

REGULATION OF VLDL TRAFFICKING BY ORP 10

by

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Abstract

Of the challenges facing the improvement of human health, none has taken the forefront quite like the endeavor to discover novel treatments for heart disease. As heart disease has now become the leading cause of death throughout the world [1], the medical community has made incredible strides in the mission to treat atherosclerosis which is the major contributor to heart disease. Very Low Density Lipoproteins (VLDL) are secreted by the liver and subsequently converted to Low Density Lipoproteins (LDL). Many factors contribute to the narrowing of the arterial walls, however oxidized LDL is the main factor that leads to the deposition of plaque, leading to atherosclerosis pathologies. Recently, a main focus of research into atherosclerotic processes has been the synthesis and trafficking of VLDL in hepatocytes. The rate-limiting step for the secretion of VLDL from the liver has been determined to be the transport of VLDL from the endoplasmic reticulum (ER) to the Golgi apparatus. VLDL molecules are transported in a specialized transport vesicle the Very Low Density Lipoprotein Transport Vesicle (VTV) [2]. VLDL's core protein, apolipoproteinB-100 (apoB100), is initially lipidated in the ER, and then subsequently delivered to the Golgi apparatus where the VLDL molecule undergoes maturation involving further lipidation and glycosylation of apoB100. Oxysterol Binding Proteins (OSBP) and the sub family OSBP Related Proteins (ORP) have been implicated in many different trafficking processes, mainly the trafficking of sterols, cholesterol, and lipids. Recently, ORP 10 was shown to be a negative regulator of apoB100 secretion in growth medium [3]. Using co-immunoprecipitation, the current study shows that ORP 10 interacts with VLDL's core protein apoB100 directly. Employing an *in vitro* budding assay, we show that the blocking of ORP 10

with a specific antibody against ORP10 increases VLDL formation from the ER. Given that the ER to Golgi pathway is the rate-limiting step in overall VLDL secretion, these findings support the conclusion that ORP 10 is a negative regulator of VLDL trafficking between the ER and Golgi, and that this process is mediated by the ORP 10 protein binding with apoB100.

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I am blessed to have had these individuals in my life, and will never forget the impact that they have had on my education. As Mahatma Gandhi once said, “be the change you wish to see in the world.”

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Chapter 1: Literature Review and Aims

Introduction

Heart disease has complicated human health for centuries. The Center for Disease Control reports that 610,000 lives are claimed annually in the U.S. due to Heart Disease, which makes it the leading cause of death amongst men and women [4]. Although several risk factors have been implicated in the genesis of heart disease, none has been more elucidated and supported by research than the atherosclerotic mechanisms that result from the buildup of plaque within the arterial wall. In atherosclerosis, the pathogenic process of plaque deposition in the arterial wall is countered by the uptake of this plaque by the macrophages [5]. These macrophages when filled with cholesterol are then termed “foam cells” and contribute to the narrowing of the blood vessels [6]. The excess plaque in the sub-endothelium is made up of oxidized low-density lipoproteins (LDL), and consists of cholesterol, triglycerides, and lipoproteins [6, 7]. The LDL molecules, also known as the “bad cholesterol,” are products of very-low density lipoproteins (VLDL) that are secreted from the liver.

Very Low Density Lipoproteins

Lipid metabolism is a vital, and complex process within the human body. Its regulation internally is responsible for the transport, usage, and storage of fat and cholesterol that has been ingested from the external environment. Substantial amounts of data support the claim that dyslipidemia can lead to metabolic syndromes, and diabetic disease processes [8]. Chylomicrons are the lipid carriers of the small intestine, and have several parallel functions to their hepatic

counterparts, the VLDL [7]. However, the VLDL is characterized by its unique size and cargo that make it quite different from the Chylomicron [2]. Furthermore, VLDL is transported from the ER to Golgi in a COPII dependent manner [9], whereas the Chylomicrons transport vesicle, the PCTV (pre-chylomicron transport vesicle), contain the COPII proteins but are not dependent on them [7, 9]. COPII proteins are associated with several vesicles involved in transport of molecules in the body. For instance, the PTV (Protein Transport Vesicle) is dependent on the COPII proteins [10].

Due to the cellular toxicity of free fatty acids, the human hepatocyte uses the VLDL molecule to package cholesterol and triacylglycerol (TAG), and transport them to tissues for metabolism [9]. VLDL synthesis begins in the ER when the main component of VLDL, apoB100, is partially lipidated by MTP (Microsomal TAG-Transfer Protein) [2]. The VLDL is then transported to the Golgi in a specialized transport vesicle known as the VTV (Very Low Density Lipoprotein Transport Vesicle) [2, 9]. In the Golgi, the VLDL molecule undergoes maturation, and then is secreted in a PG-VTV (Post Golgi-VTV) to the plasma membrane [9]. The ER to Golgi pathway in hepatocytes has been the focus of research looking into the disease mechanisms that are behind dyslipidemia and metabolic disorders. The potential to target rate-limiting steps in this pathway may lead to the development of medications treating the pathological processes resulting in cardiovascular and metabolic disease.

Very Low Density Lipoprotein Transport Vesicles

VLDL is secreted from the ER in an exclusive transport vesicle known as the VTV [2].

The VTV delivers VLDL by docking with the Golgi in a COPII dependent manner [2]. The VTV-Golgi fusion, is facilitated by specific SNARE proteins, and the blocking of these proteins by specific antibodies, results in the failure of VTV-Golgi binding [11]. VLDL is comprised of the core component apoB100, triglycerides, and cholesterol. The delivery of this cargo to other tissues is vital to the overall health of the organism. In vitro budding assays, immunoblots, and electron microscopy are some of the methods that have been used to show that the VTV contains only VLDL and not albumin, a protein found in PTVs [2].

OSBP and ORP Proteins

The OSBPs (Oxysterol Binding Proteins) are of particular interest to those investigating lipid and cholesterol metabolism. OSBPs and the sub-family ORPs (OSBP- related proteins) have been found to play crucial roles in lipid, and sterol, metabolism and trafficking [12]. For example, mammalian ORP8 is involved with macrophage lipid homeostasis, and HDL (High Density Lipoprotein) metabolism, and ORP 11 is implicated in the generation of adipose tissue [13]. In the context of VLDL trafficking, ORP 10 has now become a focal point in research concentrated on this pathway. Several studies have shown data that may correlate how the function of ORP10 is intricately tied to the VLDL trafficking pathway. Based on research that has included (i) associating OSBPL10 SNPs (single-nucleotide polymorphisms) with high-end triglyceride levels in dyslipidemic subjects [14], (ii) and ORP10 regulating apoB100 secretion [3], as well as other findings, this project was focused on explaining the role of ORP 10 in VLDL trafficking. Furthermore, the research was primarily concentrated on showing that ORP10 is

regulating the VLDL trafficking pathway between the ER and Golgi, which is the rate-limiting step of VLDL secretion into the bloodstream. The implications of this research could assist future studies into the role of ORP10 in lipid metabolism.

In 2012, Nissila et al. found that ORP 10 associates with microtubules (MT) and is regulating the secretion of apoB100 from primary hepatocytes of HuH7 cells [3]. By using pulse-chase analysis of [35S] apoB-100 in the cell, they demonstrated the increased secretion of apoB100 from cells expressing ORP10-specific shRNA [3]. The absence of enhanced or modified albumin secretion in the shRNA containing cells indicates a role of ORP10 in the negative regulation of apoB100 molecules exclusively, and not the regulation of protein trafficking [3].

It is now known that OSBPL10 SNPs are correlated with high-end triglyceride levels in dyslipidemic subjects [14, 15]. Pertilla et al. after discovering this data then compared it to a subcohort examination survey on metabolic syndrome, which revealed similar results in terms of high-end triglyceride levels, and further substantiated the claim that OSBPL10 SNPs are correlated with dyslipidemia [14]. They further investigated this correlation by silencing the OSBPL10 gene. This resulted in the increased integration of [³H] acetate into cholesterol, and both [³H] acetate and [³H] oleate into triglycerides [14]. The silencing also increased the secretion of apoB100 in the growth medium [14]. This led to the assumption that VLDL trafficking and lipogenesis in the liver is being suppressed by ORP10 [14].

ORP10 is present at ~80 kDa according to western blot analysis (Figure 1). The protein consists of 764 amino acids, and is found distributed mostly in kidney and placenta, and less in

liver, skeletal muscle, spleen, small intestine, heart, lung, and testis [12]. Preliminary results have shown ORP10 is located in the ER and cis-Golgi, and found in whole cell lysate (WCL) of purified cellular fractions of rat primary hepatocytes, however is absent from the trans-Golgi and cytosol (Figure 1). It is also found associated with MTs, which is consistent with its possible role in subcellular trafficking [3]. Although, the exact mechanism behind VLDL/VTV ER to Golgi transport is yet to be fully elucidated, it is known that it moves in a COPII dependent fashion [2]. Whether this transport involves MTs or not, is yet to be determined. However, a fluorescence recovery after photo-bleaching (FRAP) analysis, was conducted by Pertilla et al. on live HuH7 cell types utilizing the EGFP-ORP10 on microtubules [14]. The results found two moving elements, one being a fast component, with a half-life of 4.5s, and the other a slow component, reported at a half-life of 64.3s [14]. The group reported that the former component may be the version of ORP10 that is distributed in the cytosol, and the slower time is ORP10 associating with the MTs [3]. They also stated that the slower component is in the same time range of half-life, as other transport vesicles that are involved with trafficking via MTs, from ER to Golgi [14, 16], and Golgi to plasma membrane [17]. This data supports the idea that ORP10 is involved in cellular trafficking between organelles. It could be that ORP10 is functioning at membrane contact sites (MCS) between the ER and Golgi, similar to other ORPs [18]. However, these findings have yet to be correlated with any transporting role of ORP10 in VLDL trafficking.

ORP10 contains a C terminal ligand-binding domain that binds to cholesterol and acidic phospholipids [3]. It could be that ORP10 interacts with the VLDL molecule directly or indirectly through this interaction, or that ORP10 is interacting with other proteins essential for VTV /VLDL trafficking. Our research now reveals that the ORP 10 molecule is binding directly

to apoB100. Furthermore, the C terminal ligand-binding domain accompanies a PH (pleckstrin homology) domain that specifically interacts with phosphatidylinositol-4-phosphate (PIP4) [3], which is found concentrated in the Golgi membrane [19]. The phosphoinositides (PIs), of which PIP4 is classified under, are phospholipids found in cellular membranes that facilitate vital functions in the membrane, as well as trafficking and signaling within the cell [20]. The significance of the interaction specifically to PIP4 is due to the decreased affinity of ORP10's PH domain to other PIPs, relative to its affinity to PIP4 [3]. Although, there are reports that ORP10 was found to specifically bind with PIP3 (another PI that is found in biological membranes), the Nissila group reported that they were unable to replicate these findings [3, 21]. Therefore, the specific interaction of ORP10's PH domain and PIP3 is still yet to be verified. Additionally, the C terminal ORD (OSBP-related binding domain/C terminal) domain was shown to have strong affinity to PIP3, 4, and 5, as well as other acidic phospholipids [3]. The affinity of these interactions varied amongst the different phospholipids [3]. It was also reported that ORP10 will localize on the microtubules, and will target the Golgi, facilitated by an N-terminal associated PH domain [3]. This may indicate a crucial aspect of ORP10 in its affinity to the Golgi that may also be a key to the determination of its overall function. Recent data has shown that ORP10 was also reported to interact with Diaphanous 1, which is a protein that regulates the rate of cortisol synthesis in the adrenocortical cells, and the movement of the mitochondria, as well as regulating microfilament and microtubule functions [22]. Other interactions by ORP10 that have been recently reported, is its ability to transport phosphatidylserine between the ER and the PM in yeast, by a yeast homologue of ORP10, Osh6 [23]. These interactions, by ORP10, are still in need of further investigations.

Further data has shown ORP10 does not bind oxysterols but does bind cholesterol and acidic phospholipids [3]. The lack of binding oxysterol by ORP10 is interesting because other OSBPs and ORPs do bind oxysterols (hence the name Oxysterol Binding Proteins) [18]. This may indicate that ORP10 has a completely different primary ligand than ORPs in the same subfamily whose roles are more established [3]. The findings however, are consistent with some ORPs. For example, ORP9L is found to bind cholesterol and not oxysterols [3, 24]. Nissila et al reported that ORP10 and ORP9L formed a complex, and that this interaction is located around the nuclear membranes where the Golgi is located [3]. However, the silencing of ORP9L did not result in increased apoB100 secretion within the HuH7 type cells [3]. Therefore, the group proposed two theories pertaining to the negative regulatory functions of ORP10 on apoB100 secretion; It is either performed by a surplus of ORP10 that is presumed to be associated with MTs, and not in complex with the ORP9L, or (ii) the remaining ORP9L that is in the ORP10 silenced cells, is enough to execute the negative regulatory process [3]. The most likely scenario is that an ORP10 surplus is carrying out the negative regulatory function on apoB100, however this is also yet to be confirmed.

Aims

Aim 1: Demonstrate that ORP10 is regulating VLDL trafficking between the ER and Golgi. The primary objective of this project was to show that ORP10 is a regulator of VLDL trafficking. Given that ORP10 is negatively regulating apoB100 secretion, which is the main component of the VLDL molecule, the general thought is that it will negatively regulate

VLDL trafficking during certain steps of the VLDL trafficking pathway. This objective was executed by employing a budding assay using an antibody specific to ORP10, in order to block ORP10's function with VLDL. This allowed us to quantify the level and rate of VLDL/VTV budding from subcellular membranes, specifically the ER. By comparing this data to the budding assay of normal, non-antibody ORP10 samples, an analysis was made on how ORP10 regulates VLDL trafficking. Western Blot analysis, and SDS-PAGE was conducted in this project to support the hypothesis. It is of interest to note that based on analysis that ORP10 negatively regulates apoB100 secretion, proven by increased amounts of apoB100 in growth medium of ORP10 silenced cells [3], the conjecture can be made that ORP10 is not necessary for VLDL synthesis. This was point to consider when investigating the effect of ORP10 on VLDL trafficking. The budding assay results contributed vastly to the overall project and analysis of ORP10's function in hepatocytes. Furthermore, the central focus of the project was on the ER to Golgi pathway, which is the rate-limiting step in VLDL secretion [25].

Aim 2: Determine if ORP10 is interacting with the VLDL or VTV. The secondary objective to this project was to determine if ORP10 was interacting with either the VLDL molecule or the VTV. Co-immunoprecipitation assays were used to show if these interactions are present. The results of the Co-Immunoprecipitation assay with ORP 10 and apoB100 revealed a direct interaction between the two proteins. A subsequent co-immunoprecipitation was carried out with ORP 10 and Cide B and revealed no interaction between the two proteins (Refer to discussion for further analysis of these results).

Chapter 2: Materials and Methods

Isolation of ER, Cytosol and Golgi

Cytosolic fractions were collected from dissected rat liver, and then processed using the same method described by Siddiqi [2]. The fractions were then washed with 0.25 M cold sucrose that was mixed with 10mM of Hepes Buffer. The fractions were then homogenized using a Potter Elvehjem serrated tissue homogenizer in cold buffer B containing 25mM Hepes, 125 mM KCL, 2.5 mM MgCl₂ 0.5 mM DTT (Dithiothreitol), 0.5 mM EGTA and mixed with protease inhibitors. The hepatocytes were then subjected to continued homogenization using a Parr bomb at between 1100 psi for 40 min. The cells were then centrifuged at 13,500 rpm for 10 min and the post-nuclear supernatant (PNS) was collected. After further centrifugation at 40,000 rpm for 95 min at 4° C, the supernatant from the samples was gathered as the cytosol. The cytosol was then incubated overnight in dialysis with a cold buffer C which included 25 mM Hepes (pH 7.2), 125 mM KCl, 2.5 mM MgCl₂, 0.5 mM DTT, and protease inhibitors.

The ER and Golgi were separated in a similar fashion, but cells were washed in cold buffer A that prepares the post nuclear supernatant. A sucrose step gradient overlay was used to isolate cis and trans Golgi fractions, and that sample was further subjected to a second isolation using another sucrose gradient overlay to ensure purification. After sucrose density gradient is completed, the samples are centrifugated 25,900 rpm for 3 hrs at 4° C. After centrifugation, the trans Golgi is aspirated from the top fraction, followed by the cis-Golgi being aspirated, then finally the ER is pelleted at the bottom layer of the tube. Once the fractions are removed they are

washed by 10mM cold Hepes, and each sample is separately centrifugated at 35,000 rpm for 40 min, and the pellet is re-suspended in transfer buffer and stored at -80° C.

Western Blotting

Determining the concentration of protein in each sample was executed by conducting the Bradford Method [11]. Each sample contained 2X Lammelis Buffer (4X for trans-Golgi), the protein sample, and PBS-T to establish exact volume for each sample. The samples were prepared and then boiled to linearize the proteins in preparation of the SDS-PAGE step. The samples were added into the wells, and were separated in a 12% polyacrylamide resolving gel using electrophoresis at 21mAmps for roughly 3 hours. Once SDS-PAGE was conducted, the proteins were transferred to a nitrocellulose membrane in order to probe with specific antibodies. The transfer was conducted overnight at 4 degrees Celsius. After the transfer, the membrane was incubated at 37 degrees Celsius in 10% dry non-fat milk in 50µl of PBS-T overnight for blocking. Following the blocking step, the membrane was washed 2 times with PBS-T and then incubated with the primary antibodies overnight at 4 degrees Celsius (ORP10 and apoB100 antibodies were purchased from the Santa Cruz Biotechnology company [11]). The ORP10 antibody was specifically an anti-goat antibody, and the apoB100 was an anti-rabbit antibody. After the primary antibody incubation step, the membrane was washed 3 times with PBS-T, and then was incubated at 37 degrees Celsius for 1 hour, with 3µl of the respective secondary antibody in 50µl of PBS-T with 0.1% dry non-fat milk. After the secondary antibody step, the membrane was washed 3 times with PBS-T, and then 2 times with PBS. Following the washes, the membrane was coated with ECL and then developed for results.

Co-Immunoprecipitation

10% Triton X-100 (v/v) was used in order to solubilize 300µg of the ER membranes. This procedure was conducted at 4° Celsius for 15 min. Anti-Goat polyclonal, anti-ORP10 polyclonal antibodies were then added and the mixture was incubated at 4° Celsius for exactly 4 hrs. Anti-Rabbit, anti-apoB100 and anti-Cide-B were then incubated using the same process as above. Following 4hrs of incubation, the anti-Goat or anti rabbit IgGs that are bound to the co-immunoprecipitation agarose beads were then added and washed 2 times using cold PBS. Following the wash, the samples were incubated at 4° Celsius overnight. The following day the beads, which then were bound to the immune complexes, were washed 10 times with cold PBS to remove any unbound proteins. The samples were then subjected to a western blot (4% stacking gel, and 12% resolving gel) to confirm the protein-to-protein interaction.

VTV Budding Assay

The VTV budding assay was conducted by using rat liver purified ER. A positive control that consisted of all the cellular components within the cytosol necessary to accomplish basal level *in-Vitro* VTV budding from the ER, and also incubated with 20µl of IgG antibody. The negative control did not contain cytosol, which is necessary for VTV budding from the ER, and did not contain antibody. The independent variable was the sample containing 20µl of ORP 10 antibody. Both IgG and ORP 10 antibodies were acquired from Santa Cruz Biotechnology and were polyclonal anti-Goat primary antibodies. All samples were incubated with 500µg of [³H]-ER, which subsequently labeled all TAG within VLDL molecules. The reaction mixture also

contained 1000µg of cytosol, 50µl of Calcium (Ca), 50µl Magnesium (Mg), 50µl Dithiothreitol (DTT), 10µl E600, 10µl guanosine triphosphate (GTP), and 100µl adenosine triphosphate (ATP). 94µl of transport buffer for positive control and independent variable was added, and 114µl was added into the negative control, so that all samples had equal volume of 500µl. The reaction mixture was incubated @ 37° Celsius for 30 min. After incubation, the addition of 700µl of cold HEPES (10mM) was added to all samples, and the tubes were incubated on ice for 5 min to stop the reaction. The samples were then overlaid a continuous sucrose gradient (0.2M-1.15M) and subsequently centrifuged @ 25,900 rpm for 2:05 hrs. The centrifuge used was a Beckman SW41 rotor. After centrifugation, collection of 500µl of each sample was conducted and d.p.m. was determined using a liquid scintillation counter (2900 Tri-carb).

Chapter 3: Results

ORP 10 binds to apoB100

The first step was to determine where ORP 10 was interacting with the VLDL or VTV molecule itself. A co-immunoprecipitation was employed with ORP 10 and apoB100 samples. Using a specific antibody for ORP 10, the results show a clear binding of apoB100 and ORP 10 at 80kDa in the apoB100 sample. In the results of the experiment (Figure 3), ORP 10 is clearly shown concentrated in the ORP 10 lane at 80kDa mark, and in the apoB100 lane ORP 10 is binding at 80kDa. Given that ORP10 has been shown to interact with acidic phospholipids at the ER membrane [3], and that apoB100 contains a cytosolic domain at the ER membrane [26], this result and binding is logical.

ORP 10 does not bind to Cide-B

Following the co-immunoprecipitation with apoB100, we wanted to know whether the ORP10 protein would bind with any other essential proteins for VTV synthesis. Given that Cide-B is necessary for VTV biosynthesis, and that the blocking of Cide-B reduces VTV trafficking significantly [10], we decided to determine whether the ORP 10 protein was interacting with the Cide-B protein. This not only would have shown whether there is a more intricate mechanism behind the ORP 10 regulation, but would also reveal how significant the apoB100 interaction with ORP 10 truly is. As shown above (Figure 4), the ORP 10 lane of the co-immunoprecipitation revealed no interaction with the Cide-B protein. If any interaction were to

be happening at the ER level, there would have been a band revealed above the 25kDa mark at roughly 27kDa. The results show that Cide B and ORP 10 are not interacting. This evidence strengthens the notion that apoB100 is an integral component to ORP10 facilitated regulation of VLDL and VTV trafficking.

Blocking ORP10 with a specific antibody increases VLDL and VTV budding from the ER

A VTV budding assay was conducted using an ORP-10 specific antibody to block the function of ORP 10. Since it had already been established that the use of shRNA on ORP 10 increased the secretion of apoB100 in growth medium [3], the general thought was that the blocking of ORP 10 function between the ER and Golgi would increase VTV and VLDL formation from the ER membrane. Results show (Figure 5) that a significant increase in VTV budding was established in the sample that contained the ORP 10 specific antibody. The exact increase of VTV formation in this sample was an 8.2% increase from the ER membrane as compared to the control. Given that the efficacy of antibody induced blockage of a protein is usually not 100%, the results are evident in showing that ORP 10's function is essential to the negative regulation of VLDL trafficking between the ER and Golgi, which is the rate-limiting step in the VLDL secretion pathway [2].

Western Blotting

ORP 10

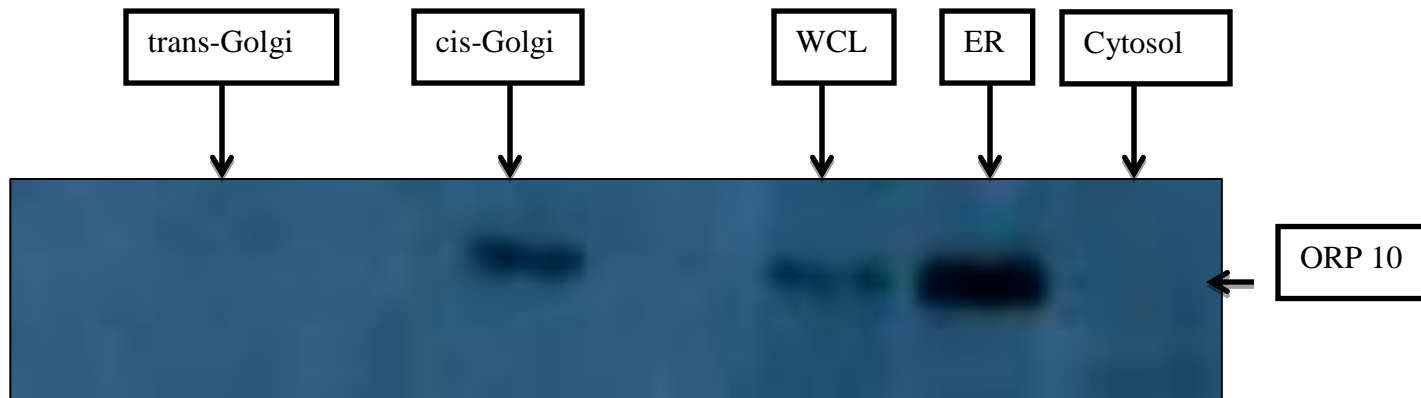


Figure 1

In this figure a western blot analysis was conducted using anti-goat ORP10 primary antibody from Santa Cruz Biotechnology, and anti-goat secondary antibody. Note: ORP10 was found concentrated at the cis-Golgi, ER, and WCL of primary rat hepatocytes. Of consideration, is the absence of ORP10 in cytosol, but more importantly its absence in trans-Golgi samples. ORP10 is showing up in western blot analysis at ~80 kDa.

Apolipoprotein-B100

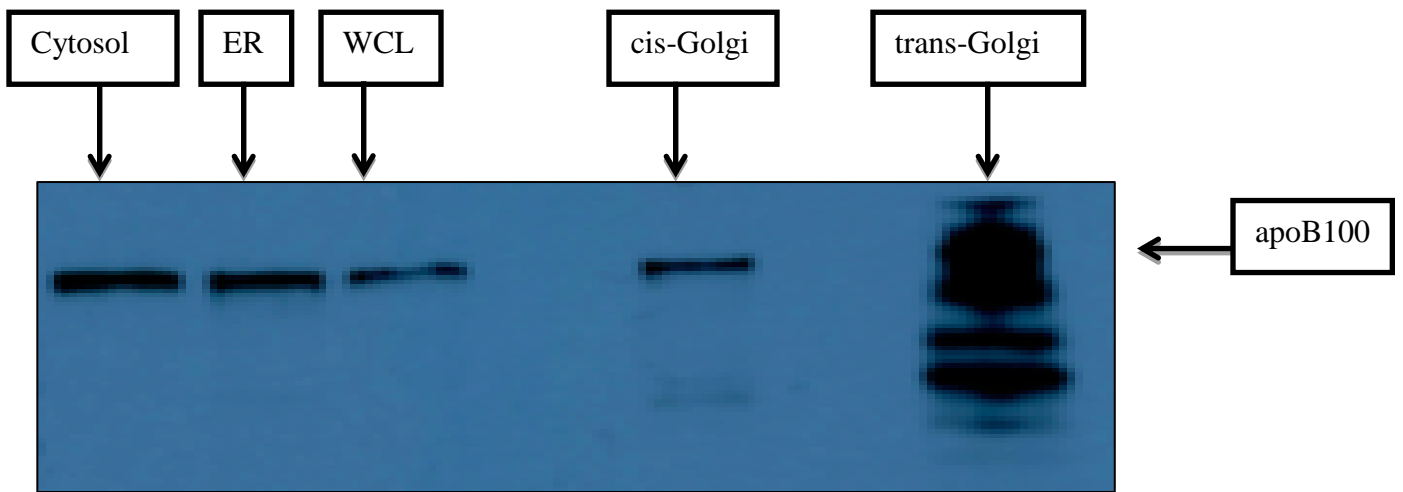


Figure 2

In this figure a western blot analysis was conducted using anti-rabbit apoB100 primary antibody from Santa Cruz Biotechnology, and anti-rabbit secondary antibody. Note: apoB100 is the main component of VLDL and is localized to the cytosol, ER, WCL, cis-Golgi, and trans-Golgi of primary rat hepatocytes. ApoB100 shows up at different molecular weights, but is primarily shown at ~250 kDa in Western Blot analysis.

Co-Immunoprecipitation

ORP 10 and ApoB100

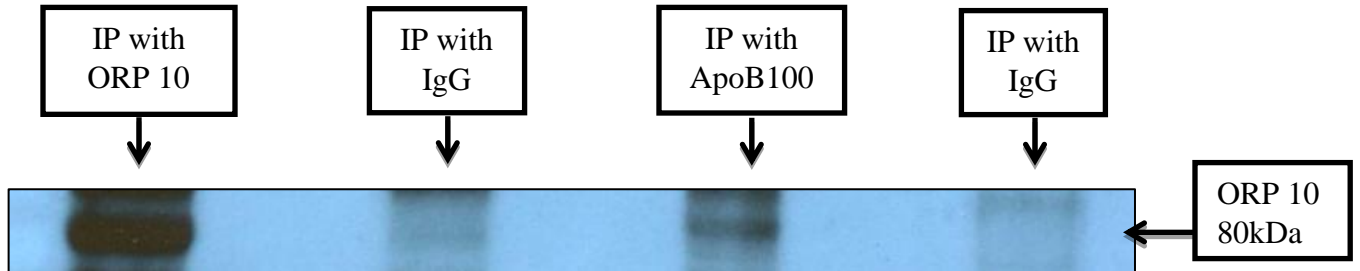
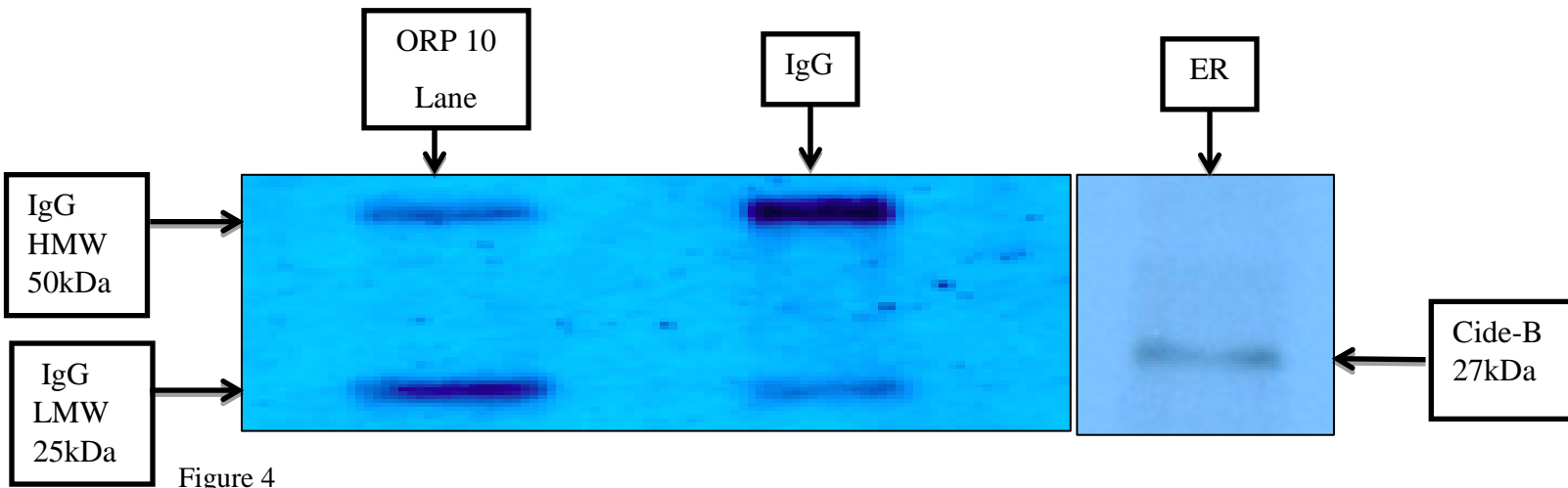


Figure 3

In this figure a Co-Immunoprecipitation was conducted with ORP 10 and Apolipoprotein B100 was performed. A specific antibody was used to bind ORP 10 at 80kDa. As the blot clearly shows, ORP 10 is found concentrated in the IP with ORP 10 lane at 80kDa, and also found bound in the IP with apoB100 lane. There is no ORP 10 band in both IgG samples. This provides strong evidence of direct ORP 10 and apoB100 binding.

ORP 10 and Cide B



In this figure a Co-Immunoprecipitation was conducted with ORP 10 and Cide-B protein. The bands on the gel are from the IgG antibody, shown at ~50kDa, and ~25 kDa. This assay was conducted to observe if ORP 10 interacts with Cide-B, which is a necessary protein for ER to Golgi trafficking of ORP 10. Cide-B is a 27 kDa protein, and did not precipitate in the ORP10 lane. On the right, is an image of the positive control for Cide-B, showing bands at 27 kDa in Western Blot analysis.

VTV Budding Assay

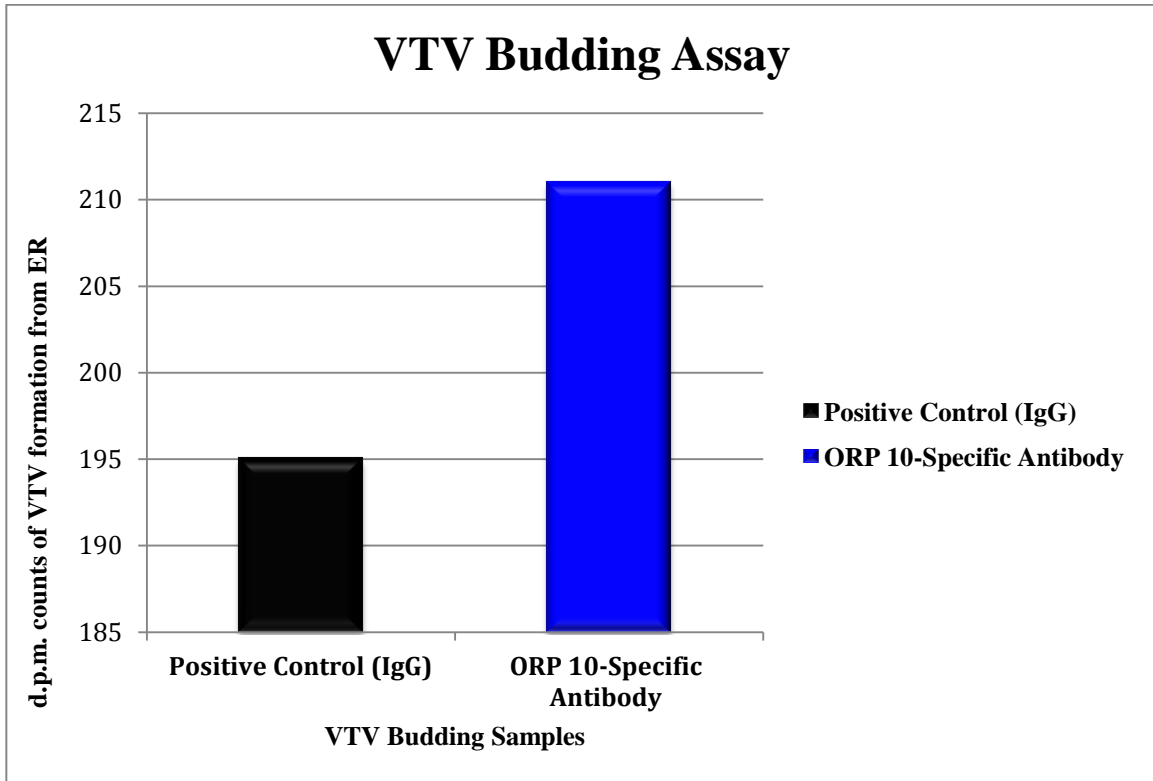


Figure 5

In this figure the d.p.m. count of both the positive control and ORP 10 – Specific Antibody samples are compared. The positive control (IgG) exhibited a 195 average d.p.m for the formation of VTV from the ER, as compared to the ORP 10 – Specific Antibody sample, which displayed a 211 d.p.m. The increase in VTV formation was 8.2% when compared to the positive control (IgG). This further supports the conclusion that ORP10 is a negative regulator of VLDL trafficking between the ER and Golgi.

Chapter 4: Discussion

It has been shown that the increase in VLDL secretion from hepatocytes is expressed in patients exhibiting atherosclerotic disease processes and metabolic syndrome [27]. As heart disease continues to increase and remains the major cause of death throughout the world, it is vital that the biomedical science community continues to contribute to the research into novel therapies and understanding of the molecular pathways involved in the pathogenesis of cardiovascular disease. Groundbreaking research has begun on VLDL and other lipid trafficking pathways that continue to meet this demand.

The ORP 10 protein is of particular interest. It was shown previously to be a negative regulator of VLDL secretion, due to when hepatocytes were subjected to shRNA specific to ORP 10; an increase of apoB100 was shown in growth medium [3]. This data was supported by further studies that showed subjects with SNPs in the OSBPL-10 gene had increased triglyceride counts [14], which is directly correlated to dyslipidemia. Another study substantiated these findings showed that specific SNPs in the OSBPL10 gene of Japanese subjects were found to possibly increase their chances of acquiring hyper - LDL cholesterolemia [15]. This data formed the basis for our current study into how ORP 10 was regulating VLDL secretion between the ER and Golgi.

Given that the ER to Golgi pathway is the rate limiting step in overall VLDL secretion [2], we found it pertinent to begin our study on this pathway. The initial western blotting analysis showed that ORP 10 and apoB100 are both found in the ER, whole cell lysate, and the cis-Golgi of hepatocytes. Following this confirmation, the co-immunoprecipitation of ORP 10 and

apoB100 indicated the specific binding and interaction of these two proteins at the ER level (Figure 3). We followed this evidence by conducting a co-immunoprecipitation on ORP 10 with Cide-B. Given that Cide-B is necessary for VTV biosynthesis [10], if it were to interact directly with ORP 10 it could show a more intricate cascade of protein signaling for the regulation of VLDL trafficking. However, the co-immunoprecipitation revealed no binding of ORP 10 and Cide-B at the ER level (Figure 4).

The lack of ORP 10 and Cide-B binding, and the presence of ORP-10 and apoB100 binding provides further evidence that the former interaction plays a crucial role in the negative regulation of ORP 10. Since apoB100 is the core protein of VLDL molecules [2], and it also contains a cytosolic domain [26], the binding of ORP 10 to apoB100 is a logical conclusion in being a direct step in the regulation of VLDL secretion at the ER level.

The final experiment conducted was a VTV budding assay from the ER. Our thought process for performing this experiment was due to the past evidence of ORP 10 being a negative regulator of VLDL secretion, primarily the secretion of apoB100 into growth medium [3], and given that the ER to Golgi trafficking is the rate limiting step in overall VLDL secretion [2], then surely if ORP 10 were to be blocked by a specific antibody at the ER level we should observe an increase in overall VTV formation. As expected, an 8.2% increase in VTV formation was shown in the sample containing the ORP 10 specific antibody. We can now confirm that ORP 10 is regulating the VLDL molecule at the ER level, and is indeed a negative regulator of VLDL secretion.

Given all the data, it is interesting to note that ORP 10 is not necessary for the synthesis of the VTV or the secretion of VLDL. This is noteworthy because it raises the question of what is regulating the activity of ORP 10? The regulating activity of VLDL is likely being carried out by a pool of ORP 10 within the cell [3]. However, the question remains of what is signaling the ORP 10 protein to modify the rate of VLDL trafficking? It is possible that ORP 10 is binding to the apoB100 cytosolic domain at the ER level either blocking the biosynthesis of the VTV, or restricting the VLDL cargo from being loaded into the VTV itself. Another possibility is that ORP 10 could be marking the VLDL molecule to be delivered to a proteasomal degradative pathway. This regulation could be facilitated via various kinases or other protein-protein interactions coming from within the cell.

An alternative possibility is that the ORP 10 molecule could be acting as a type of chaperone protein that may be escorting the VTV to the cis-Golgi. Given that ORP 10 is found at the cis-Golgi in western blot analysis (Figure 1), and has been reported to have affinity to the Golgi through its N-terminal domains affinity for GM130 (Golgi Matrix Protein) [3], and its PH domains binding capacity and increased affinity for PIP4, which is concentrated in Golgi membranes [3], these findings must be considered when investigating ORP 10's regulatory function in the future. It may be that ORP 10 has another function at the cis-Golgi level, but Nissila et al. reported the inability to elicit "full length" ORP 10's targeting of the Golgi with any "physiological mechanism" [3]. The VLDL and VTV components could quite possibly be the stimulus that is needed for this to occur.

With all the information and evidence pointing to the negative regulation of VLDL trafficking by ORP 10, it is apparent that further research must be conducted in order to fully

elucidate the mechanism behind this intricate molecular process. Further studies such as knockout of ORP 10 should prove worthy in the discussion of ORP 10's regulatory function. Nevertheless, we can now conclude that ORP 10 interacts directly with VLDL's core protein apoB100, and that it negatively regulates the formation of the VTV at the ER level. Inspection into how other ORP proteins are being utilized within the human body could also assist in the revealing of ORP 10's complete function. We believe it is imperative for the endeavor of finding treatments for cardiovascular disease.

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