery that this vesicle contains hepatic lipase after the enzyme forms an active dimer is of particular interest. Thus, attention should be given to determining what impact is elicited by the presence of dimeric hepatic lipase within the VTV. The significance of the described interaction between hepatic lipase and LRP in the ER and post-Golgi vesicles should also be investigated. Previous studies have described mechanisms by

which the LDL receptor facilitates degradation of ApoB containing particles at the Golgi level [44], thus it is not farfetched to suggest that the interaction between hepatic lipase and LRP may facilitate a similar process.

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