Differential Expression Of Proteins Involved In VLDL Trafficking Causes Reduced VLDL Secretion In Male Ames Dwarf Mice

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DIFFERENTIAL EXPRESSION OF PROTEINS INVOLVED IN VLDL TRAFFICKING CAUSES REDUCED VLDL SECRETION IN MALE AMES DWARF MICE

by

FAISAL AHMED MOINUDDIN
B. Tech. Biotechnology
Jawaharlal Nehru Technological University, 2012

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology in the Department of Biomedical Sciences in the College of Medicine at the University of Central Florida Orlando, Florida

Fall Term
2015

Major Professor: Shadab A. Siddiqi
ABSTRACT

Cardiovascular diseases (CVDs) have been recorded as the number one cause of death worldwide, accounting for 32% of total deaths annually. More than two-thirds of all CVD cases are associated with atherosclerosis, which is the accumulation of fats and other substances causing plaque formation in the interior walls of major arteries. This leads to narrowing of the lumen and hardening of the arteries, ultimately resulting in angina, heart attack and/or stroke. Studies have shown that the pathogenesis of atherosclerosis and associated CVDs is strongly linked to elevated secretion of liver-specific lipoproteins called very-low-density-lipoprotein (VLDL). VLDLs are crucial lipoproteins responsible for transportation of triacylglycerides (TAGs), chemically inert particles that are physiologically significant for their energy storing capacity, from the liver to peripheral tissues. These VLDL particles are synthesized in the lumen of the endoplasmic reticulum (ER) of hepatocytes, transported from the ER to the cis-Golgi in special transport vesicles called VLDL-transport-vesicles (VTVs) and secreted into plasma through a highly regulated secretory pathway. Previous studies from our laboratory have shown that VTV-mediated ER-to-Golgi VLDL trafficking is the rate-limiting step in overall VLDL secretion from hepatocytes into plasma. In this project, we investigated intracellular VLDL trafficking and VLDL secretion in Ames dwarf (Prop1df, df/df) mice, a mutant mouse model homozygous for a recessive mutation at Prop1 gene locus (Prop1df) having deficiency of growth hormone (GH), thyroid stimulating hormone (TSH) and prolactin (PRL). This model is characteristic of prolonged longevity (~50% longer) and improved insulin sensitivity in comparison to their wild-type (N) counterparts. Ames dwarf (df/df) mice have recently been shown to have highly reduced plasma TAG levels, associating them with reduced susceptibility to atherosclerosis and associated CVDs.
The underlying mechanism responsible for reduced VLDL secretion in Ames dwarf mice is yet to be characterized. We hypothesize that VTV-mediated trafficking of VLDL is reduced in Ames dwarf mice because of reduced expression of proteins regulating VLDL and VTV formation. To test our hypothesis, we first performed VTV-budding assay using cellular fractions isolated separately from Ames dwarf (df/df) and wild-type (N) mice livers. Our results show a significant (45%) reduction in VTV-budding process in Ames dwarf (df/df) mice compared to wild-type (N). Next we performed 2-dimensional differential gel electrophoresis (2-DIGE) on VTV and whole cell lysate (WCL) samples in order to examine the differences in protein expression and to have highly specific protein separation. ExPASy database was used to analyze protein spots that allowed us in identifying proteins specifically expressed in each of the mouse groups. Employing western blotting, samples (ER, cytosol, VTV and WCL) from both sets of mice were tested for expression levels of VLDL and VTV associated proteins (ApoB100, Sec22b, CideB, MTP, Apo-A1 and Apo-AIV) with β-actin as the loading control. Significant differences in expression level of these proteins were observed which strongly suggest that the formation of VTV from ER in male Ames dwarf (df/df) mice is reduced compared to wild-type (N). Overall, we conclude that the differential expression of proteins required for VLDL transport causes reduced VLDL secretion in male Ames dwarf (df/df) mice.
I dedicate this thesis to myself and everyone who has ever helped me come so far in life.
ACKNOWLEDGMENTS

I would like to acknowledge Mrs. & Mr. Ghulam Qader Khusrow, Mrs. & Mr. Waseem Ghulam Ahmed, Mrs. & Mr. Iqbal Ahmed Aasim, Abdul Basit, Maryam Waseem Ahmed, Zoya Waseem Ahmed, David P. Anurag, Ghali Manogna, Suraj Singh, Heena Shiekh, Dishanth M. Kumar, Siddhanth Mohapatra, Atul Dhall, Rishav Palchoudhuri, Veethika Pandey, Ravi N. Shanker, Vivek Krishna, Manoj Reddy Gopu, Akbar, Siddharth, Vidhur Goyal, Aman Goel, Connie Mitra Sen, Bhavesh Kumar and everyone who have stood by my side through thick and thin providing unconditional love and support.
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**LIST OF ACRONYMS (or) ABBREVIATIONS**

**ABBREVIATION**

D.P.M / d.p.m – disintegrations per minute

**ACRONYM**

<table>
<thead>
<tr>
<th>ABBREVIATION</th>
<th>ACRONYM</th>
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<tbody>
<tr>
<td>D.P.M / d.p.m</td>
<td>d.p.m – disintegrations per minute</td>
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<tr>
<td>APOB</td>
<td>Apolipoprotein B</td>
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<tr>
<td>APOAI</td>
<td>Apolipoprotein AI</td>
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<tr>
<td>APOAIV</td>
<td>Apolipoprotein AIV</td>
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<tr>
<td>CideB</td>
<td>Cell-death Inducing DFFA-like Effector B</td>
</tr>
<tr>
<td>COPII</td>
<td>Coat protein complex II</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>MTP</td>
<td>Microsomal triglyceride transfer protein</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
</tr>
<tr>
<td>TAG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoproteins</td>
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<td>VTV</td>
<td>very-low-density-lipoprotein (VLDL) transport vesicle</td>
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CHAPTER ONE: INTRODUCTION

Atherosclerosis

In 2014, World Health Organization (W.H.O) statistics (source: W.H.O factsheet fs317) showed that 32% of total deaths annually recorded were due to cardiovascular diseases (CVDs) making it the number one cause of death worldwide. Of all the CVD cases, 68% have been recorded to be associated with atherosclerosis. Prevalence of atherosclerosis in U.S.A alone has resulted in an estimated economic cost of $350 billion and it is only going to increase gradually. The pathogenesis of atherosclerosis and associated CVDs, as shown by studies [1, 2], has been strongly linked to elevated secretion of liver-specific lipoprotein particles called very-low-density-lipoprotein (VLDL) particles.

Figure1: Deaths caused by Cardiovascular Diseases (CVDs) globally, 2014. [W.H.O factsheet fs317]
Very-Low-Density-Lipoprotein (VLDL)

(A) Biogenesis

Liver is one of the most important organs in the human body being capable of performing numerous functions singlehandedly. One of the significant functions of liver involves its ability to transport triglycerides (TAGs) throughout the body. These TAGs are concentrated form of fats required for cell survival significant for storage and yielding high energy. TAGs are chemically inert biomolecules. Therefore they cannot be secreted into blood directly. Liver also solves this problem by packaging TAGs into lipoprotein particles called VLDL particles. This signifies the role of VLDLs as crucial lipoproteins responsible for transportation of TAGs from liver to peripheral tissues. VLDLs are synthesized in the luminal cavity of endoplasmic reticulum (ER) of hepatocytes (liver cells) and secreted into plasma through a highly regulated secretory pathway. The biogenesis process starts post translation when the core VLDL protein, Apolipoprotein B (APOB), is being translocated into the luminal cavity of the endoplasmic reticulum (ER). APOB is received inside the ER lumen by microsomal triglyceride transfer protein (MTP) and its heterodimer PDI concomitantly. Once 28 kilo Dalton (kDa) long APOB has been received, it undergoes protein folding along with initial lipidation by MTP. MTP has the ability to transfer both neutral and polar lipids. This leads to the development of a nascent-VLDL particle and is dependent on the availability of TAGs [3, 4]. It has been shown by recent studies [5] [6] that CideB (cell death inducing DFFA45-like effector) protein also participates in formation of nascent-VLDL particles by lapidating APOB molecule. Formation is following by transport of nascent VLDL particles in special vesicles called VLDL-transport-vesicle (VTV) from the surface of ER to cis-Golgi [7].
(B) **Vesicle Formation**

Nascent VLDL particles are secreted from the ER in a coat protein complex II (COPII)-dependent vesicles [7, 8]. The COPII-dependent VTV vesicle formation is initiated when Sar1b protein in its GTP form is recruited by the ER membrane which consequently is followed by recruitment of Sec23-24 and Sec13-31 [9, 10]. Studies have proven that Sar1b protein expression is critical to the formation of VTVs and any mutation in the Sar1b gene suppresses the overall secretion of VLDL significantly. Another crucial protein in this process is CideB which plays an essential role in VTV formation [5]. It was shown that CideB interacts with APOB along with the COPII proteins facilitating the formation of VTV around the nascent-VLDL particle [5].

(C) **Trafficking**

Once VTVs bud off from the ER membrane they recruit soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein [11-13]. The SNARE proteins which associate with vesicles are termed vesicle-SNARE (v-SNARE) proteins and those which are present on the surface of the target membrane are called target-SNARE (t-SNARE) proteins. Both v-SNARE and t-SNARE form a complex to mediate the fusion of the membranes and delivery of the cargo form the vesicle. Sec22b has been shown by studies [14], as the distinctive VTV v-SNARE protein, crucial in the fusion of VTV with cis-Golgi. The corresponding t-SNARE proteins for Sec22b to form a complex have also been identified by the same study as rBet1, Syntaxin5 and GOS28 [14]. This VTV-mediated trafficking of VLDL from ER to Golgi has been identified as the rate limiting step in the overall secretion of VLDL into plasma from hepatocytes [15, 16].
Figure 2: Schematic representation of the rate-limiting step, VTV-mediated ER to Golgi transport of VLDL, in overall VLDL secretion from hepatocytes.

Ames Dwarf (df/df) Mice

Ames dwarf (df/df) mice first discovered in the 1950’s in a colony of laboratory mice at University of Iowa in Ames. These are a mutant model homozygous for a recessive single point mutation at Prop1 gene locus (Prop1<sub>df</sub>), directly upstream from homeotic factor Pit-1. Pit-1 is responsible for the development of a specific region in the anterior pituitary gland which synthesizes growth hormone (GH), prolactin (PRL) and thyroid stimulating hormone (TSH). Therefore these dwarf mice are deficient in GH, PRL and TSH [17]. The deficiency of GH affects insulin, IGF-1 and GH signaling pathways and this study is performed in search of better understanding between GH deficiency and VLDL secretion in hepatocytes.
Characteristics of Ames Dwarf (df/df)

Earlier it was understood by the scientific community that deficiency of growth hormone (GH) was the reason behind the pathogenesis of cardiovascular diseases in mammals. As GH production decreases overtime, the development of diseases due to inefficiency of immune system in humans and mice was correlated to GH deficiency. However, research in the past few decades has given us a much better and concrete understanding about the notion behind GH and its role in human physiology. And this understanding has been contributed majorly by Ames dwarf (df/df) mice as research is continuously helping us understand more about its prolonged longevity (~50% longer than wild-type (N)) in relation of being GH deficient. These Ames (df/df) mice have been observed to exhibit phenotypic characteristics such as delayed aging, prolonged longevity (~50% longer), improved insulin sensitivity etc.

VLDL Secretion in Ames Dwarf (df/df)

It has been well documented that higher TAG levels in hepatocytes corresponds to higher VLDL secretion in subjects which show higher risk association to atherosclerosis and associated CVDs. Recently, it was shown that Ames dwarf (df/df) mice were found to have reduced plasma TAG levels along with elevated liver TAG levels in comparison to their wild-type (N) siblings [18]. This observation seems to indicate a unique regulatory mechanism in Ames dwarf (df/df) mice which helps them retain TAG inside hepatocytes. Another important thing to consider is that there is no indication of liver steatosis in Ames dwarf (df/df) mice as a result of high TAG levels in the liver. The presence of such a regulatory mechanism needs to be investigated as it could possibly lead us to better understanding of the signaling mechanism behind reduction in VLDL secretion in these dwarf mice.
CHAPTER TWO: METHODOLOGY

Materials

For cell culture experiments, *McA-RH7777* cells, DMEM media and fetal bovine serum (FBS) were purchased from American Type Culture Collection (ATCC); rat growth hormone (r-GH) was purchased from Abcam Plc; $[^3]$H-Oleic acid (45.5 Ci/mM) was purchased from PerkinElmer Life Sciences; BSA-Oleic acid was purchased from Sigma Aldrich. For preparing whole cell lysate (WCL), radio immunoprecipitation (RIPA) buffer was obtained from Thermo Fisher Scientific; protease inhibitor cocktail tablets (catalogue number 04693116001) were purchased from Roche Applied Sciences. For 2D-DIGE experiments, all the reagents and materials were purchased from GE Healthcare. For gel electrophoresis experiments, immunoblotting reagents were obtained from Bio-Rad (Hercules, CA); enhanced chemiluminescence (ECL) reagents were purchased from GE Healthcare; other reagents used were of analytical grade and purchased from local companies. Liver tissues from Ames dwarf (df/df, PROP$^{df}$) mice and their wild-type (N) controls were acquired as a gift from Dr. Michal M. Masternak (Associate Professor, UCF). All procedures involving mice were conducted according to the guidelines of University of Central Florida Institutional Animal Care and Use Committee (IACUC) and strictly followed IACUC approved protocol.

Antibodies

Rabbit polyclonal anti-ApoB (Apolipoprotein B) antibody was made commercially (Protein Tech Group) via a synthetically produced peptide corresponding to amino acids 2055-2067 of rat ApoB. Mouse polyclonal antibodies to microsomal triglyceride transfer protein (MTP), SEC22B, Apolipoprotein AI (ApoAI) and β-Actin; rabbit polyclonal anti-ApolipoproteinAIV (ApoAIV)
antibody; goat polyclonal anti-CideB antibody were all purchased from Santa Cruz Biotechnology, Inc.

**Culturing of McA-RH7777 Cells**

Briefly, McA-RH7777 cells (0.2 – 0.3 million per well) were plated in a 6-well plate in 10% FBS/DMEM media overnight. Cells were then incubated with $[^3\text{H}]-\text{labelled OA} + \text{BSA-OA} + 0.5\% \text{FBS/DMEM}$ media for 60 minutes; washed with PBS. This was followed by the incubation of cells with rat-GH (100ng/ml) + DMEM media for 24 hours. Media (200 µl) was collected at time points of 0 mins, 30 mins, 1, 2, 4, 24 hours.

**Measurement of Radioactivity**

Radioactivity pertaining to VLDL particles (comprising of $[^3\text{H}]-\text{TAG}$) in media samples were measured in terms of disintegrations per minute (d.p.m.) using Tri-Carb 2910TR liquid scintillation analyzer (PerkinElmer Life Sciences) [5].

**Preparation of Hepatic Endoplasmic Reticulum (ER)**

Hepatic ER was prepared from the same protocol described previously [5, 7]. Briefly, mice hepatocytes obtained via perfusion of mice liver were incubated in Buffer B (136 mM NaCl, 11.6 mM KH$_2$PO$_4$, 8 mM Na$_2$HPO$_4$, 7.5 mM KCl, 0.5 mM DTT; pH 7.2) along with BSA bound [3H]-oleate (100 µCi) for 35 minutes at 37°C. Cells were then washed with 2% BSA in PBS to remove excess of $[^3\text{H}]-\text{oleate}$. 0.25 M sucrose in 10 m M Hepes along with 50 mM EDTA and protease inhibitor (Roche Diagnostic GmbH, Germany) was used to homogenize the cells in a Parr bomb at 1100 psi for 40 minutes. This was followed by isolation of ER via a sucrose step gradient which
was repeated to enhance the purity of ER [5, 7]. After procurement of ER, the protein concentration was determined using Bradford method and the purity was tested by Western blotting method using marker proteins as described earlier.

**Preparation of Hepatic Cytosol**

We prepared hepatic cytosol following previously described protocols [5, 7]. Briefly, cytosol buffer [25 mM Hepes, 125 mM KCl, 2.5 mM MgCl2, 0.5 mM DTT and protease inhibitor cocktail (pH 7.2)] was used to wash freshly isolated mice hepatocytes, which were homogenized using a Parr bomb at 1100 psi for 40 mins. The sample was subjected to ultracentrifugation at 100,000 g for 95 min (Beckman 70.1 Ti rotor) and the supernatant (cytosol) was collected. Dialysis of the cytosol against cold cytosol buffer was performed at 4°C overnight. Finally, a centricon filter (Amicon) and ultra-filtration membrane (Millipore) with a 10 kDa cut-off was used to concentrate the cytosol sample to a final concentration of ~20 mg/ml. The concentration was determined via Bradford assay and the purity of hepatic cytosol was determined via western blots using marker proteins for ER.

**Preparation of Hepatic Whole Cell Lysate (WCL)**

Mice hepatic whole cell lysate was prepared by following the exact same protocol mentioned previously [5]. Briefly, primary mice hepatocytes were subjected to lysis using RIPA buffer along with protease inhibitor cocktail and subjected to centrifugation at 13,000 g for 15 minutes. The resultant supernatant was collected and its protein concentration was calculated via Bradford method.
In vitro VTV Budding

The in vitro VTV budding was performed exactly the same as previously established protocol in our laboratory [7, 19, 20]. Briefly, 500 µg protein of hepatic ER containing [³H]-TAG was incubated with 1000 µg protein of hepatic cytosol along with 1 mM GTP and an ATP-regenerating system (1mM ATP, 5mM phosphocreatine and 5 units of creatine phosphokinase) for 30 minutes at 37°C. Post-incubation, continuous sucrose density gradient (0.1 - 1.15 M sucrose) was used to resolve the mixture. Fractions of 0.5 ml volume were collected and the [³H]-TAG associated radioactivity was determined using the protocol mentioned earlier.

Two-Dimensional Differential Gel Electrophoresis (2D-DIGE)

2D-DIGE experiment was performed following the same protocol described previously [19]. Briefly, we used a total of 100 µg protein for sample preparation. First, 50 µg protein each from both group of mice were solubilized separately in equal volume of lysis solution [7M urea, 2M thiourea, 4% CHAPS, 2mM tri-butyl phosphine (TBP), 0.5% carrier ampholyte (pH 3–10), 40mM Tris] for 15 minutes. Proteins from wild-type (N) and Ames (df/df) were stained with Cy5 and Cy3 dye respectively by mixing with 1 µl of diluted CyDye. This mix was incubated for 30 minutes in the dark at 4°C. 1 µl of 10mM lysine was then added to stop the reaction. Both samples were then mixed together and 2D rehydration buffer was added to result in a final volume of ~200 µl. This mixture was then incubated at 37°C for 1 hour and finally loaded onto immobilized pH gradient gel (IPG) strips (pH 3–10) by overnight incubation at room temperature.

The next step was isoelectric focusing (IEF) and was performed following the protocol provided by GE Healthcare. A total of 50,500 volt-hours are applied to each IPG strip as the complete IEF process lasts for 21 hours. After IEF, the IPG strip was subjected to 10 minute washes, first with
equilibration buffer I [0.5M Tris–HCl pH 6.8, 6M urea, 30% glycerol (v/v), 2% SDS, 25mM DTT] and then with equilibration buffer II [0.5M Tris–HCl pH 6.8, 6M urea, 30% glycerol (v/v), 2% SDS, 12mM iodoacetamide].

IEF was followed by separation of proteins via a gradient gel (8–18% SDS-polyacrylamide gel) on top of which the IPG strip was loaded. The SDS-gel was run at a constant current of 20 mAmps for 6-7 hours at 15 °C until the dye front was running out of the gel.

The final step was gel image scanning which was performed using Typhoon TRIO (GE Healthcare) immediately after gel electrophoresis ended. Consequently, the protein spots on the scanned images were further analyzed using the online portal of ExPASy’s SWISS-2DPAGE (Swiss Institute of Bioinformatics).

**Immunoblot Analysis and SDS-PAGE**

Bradford assay was used to determine the concentration of protein in ER, Cytosol, VTV and WCL samples [7]. Protein samples (40 µg) from Wild-type (N) and Ames (df/df) were separated via SDS-PAGE (4%-20%) and trans blotted onto a nitrocellulose membrane (Bio-Rad) overnight at 4 °C. 10% (w/v) non-fat dried skimmed milk powder in PBS-T (PBS with 0.05% Tween 20) was used for blocking the membrane, followed by incubation with specific primary antibodies and relevant HRP-conjugated secondary antibodies [21]. ECL Western blot detection reagent (GE, Healthcare) and autoradiography film (MIDSCI, MO) were used for detection of proteins.
CHAPTER THREE: FINDINGS

In vitro Effect of Rat Growth Hormone (r-GH) on VLDL Secretion

To test the association of reduced plasma TAG levels to smaller liver size in Ames (df/df) mice, we investigated the effect of growth hormone on VLDL secretion in vitro. McA-RH7777 cells were subjected to 1 hour incubation with [3H]-labelled OA + BSA-OA + 0.5% FBS/DMEM, followed by incubation with rat growth hormone (100ng/ml) for 24 hours. Media was collected at time points of 0 mins, 30 mins, 1, 2, 4, 24 hours and subjected to d.p.m count. Results obtained suggest that under in vitro conditions GH does affect VLDL secretion [Fig. 1]. This result is in accordance with previous studies [22, 23] on effects of GH on production and secretion of ApoB, structural protein of VLDL particles. Therefore, we have ruled out the association of reduced plasma TAG levels in Ames (df/df) mice to its smaller liver size.

Reduced VTV Budding in Ames Dwarf (df/df) Mice

The rate limiting step in the overall secretion of VLDL from hepatocytes is its transport from its site of synthesis, ER, to cis-Golgi in VTV [7, 15]. In order to test any alterations in this rate limiting step, we compared the degree of VTV biogenesis in both groups of mice via a VTV budding assay. For this assay, equal quantities of protein samples were used from both groups of mice. To initiate budding of VTV, ER sample was incubated in cytosol along with an ATP regenerating system at 37°C for 1 hour. After this incubation, a continuous sucrose gradient was used to resolve the reaction mixture through ultracentrifugation. The complete sucrose gradient had been fractionated by aspiration into fractions of 0.5 ml volume each, and [3H]-TAG d.p.m counts associated with every fraction was determined. Lighter-density fractions with higher [3H]-TAG d.p.m indicated higher VTVs.
Results showed that the average of D.P.M counts of fractions containing VTVs from Ames (df/df) mice samples were found to be 45% less in comparison to the same fractions from wild-type (N) mice [Fig. 2]. This difference is highly significant and provides crucial evidence illustrating the fact that there exists a regulatory mechanism causing reduced VLDL secretion in Ames (df/df) mice. It also helps in establishing a link between our hypothesis and the reduced TAG levels observed in Ames (df/df) mice. This result provides us the platform to further analyze and identify the cellular mechanisms/proteins/other factors behind this regulation in relation to reduced VLDL secretion.

**Analyzing Protein abundance**

In order to have higher specificity while analyzing protein expression in both wild-type (N) and Ames (df/df) mice hepatocytes, we performed 2D-DIGE where separation of proteins takes place through two parameters [Isoelectric point (pI) and molecular weight (Mw)]. 2D-DIGE was first performed on hepatic WCL samples and then on VTV samples from both groups of mice. Interestingly, the results showed us protein spots specific to each sample [Fig. 3, 4]. Consequently the pI and Mw axial coordinates for these spots were noted carefully and these coordinates became the feeder information for the online protein database ExPASy SWISS 2D-PAGE. Table 1 shows the list of proteins identified specific to samples of each mouse group. This list of proteins were further investigated to find any cellular mechanism in which they played a role with a possible link towards VLDL and VTV biogenesis. We found out Cathepsin B (CATB), protein involved in secretory pathway of pancreatic and chromaffin cells [24, 25], to be expressed only in wild-type (N) samples and either highly suppressed or not expressed in Ames dwarf (df/df). This is
interesting based on the fact that CATB has not yet been investigated pertaining to VLDL secretion from hepatocytes and this result gives us evidence to test it.

Comparing Basal Expression Level of VLDL-Synthesis and VTV-Biogenesis associated Proteins

Having found differences in protein expression through our 2D-DIGE results, it was imperative for us to test the basal expression level of VLDL and VTV biogenesis specific proteins in both groups of mice. Samples (40 µg each) used to compare the basal expression levels of proteins include ER, Cytosol, VTV and WCL. The protein bands visible on the autoradiography film were quantified using ImageJ software. All the results were quantified using β–Actin as the loading control.

We started with testing Apolipoprotein B (ApoB), principal lipoprotein mandatory for synthesis and secretion of VLDL [26]. ApoB has earlier been shown to play a central role in hepatic lipoprotein metabolism [27]. Elevated secretion of ApoB from hepatocytes or decreased removal from plasma causes increased plasma concentration of ApoB, a highly lethal condition causing increased risk for pathogenesis of atherosclerosis [28-30]. The comparative results obtained showed ~50% elevated expression in Ames (df/df) ER; ~50% reduced expression in Ames (df/df) Cytosol; ~80% reduced expression in Ames (df/df) VTV and no differences in WCL samples [Fig. 7 (a)]. From these results we comprehend that VLDL at its site of synthesis, i.e. ER, in Ames (df/df) mice is getting accumulated.

Next, we tested for microsomal triglyceride transfer protein (MTP), a molecular chaperone residing specifically in luminal cavities of ER and Golgi. MTP along with its heterodimer, PDI, plays a crucial role in translocation and assembly of ApoB [31]. It has also been shown to assist in
the early-stage lipidation of ApoB [32]. Defects in MTP gene result in an autosomal recessive disorder in hepatocytes called Abetalipoproteinemia [33], causing deficiencies in ApoB and ultimately interferes with the normal absorption of fat and fat-soluble vitamins from food. Protein expression comparative results show us no difference at ER level in both groups of mice whereas we found ~35% lower expression in Ames (df/df) WCL [Fig. 5 (b)]. MTP expression at the ER level in both groups of mice is unaltered meaning steps involving translocation and assembly of ApoB are similar. However on an overall cellular level, the reason behind reduced expression in Ames (df/df) mice still needs to be investigated.

MTP was followed by Cell-death Inducing DFFA-like Effector B (CIDE-B), another crucial protein which not only mediates ApoB lipidation [6] but also triggers formation of VLDL-transport vesicle (VTV) [5]. Our lab has already shown that knockdown of CideB results in significant reduction in VTV biogenesis [5]. Results for CideB expression reveal ~35% reduced expression in Ames (df/df) ER; ~50% reduced expression in Ames (df/df) Cytosol, ~60% reduced expression in Ames (df/df) VTV and ~40% reduced expression in Ames (df/df) WCL sample [Fig. 7 (c)]. Reduced CideB expression strongly supports our hypothesis of an existing regulatory mechanism for reduced VLDL secretion via reduction in expression of crucial proteins in Ames (df/df) group. After CideB we tested for SEC22B, distinctive vesicle SNARE protein of VTVs mandatory for fusion of VTV with cis-Golgi membrane [14]. The association of SEC22B to the surface of VTV is crucial in both VTV-Golgi fusion and also in the delivery of VLDL particles to Golgi lumen [14]. Our western blot ImageJ results show ~25% reduced expression in Ames (df/df) ER; ~70% elevated expression in Ames (df/df) Cytosol; ~75% elevated expression in Ames (df/df) VTV and ~60% reduction in Ames (df/df) WCL sample [Fig. 7 (d)]. We comprehend that the highly reduced and elevated expression of SEC22B in Ames (df/df) ER and Cytosol samples respectively possibly
correlates to reduction in VTV budding in dwarf mice. However, further evidence is needed in order to explain the actual mechanism.

As mature VLDL particles acquire ApoAI after VTV-mediated ER to Golgi transport, we next tested for ApoAI protein expression. Apart from association with VLDL, ApoAI is also the structural core protein for high-density-lipoprotein (HDL) particles which help in reverse cholesterol transport, a crucial step in maintaining plasma TAG levels [34]. Results for ApoAI show ~55% increased expression in Ames (df/df) ER; ~30% increased expression in Ames (df/df) Cytosol; ~30% increased expression in Ames (df/df) WCL sample [Fig. 7 (e)]. ApoAI was not observed in VTV sample as expected. These results strongly suggest us that Ames (df/df) mice are efficient in the synthesis of ApoAI. However the correlation of ApoAI levels to overall VLDL secretion needs further analysis at the Golgi and post-Golgi level.
Figure 3: The *in vitro* effect of rat-GH on *McA-RH7777* cells, 24 hour experiment. GH was introduced at the start of the 24 hours and no supplemental supply of GH was given. 3H-TAG were used to track the degree of VLDL secretion. † † indicates significant difference in 3H-TAG count pertaining to VLDL secretion. \( p < 0.05 \).
Figure 4: VTV budding assay result; significant (45%) reduction in Ames dwarf (df/df) in comparison to wild-type (N). ($p<0.01$)
Figure 5: 2D-DIGE WCL results. Scan of Cy5 stained wild-type (N) WCL sample (A); Scan of Cy3 stained Ames dwarf (df/df) WCL sample (B); Overlap scan (C).
**2D-DIGE whole cell lysate (WCL) ExPASy results**

**Table 1:** 2D-DIGE whole cell lysate (WCL) ExPASy results. Molecular weight (Mw) range and isoelectric point (pl) range were used to obtain the results from ExPASy.

<table>
<thead>
<tr>
<th>Present only in Wild-type (N)</th>
<th>Present only in Ames dwarf (df/df)</th>
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<tbody>
<tr>
<td><strong>Molecular weight (Mw) Range</strong></td>
<td><strong>Molecular weight (Mw) Range</strong></td>
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<tr>
<td>18 – 24</td>
<td>43 – 49</td>
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<tr>
<td>47 – 53</td>
<td>42 – 48</td>
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</tbody>
</table>

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Figure 6: 2D-DIGE VTV results. Scan of Cy5 stained wild-type (N) VTV sample (A); Scan of Cy3 stained Ames dwarf (df/df) VTV sample (B); Overlap scan (C).
2D-DIGE VTV ExPASy results

Table 2: 2D-DIGE VTV ExPASy results. Molecular weight (Mw) range and isoelectric point (pI) range were used to obtain the results from ExPASy.

<table>
<thead>
<tr>
<th>Molecular weight (Mw) Range</th>
<th>Isoelectric point (pI) Range</th>
<th>Proteins (Accession number)</th>
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</thead>
<tbody>
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<td><strong>Present only in Ames dwarf (df/df)</strong></td>
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<tr>
<td>19 – 25</td>
<td>4.9 – 5.5</td>
<td>CATB (P10605)</td>
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<td></td>
<td></td>
<td>PSB4 (P99026)</td>
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<tr>
<td>21 – 27</td>
<td>4.6 – 5.2</td>
<td>COX2 (P00405)</td>
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<tr>
<td></td>
<td></td>
<td>TCTP (P63028)</td>
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<tr>
<td></td>
<td></td>
<td>TPM3 (P21107)</td>
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<td>26 – 32</td>
<td>6.0 – 6.6</td>
<td>PRDX6 (O08709)</td>
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<td>28 - 34</td>
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<td>63 - 69</td>
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<td>TKT (P40142)</td>
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<td>37 – 43</td>
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<td>6.0 – 6.6</td>
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<td>ARG1 1 (Q61176)</td>
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<tr>
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<td>CAH3 (P16015)</td>
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</table>
Western blot results

Figure 7: Overview of Western Blot results
a. Apolipoprotein B (APOB)

**Figure 7 (a):** Immunoblot results of ApoB samples from wild-type (N) and Ames dwarf (df/df) samples

(A) ER samples; (B) Cytosol samples; (C) VTV samples; (D) WCL samples \( p < 0.05 \)
b. Microsomal TAG Transfer Protein (MTP)

Figure 7 (b): Immunoblot results of MTP samples from wild-type (N) and Ames dwarf (df/df) samples
(A) ER samples; (B) WCL samples. $[p<0.05]$
c. Cell-death Inducing DFFA-like Effector B (CideB)

Figure 7 (c): Immunoblot results of CideB samples from wild-type (N) and Ames dwarf (df/df) samples

(A) ER samples; (B) Cytosol; (C) VTV; (D) WCL samples [$p<0.05$]
d. Sec22b

**Figure 7 (d):** Immunoblot results of Sec22b samples from wild-type (N) and Ames dwarf (df/df) samples

(A) ER samples; (B) Cytosol samples; (C) VTV samples; (D) WCL samples \( p<0.05 \)
e. Apolipoprotein AI (APOAI)

Figure 7 (e): Immunoblot results of ApoAI samples from wild-type (N) and Ames dwarf (df/df) samples

(A) ER samples; (B) Cytosol samples; (C) WCL samples [$p<0.05$]
f.  Apolipoprotein AIV (APOAIV)

Figure 7 (f): Immunoblot results of APOAIV samples from wild-type (N) and Ames dwarf (df/df) samples

(A) ER samples; (B) Cytosol samples; (C) VTV samples; (D) WCL samples [p<0.05]
CHAPTER FOUR: CONCLUSIONS

Maintenance of energy homeostasis in humans is crucial as any alterations could chronically lead to complications and disorders. One of the most efficient mechanisms through which our body transports and utilizes energy in our body is through fats, in absence of which our body would heavily rely on carbohydrates and proteins ultimately leading to complications in overall physiology and metabolism. Fats being physicochemically inert in nature cannot be transported throughout the body through blood (polar in nature). This problem is solved in two steps: First, fats are transported throughout our body in its most concentrated form of triacylglycerides (TAGs); second, these TAGs are packaged into lipoprotein particles which can be directly secreted into blood plasma. The two main organs of our body responsible for synthesis and secretion of lipoprotein particles are small intestine and liver. In basal post absorptive state, majority of lipoprotein synthesis in our body is carried out by liver [7] [26].

We have seen that GH does affect VLDL secretion from hepatocytes in rodents. However, we have yet to establish complete understanding of the signaling mechanisms behind this. In this study, we have investigated the rate-limiting step, VTV-mediated transport of VLDL from ER to Golgi, in overall VLDL secretion from hepatocytes of long-lived Ames dwarf (df/df) mice. We found out that the initiation of this rate limiting step, budding of VTV from the surface of ER, is significantly (45%) reduced in Ames dwarf providing us evidence that a regulatory mechanism does exist in Ames dwarf (df/df), in comparison to wild-type (N) mice, pertaining to overall VLDL secretion. Consequently we wanted to test the abundance of hepatocyte proteome in both the mice groups and in order to attain this we performed 2D-DIGE. Results from 2D-DIGE showed us proteins which were highly suppressed or absent in Ames dwarf (df/df) mice. Interestingly we observed Cathepsin B (CATB), protein shown by studies to be involved in intracellular trafficking in the
secretory pathway of pancreatic cells and chromaffin cells [24, 25], only in wild-type (N) samples. Although CATB has not been investigated yet to see if it plays any crucial role in VLDL secretion from hepatocytes, the result gives us substantial reason to test it.

From our 2D-DIGE results, we also noticed that there were proteins pertaining to VLDL synthesis and VTV formation whose expressions looked to be suppressed. And in order to test our observations, we next resorted to Western Blot analysis to test the basal expression level of established proteins known to regulate VLDL synthesis and VTV formation. Western blot results were quantified using ImageJ software and we found out significant difference in expression of each protein. Results showed us that APOB at the ER level in Ames dwarf (df/df) is either getting accumulated or not being processed into nascent-VLDL particles as efficiently as compared to wild-type (N) mice. Results from MTP show no difference meaning the translocation of APOB into the ER lumen is the same in both groups of mice. It has been shown that knockdown or knockout of CideB protein expression leads to suppression of VTV formation and overall VLDL secretion from hepatocytes [5]. Significantly reduced CideB protein expression in Ames dwarf (df/df) mice gives proof and shows us how VLDL synthesis and VTV budding are affected at the ER level causing increased reduction in overall VLDL secretion.

In conclusion, we now know that there are differences in the hepatocyte proteome of Ames dwarf (df/df) mice compared to wild-type (N) mice. We have also observed reduced (45%) VTV budding at the ER level supported by reduced expression of CideB protein in Ames dwarf (df/df) mice. Moving forward, the next step would be to characterize the role played by CATB in the overall secretion of VLDL from hepatocytes.
LIST OF REFERENCES


