ALPHA-TOCOPHEROL REDUCES VLDL SECRETION THROUGH MODULATION OF THE VLDL TRANSPORT VESICLE

by

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ABSTRACT

The liver distributes serum triacylglycerol (TAG) via the very low-density lipoprotein (VLDL), and an increase in VLDL production may result in hyperlipidemia. VLDL synthesis consists of lipidation of Apolipoprotein B100 (ApoB) as it is co-translationally translocated across the endoplasmic reticulum (ER) membrane, and this nascent VLDL particle must undergo subsequent maturation and post-translational modification in the Golgi. The ER-to-Golgi trafficking of VLDL represents the rate-limiting step in VLDL secretion and is mediated by the VLDL Transport Vesicle (VTV). Many in vivo studies have indicated that vitamin E (alpha-tocopherol) supplementation protects against atherosclerosis and can reduce hepatic steatosis in nonalcoholic fatty liver disease (NAFLD), but its effects at the molecular level on hepatic lipid metabolism are poorly understood. To investigate the effects of alpha-tocopherol on hepatic VLDL secretion and cellular lipid retention, we performed several experiments in HepG2 (human) and McARH-7777 (rat) hepatoma cell lines including pulse-chase experiments using ^{3}H-oleic acid (^{3}H-OA), confocal microscopy with BODIPY lipid droplet staining, and an in vitro VTV budding assay. Our results demonstrate a significant reduction of ^{3}H-TAG secretion and ApoB media expression in response to 100 μM alpha-tocopherol, with a corresponding decrease in markers of VTV biogenesis in western blots of whole cell lysates (WCL) and retention of ApoB within the cell, indicating disruption of an early step in VLDL biogenesis. Further evidence indicates an increase in size and lipidation of the VTV and VLDL particle. BODIPY staining as well as 3H-TAG retention in WCLs was also sharply reduced. Overall, these

results indicate that alpha-tocopherol reduces VLDL secretion, partially disrupts hepatic VLDL synthesis and VTV biogenesis, increases the lipidation of remaining VLDL particles, and diminishes overall cellular lipid droplet retention.

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Bovine Serum Albumin-Conjugated Oleic Acid (BSA-OA)

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Dimethyl Sulfoxide (DMSO)

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Low-Density Lipoprotein Receptor (LDLR)

Microsomal Triglyceride Transfer Protein (MTP)

Nonalcoholic Fatty Liver Disease (NAFLD)

Nonalcoholic Steatohepatitis (NASH)

Oleic Acid (OA)

Oxidized LDL (ox-LDL)

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Tocopherol Binding Protein (TBP)

Tocopherol Associated Proteins 1/2/3 (TAP1/2/3, aka Sec14L2/3/4),

Tocopherol Transfer Protein (TTP)

Trans-Golgi Network (TGN)

Triacylglycerol (TAG)

Tritium (^{3}H)-Tagged Oleic Acid (^{3}H-OA)

Very Low-Density Lipoprotein (VLDL)

VLDL Transport Vesicle (VTV)

Whole Cell Lysate (WCL)

CHAPTER 1: INTRODUCTION

The liver is tasked with maintaining a delicate homeostasis between the import, export, de novo synthesis, and catabolism of lipids in order to maintain an appropriate concentration of serum lipids while simultaneously avoiding steatosis.[1] Very low-density lipoprotein (VLDL) biogenesis plays a central role in this function, and one of the mechanisms by which lipid homeostasis is reflected in this process is through synchronization of the rate of hepatic VLDL synthesis with that of its secretion.[1] VLDL synthesis occurs in a highly ordered and controlled manner that requires the incorporation of many processes within the cell: exogenous lipid import is tied to the synthesis of triacylglycerol (TAG) and other lipid species, which are in turn necessary for apolipoprotein translation and maturation; all of which come together in the intracellular vesicular trafficking and eventual secretion of the mature VLDL particle, which is in turn responsible for the distribution of lipids throughout the peripheral tissues.[1] Investigating the key aspects of this intricate process is crucial for understanding the pathogenesis of many prominent metabolic disorders, such as hypercholesterolemia, atherosclerosis, cardiovascular disease, fatty liver disease, and many others.[1]

One such aspect of lipoprotein metabolism that has received relatively little attention to date is the role vitamin E (alpha-tocopherol) plays in VLDL synthesis and secretion. Researchers have spent decades focused on the role played by vitamin E on the end results of aberrant VLDL metabolism, such as atherosclerotic plaque formation and cardiovascular disease.[2-8] Few, in comparison, have investigated the impact of this

compound on the origin of these particles, primarily due to the difficulty in studying VLDL biosynthesis and the challenge of solubilizing tocopherols for cell culture-based molecular study. As a result, there remains a lack of understanding regarding the molecular mechanisms by which vitamin E regulates hepatic lipoprotein metabolism.

This lack of understanding is highlighted in the context of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH): while recent studies involving clinical patients and in vivo models of NAFLD/NASH have revealed that high-dose supplementation of alpha-tocopherol can reduce or even reverse markers of this disease, including steatosis, inflammation, hepatocyte ballooning, fibrosis, and markers for liver disease such as alanine aminotransferase (ALT),[4,9-14] the molecular mechanism by which vitamin E mediates these effects remains unknown. As a result, there remains no known treatment for NAFLD/NASH to date.

Rationale

In the present study, we sought to ascertain the effects of vitamin E supplementation on hepatic VLDL synthesis and secretion at the molecular level, with emphasis on the rate-limiting step in VLDL biogenesis: the generation and trafficking of the VLDL transport vesicle (VTV).[1] Clinical and animal studies have demonstrated that vitamin E can reverse steatosis and other markers of NAFLD.[4,9-14] Vitamin E's role as the principle antioxidant of serum lipoproteins is well established,[15,16] but little work has been done at the cellular and molecular level in evaluating the effects of vitamin E on hepatic lipid and lipoprotein metabolism. Further, because of factors that complicate the study of vitamin E

in vivo, such as the existing vitamin E status of individual participants, the isoform and source of vitamin E used, and the presence of vitamin E in human diets, clinical studies focused on the direct effects of vitamin E on VLDL secretion have proven inconclusive.[17-21] Because VLDL metabolism is tied to other aspects of lipid metabolism, it should reason that a change in lipid droplet accumulation will be reflected in the import, export, de novo synthesis and catabolism of lipids, and in turn, VLDL synthesis and secretion.[1] And because the ER-to-Golgi transport of the VTV is the rate-limiting step in VLDL synthesis and secretion,[1] any difference observed in VLDL secretion should in turn be reflected at the level of the VTV.

Accordingly, our hypothesis is that vitamin E affects hepatic VLDL secretion through modulation of the synthesis and secretion of the VLDL Transport Vesicle. To this end, a series of experiments were performed in accordance with the two aims of this project: to determine the in vitro hepatocellular response to vitamin E in the synthesis, secretion, and ER-to-Golgi trafficking of the very low-density lipoprotein particle, and to evaluate the effects of vitamin E on in vitro hepatocyte lipid droplet retention.

Hypothesis

Vitamin E affects hepatic VLDL secretion through modulation of the synthesis and trafficking of the VLDL Transport Vesicle.

Aims

1. Determine the in vitro hepatocellular response to vitamin E in the synthesis, secretion, and ER-to-Golgi trafficking of the very low-density lipoprotein particle.

2. Evaluate the effects of vitamin E on in vitro hepatocyte lipid droplet retention.

CHAPTER 2: BACKGROUND

VLDL Synthesis and Secretion

Free Fatty Acid Import

The liver is an essential regulator of lipid metabolism and maintains homeostasis through the secretion and recycling of lipid-rich apolipoproteins by primary hepatocytes.[1] This process occurs in a sequential manner and begins with the intake of lipid species from the circulation, either through scavenger receptor B1 (SR-B1)-mediated HDL uptake, clathrin-mediated endocytosis of LDL/IDL and chylomicron remnants via the LDL receptor (LDLR), or through the uptake of free fatty acids (FFA) or FFA bound to albumin by the CD36 fatty acid translocase.[22-24] Once imported, the hepatocyte utilizes the liver fatty acid binding protein (LFABP) to sequester the FFAs while transporting them to the endoplasmic reticulum (ER) due to the risk of FFA-induced plasma membrane (PM) cytotoxicity.[25] Upon reaching the ER, these FFA are converted into triacylglycerol (TAG) and assembled into lipid droplets (LD), which are more inert than FFA and allow for longer-term storage and utilization in other metabolic processes in the cytoplasm or may be utilized for very low-density lipoprotein (VLDL) biosynthesis.[1,26]

VLDL Synthesis, Lipidation, and ER-Associated Degradation

Once sufficient TAG is generated from FFA in the ER, these LDs are packaged into VLDL for export.[1] This multi-step synthetic process begins with the lipidation of the co-translationally translocating apolipoprotein B-100 (ApoB) molecule via the microsomal triglyceride transfer protein (MTP) [1,27-29] The ApoB100 molecule, which is dense and

poorly lipidated at this point, undergoes subsequent rounds of lipidation by enzymes including cell death-inducing DFF45-like effector B (CideB) and phospholipid transfer protein (PLTP), which enrich the particle with additional TAG, phospholipids, and other lipid species.[1,27,30,31] Here, the VLDL particle also acquires other apolipoproteins, such as ApoAIV and ApoC, which overlay these lipids in order to protect them from disruption by the hydrostatic forces of the ER lumen, with the relative enrichment of these proteins correlating to the size and lipidation of the VLDL particle.[1,32] Multiple proteins assist in the folding and lipidation of ApoB100, including chaperones Hsp110 and protein disulfide isomerase (PDI; present within the lumen of the ER and also as a heterodimer with MTP).[29,33] Should insufficient lipidation of ApoB100 occur, it will undergo ER-associated degradation (ERAD) in the 26S proteasome, a process which is mediated by Hsp70 and 90, and the trimer of VCP/p97, derlin, and the ApoB100-specific E3 ubiquitin ligase Grp78.[1,33,34] ERAD represents the primary mechanism of regulation of ApoB100 expression, rather than transcriptional- or translational-level control.[1,35] Protein expression of GRP78 is directly correlated with the rate of ERAD of ApoB100, and thus serves as a marker for ER-level VLDL degradation.[1,36,37] Small valosin-containing protein interacting protein (SVIP), which participates in VLDL transport vesicle (VTV)-specific cargo selection, also plays an important role in early VLDL synthesis by blocking ubiquitination of ApoB through disruption of this ERAD trimer.[34]

VTV Budding

The nascent VLDL that is produced in the ER must undergo further maturation in the Golgi before it is secreted into the circulation.[1] The trafficking of VLDL from the ER to the Golgi is mediated by a distinct vesicle, the VLDL transport vesicle (VTV), which represents the rate-limiting step in VLDL secretion.[1,38] The VTV is a modified COPII vesicle that contains additional VLDL-specific proteins, and its assembly and budding from the ER occurs in a highly ordered and regulated manner.[1,38] First, the small, cytosolic, Ras-related GTPase Sar1b is charged with GTP by its guanidine exchange factor (GEF) Sec12, triggering the insertion of Sar1b into the cytosolic leaf of the ER membrane and initiating vesicle budding.[39] SVIP initiates budding in a similar manner, inserting into the membrane via myristoylation and recruiting both Sar1b and ApoB100.[34] Next, activated Sar1b recruits Sec23 from the cytoplasm, and the Sec23/24C heterodimer begins assembling the inner coat of the vesicle, forming the pre-budding complex.[39,40] Sec24C functions as a cargo selector, binding VLDL-specific cargo proteins through adaptors such as SVIP and the ER outer membrane protein RTN3.[39,41] Meanwhile, the Sec13/31 heterotetramer is recruited to the budding vesicle through interaction between Sec31, Sec23, and Sar1b, forming the outer COPII coat.[39] Each sequential addition of coat complex proteins further deforms the membrane, and it is this increase in membrane curvature, combined with Sar1b's GTPase activating protein (GAP) Sec23, that triggers GTP hydrolysis of Sar1b, resulting in the fission of the vesicle from the ER membrane.[39,40] These VTVs normally range from 100-120 nm in size (compared to the 55-70 nm protein transport vesicles, or PTVs) and contain one VLDL particle including one ApoB100 molecule per vesicle.[1,38,42]

Golgi-Level Maturation and Post-Golgi Trafficking

Upon arrival at the cis-Golgi, the VTV will fuse with the Golgi membrane through the formation of a SNARE comlplex between the VTV's vSNARE, Sec22b, and the three cis-Golgi tSNAREs, syntaxin 5, rBet1, and GOS28.[43] Once in the Golgi lumen, the VLDL particle will undergo maturation, including substantial phosphorylation and glycosylation of ApoB100, further lipidation of various phospholipids, sterols, FFAs and other polar lipids, and the addition of apolipoproteins AI and E (ApoAI, ApoE), which are trafficked to the Golgi via PTVs and incorporated into the particle within the Golgi network.[1,44] These fully mature VLDL particles then exit the trans-Golgi network (TGN) and are delivered to the plasma membrane (PM) via post-Golgi VTVs (PG-VTVs) which, unlike VTVs, contain two to three mature VLDL particles and range in size from 300-350 nm.[44] Fusion of the PG-VTV with the PM culminates with the release of the VLDL particle into the extracellular space and secretion into the serum.[44] It is the highly ordered and sequential nature of VLDL synthesis, trafficking, and secretion that ultimately allows for the study of lipoprotein metabolism and its greater role in metabolic disease.

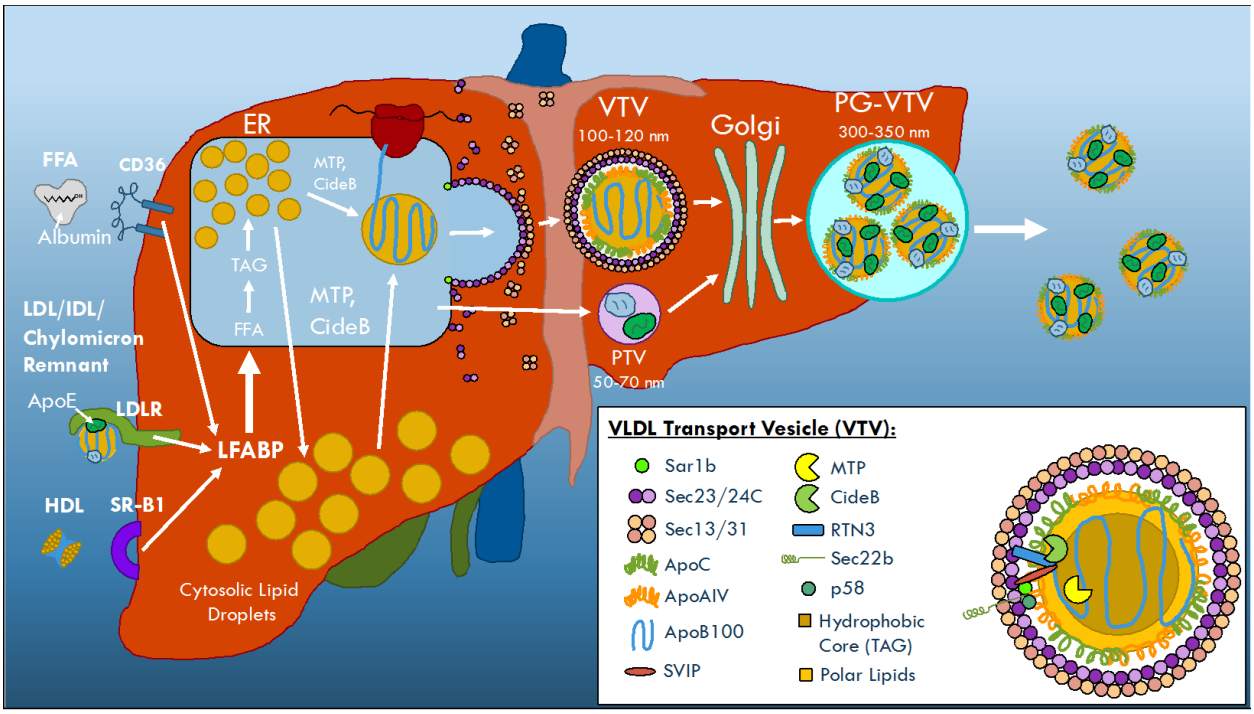

Figure 1: Overview of VLDL Synthesis, Secretion, and Key Proteins of the VLDL Transport Vesicle

Vitamin E

Background

Vitamin E is a blanket term for the eight naturally occurring tocopherols and tocotrienols that are found mostly in leafy plants, seeds, and vegetable oils.[15,45,46] Isoforms of vitamin E are labeled alpha, beta, gamma, or delta according to the position of substitutions around its chromanol ring and are characterized by a phytyl side chain that is saturated (tocopherols) or double-bonded (tocotrienols) at the 2, 4', and 8' positions, which renders vitamin E completely insoluble in water.[15,45,46] The recommended daily value for vitamin E is 15 mg (22.4 IU of natural alpha-tocopherol or 33.3 IU for synthetic), and while vitamin E is not known to accumulate to toxic concentrations from dietary sources alone like other vitamins, there are indications that long-term supplementation of high levels of

vitamin E may be associated with adverse health effects, such as bleeding, inhibition of blood clotting through interference with dietary vitamin K absorption, stroke, and an increase in overall mortality.[15,45,47] The only disease directly associated with deficiency of vitamin E is AVED (ataxia with vitamin E deficiency), which presets with loss of motor control similar to Friedreich ataxia due to the death of nerve cells, which are highly susceptible to oxidative damage.[45] Although it can rarely result from a dietary vitamin E deficiency, AVED is more commonly the result of an autosomal recessively-inherited mutation in the TTPA gene.[45]

Vitamin E Trafficking

Alpha-tocopherol is preferentially retained in cell membranes and circulating lipoproteins through the action of the alpha-tocopherol transfer protein (TTP), which is highly expressed in the liver and nervous tissue.[48] TTP exists in the cytosol of hepatocytes and selectively binds to RRR-alpha-tocopherol in the early endosome immediately following endocytosis.[49] A recycling endosome is generated containing alpha-tocopherol bound to TTP, while the late endosome containing all other isoforms of vitamin E are merged with the lysosome to be degraded via cytochrome P-450-mediated ω- and β-oxidation.[49-51] TTP directs the recycling endosome to lipid rafts containing phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) in the plasma membrane, then transfers alpha-tocopherol to the plasma membrane.[49] From there, the ABCA1 transporter translocates alpha-tocopherol out of the cell.[49] Similar tocopherol binding proteins exist both in the serum and peripheral tissues but are much less studied. Such proteins include

the tocopherol binding protein (TBP), the tocopherol associated proteins (TAP1/2/3, aka Sec14L2/3/4), afamin, and saposin B, which, interestingly, binds to gamma-tocopherol despite the preferential retention of alpha-tocopherol in most tissues.[50,52,53] The mechanism for loading alpha-tocopherol onto lipoprotein particles is unclear but appears to involve interactions between ABCA1 and ApoAI, phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP).[42,49,50,54] 100 μM is the accepted physiological concentration of alpha-tocopherol in the liver.[21]

Vitamin E and Fatty Liver Disease

Although a small store of lipids is required for various metabolic functions, an incongruity in lipid metabolism within the hepatocyte may result in abnormal aggregation of lipids.[55] Abnormal long-term accumulation of lipids within the hepatocyte may result in steatosis, which is the hallmark of nonalcoholic fatty liver disease (NAFLD).[56,57] NAFLD is a chronic and increasingly prevalent liver disorder, costing $103 billion a year and affecting up to one in three adults (64 million Americans).[56] The incidence of NAFLD is rapidly increasing and is becoming a leading cause of liver transplant.[56,57] NAFLD represents a range of pathologies that encompasses simple steatosis, representing at least 80% of cases, as well as varying degrees of inflammation, fibrosis, and ballooning of hepatocytes.[56,57] Unchecked NAFLD may eventually progress to nonalcoholic steatohepatitis (NASH), which is characterized by even greater levels of fibrosis, lipotoxicity and chronic inflammation and may lead to cirrhosis and liver cancer.[56,57]

Despite its prevalence, no direct treatment exists for NAFLD or NASH.[56-58] However, many recent clinical trials involving vitamin E supplementation of up to 1000+ IU/day for patients with varying degrees of NAFLD or NASH have yielded promising results, including reductions in steatosis, inflammation, fibrosis, ballooning of hepatocytes, and a reduction in the liver disease biomarker alanine aminotransferase (ALT).[9,12] Other groups have also used animal models of NASH to demonstrate a reduction in steatosis and inflammation in response to vitamin E supplementation,[4,9-11,13,14] as well as reduction in CD36-mediated FFA import.[14] However, the molecular mechanism by which vitamin E reverses NAFLD/NASH remains unknown, and warrants further study.

Vitamin E and Serum (V)LDL

Upon secretion from the hepatocyte, VLDL enters the serum, where it interacts with various enzymes such as lipoprotein lipase (LPL) and hepatic lipase (HL), which hydrolyzes the TAG contained within the particle back into FFA for distribution and absorption into peripheral tissues.[1,59] Other lipid transfer enzymes, such as phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP), also facilitate the transfer of lipid species such as phospholipids, cholesteryl esters, and TAG, as well as other apolipoproteins and vitamin E, between VLDL and HDL particles.[59-61] This combination of TAG hydrolysis and lipid transfer results in the transformation of VLDL into its metabolites, intermediate-density (IDL) or low-density lipoproteins (LDL), which will continue to circulate and distribute lipids to peripheral tissues until being taken back up and recycled by the liver.[1,59] Alternatively, LDL particles that become oxidized (ox-LDL) while in the circulation may

instead be aberrantly imported and retained within the arterial endothelium, leading to macrophage recruitment and uptake of ox-LDL, resulting in foam cell formation and the eventual formation of an atherosclerotic plaque.[1,59] Long-term oversecretion of VLDL, known as hyperlipidemia, is a major risk factor for atherosclerosis and cardiovascular disease.[1,59]

Vitamin E is the principle antioxidant of serum lipoproteins, including VLDL, LDL/IDL, and HDL.[15,45] Vitamin E is a single electron scavenger that requires a downstream electron acceptor, such as the water-soluble vitamin C or lipid-soluble ubiquinone, in order to protect lipoprotein particles from oxidation.[15,62] In fact, if no terminal electron acceptor is present, vitamin E will instead transfer free radicals from lipid peroxyls directly to lipoproteins, essentially becoming a pro-oxidant.[62] This is important to note when considering vitamin E supplementation, which may disrupt the redox balance within tissues.[63] Similarly, other studies have shown that atherosclerotic lesions themselves are usually not vitamin E deficient.[7,8] While many groups have sought to establish a link between vitamin E, atherosclerosis, and blood lipids, the results of these clinical trials have often been hit and miss, with many showing conflicting results. And while multiple reports have demonstrated an anti-atherogenic role for vitamin E in vascular endothelial oxLDL uptake[5,6] and inhibition of endothelial cell proliferation,[2,3] the role of vitamin E on VLDL synthesis and secretion remains largely unstudied due to the difficulty of studying both VLDL synthesis and in working with this insoluble vitamin in vitro.

CHAPTER 3: MATERIALS AND METHODS

Solubilizing Vitamin E

Two methods were used for solubilizing vitamin E for cell culture incubations. The pulse-chase experiments were performed using alpha-tocopherol-BSA-OA complexes that were generated by suspending alpha-tocopherol in a 10% ethanol solution followed by adding a portion of this suspension to an aliquot of BSA-OA. 10% ethanol (EtOH) was chosen for the initial mixing step because of the uniform suspension that was achieved after heating at 37°C and frequent vortexing for one hour, and because it was diluted to a well-tolerated 0.5% EtOH final concentration in the wells. Upon addition of the vitamin E-EtOH mixture to the BSA-OA and mixing via pipette resuspension, the mixture was clear and free of turbidity, indicating that the vitamin E was solubilized. This mixture was then added to aliquots of test media before being introduced to the cells. For pulse-chase experiments, the addition of ^{3}H-OA to the BSA-OA mixture was performed before the addition of alpha-tocopherol.

The other method for introducing vitamin E to the cells involved dissolving the alpha-tocopherol in a 44 mg/ml dimethyl sulfoxide (DMSO) stock solution, which was then added to the test media before introduction to the cells. As with the BSA-OA-vitamin E complexes, the media was ensured to be clear and free of turbidity before introduction to the cells. Analysis of western blotting of key proteins such as ApoB100, SVIP, and Sar1b immediately following incubation from both methods demonstrated that both dissolving with DMSO and complexing to BSA-OA produced the same patterns of changes in protein

expression, demonstrating that both methods were equivalent in delivering vitamin E to the cells. However, DMSO was not used for pulse-chase experiments due to the possibility of disruption of the VLDL particles within the media, and was rather used for generating samples used for some of the western blotting due to its ease of use in solubilizing vitamin E.

Other methods of dissolving vitamin E were attempted but either failed to maintain solubility of vitamin E, such as the previously reported method of dissolving vitamin E in 100% ethanol stock solutions; or proved too toxic for use in cell culture, such as with a solubilized propylene glycol-conjugated vitamin E. The previously established method of Asmis was not attempted. This method required dissolving vitamin E in hexane, coating a Teflon-coated round bottom flask at 37°C under vacuum to dissolve the hexane, then rotating the flask with fetal bovine serum (FBS) at 37°C under nitrogen for 30 hours. This method was avoided because of the uncertainty of which components of the serum were complexing with the vitamin E (for example, vitamin E is known to be transported by LDL, HDL, and albumin, and after 30 hours of incubation and perturbation, there is also a presumed possibility of vitamin E binding inappropriately to protein aggregates and thus being incapable of entering the cell) and the resulting uncertainty of the route of vitamin E into the cells. Other attempts to complex vitamin E to FBS, including by mimicking the method used to complex vitamin E with BSA-OA, were unsuccessful.

Alpha-Tocopherol Incubations

With the exception of the VTV budding assays, all experiments were performed under a standardized one-hour incubation protocol. 0.5 x 10^6 McARH-7777 (rat hepatoma) or HepG2 (human hepatoma) cells were plated in each well of a six well plate, with three positive and three negative wells per run. Positive wells received 100 μM alpha-tocopherol (ThermoFisher), 75 μM bovine serum albumin-oleic acid (BSA-OA, 100 mg/ml, Sigma), either 0.5% ethanol or 0.1% DMSO, 2.5% (McARH-7777) or 5% (HepG2) fetal bovine serum (FBS, ThermoFisher), and 1% Pen-Strep (PS, ThermoFisher) in Dulbecco's modified eagle medium (DMEM, ThermoFisher) media. Negative wells received identical treatment minus the alpha-tocopherol.

A final well concentration of 100 μM alpha-tocopherol was chosen according to previous reports that established this amount as the physiological concentration of vitamin E in hepatic tissues in vivo.[21] The concentration of 75 μM BSA-OA was chosen because preliminary studies using higher concentrations of BSA-OA resulted in the "drowning out" of the effects of vitamin E owing to a relative oversaturation of OA. The final well concentrations of 0.5% ethanol or 0.1% DMSO were settled upon as the concentration that was most well-tolerated by the cells for the duration of the 1-hour incubation while still allowing for efficient solubilization.

Metabolic Radioisotope Labeling

To determine the effects of vitamin E supplementation on VLDL secretion, we performed pulse-chase experiments using 75 μM BSA-OA to instigate VLDL secretion and

100 μM alpha-tocopherol to replicate physiological hepatic vitamin E concentration. McARH-7777 cells were incubated for one hour in triplicate using positive (vitamin E and BSA-OA) and negative (BSA-OA only) pulse media as described above with the addition of 2 μCi ^{3}H-OA per well. After one hour, the pulse media was removed, and the cells were washed three times with warm phosphate buffered saline (PBS). Then, 3 ml of 5% FBS 1% PS DMEM "chase" media was added for 48 hours, and 300 μl media fractions were collected at 1, 2, 4, 6, 24, and 48-hour time points. The cells were lysed at 48 hours using radioimmunoprecipitation assay buffer (RIPA buffer, ThermoFisher) with protease inhibitor (PI), sonicated, then pelleted at 15,000 x g for 15 minutes. ^{3}H-oleic acid (45.5 Ci/mm) was purchased from PerkinElmer Life Sciences. All samples were kept on ice immediately following collection and during use and were stored at -80°C when not in use.

Disintegrations per minute (DPM) of media, VTV, and whole cell lysate (WCL) samples containing ^{3}H-TAG was performed using a Tri-Carb 2910TR Liquid Scintillation Counter (LSC). 30 μl aliquots of media fractions, or 50 μg protein from WCL or VTV samples, were introduced to a glass LSC vial before 5 ml ScintiVerse scintillation fluid was added and the samples were capped and rigorously vortexed. The automated DPM count readings were performed by the LSC and consisted of a read time of two minutes per vial.

Confocal Imaging

In order to investigate morphological changes in the cells, 0.2 x 10^{6} McARH-7777 cells per well were plated on collagen cover slips and incubated according to the standardized protocol as described. Following the incubations of one hour for the test

media and 48 hours for the normal media, cells were fixed with para-formaldehyde and stained with DAPI nuclear stain, BODIPY lipid droplet stain, Calnexin ER stain, and/or Gos28 golgi stain, then visualized with a Zeiss confocal microscope and analyzed with Volocity imaging software.

VTV Budding Assay

The study of the effect of vitamin E on the rate-limiting step in the ER-to-Golgi trafficking of the VTV required performing an in vitro VTV budding assay which has been previously established in our lab.[38,64] For this assay, primary hepatocytes were isolated from 150-200g Sprague-Dawley rats (Harlan Laboratories), washed with cold 0.25 M sucrose 10 mM HEPES pH 7.2, and cultured with 100 μCi ^{3}H-OA-BSA for 30 minutes at 37°C in order to pre-charge the ER with ^{3}H-TAG. These cells were then homogenized via Parr cell disruption vessel and the ER was isolated via ultracentrifugation using a sucrose step gradient. Cytosol was isolated in a similar manner, but with additional steps required to prepare the cytosol buffer solution including removing ATP and GTP through dialysis. These procedures are described in detail here.[38,64]

To initiate VTV budding, cytosol buffer and other factors including GTP and an ATP regenerating system was incubated with or without vitamin E at 37°C for one hour with frequent vortexing, then this cytosol was introduced to isolated ER (500 μg protein) for 30 minutes at 37°C. The reaction was stopped by dousing with cold HEPES, and the mixture was overlaid onto a continuous 0.1 – 1.15 M sucrose gradient and spun at 82,000 x g for 2 hours at 4°C (Beckman Rotor 70.1 Ti). VTVs were collected from the top four fractions and

were concentrated using a Centricon YM-10 filter (10 μm pore size, Amicon) and Beckman Coulter ultracentrifuge. Because this assay is performed in the absence of Golgi, they resulted in the formation of VTVs that could then be quantified through DPM measurement and also characterized morphologically through western blotting in order to evaluate the effects of vitamin E on VTV vesicle budding rate, quantity, and morphology.

SDS-PAGE and Western Blotting

WCL and media samples from both pulse chase experiments and from non-pulse chase incubations, as well as VTV samples from the VTV budding experiments, were prepared and run on 12% (WCL and VTV) or 9% (media) SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gels with 4% stacking gel. Protein estimation was performed using a pre-established Bradford assay protocol (Bradford reagent, BioRad). Samples were then prepared by combining 40 μg protein (WCL and VTV) or 30 μl media fractions with Laemmli's buffer (BioRad) and β-mercaptoethanol (BioRad) reducing agent, then were boiled for three minutes before electrophoresis was performed. SDS-PAGE and western blotting reagents were purchased from Bio-Rad. Overnight western transfer was performed, and the resulting membranes were blocked in a 10% milk PBS-T solution and probed with primary antibodies (1:500 dilutions, Santa Cruz Biotech) and HRP-conjugated secondary antibodies (1:2,500 to 1:10,000 dilutions, Santa Cruz Biotech) for western blotting. Blots were developed by incubating membranes with ECL (Enhanced Chemiluminescence reagent, GE Healthcare) and developed on autoradiography film (MIDSCI).

Statistical Analysis

GraphPad Prism 7 was used to generate figures and perform statistical analysis (unpaired t test).

Animal Handling

Animal procedures related to the harvesting of murine livers for VTV budding experiments were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Central Florida.

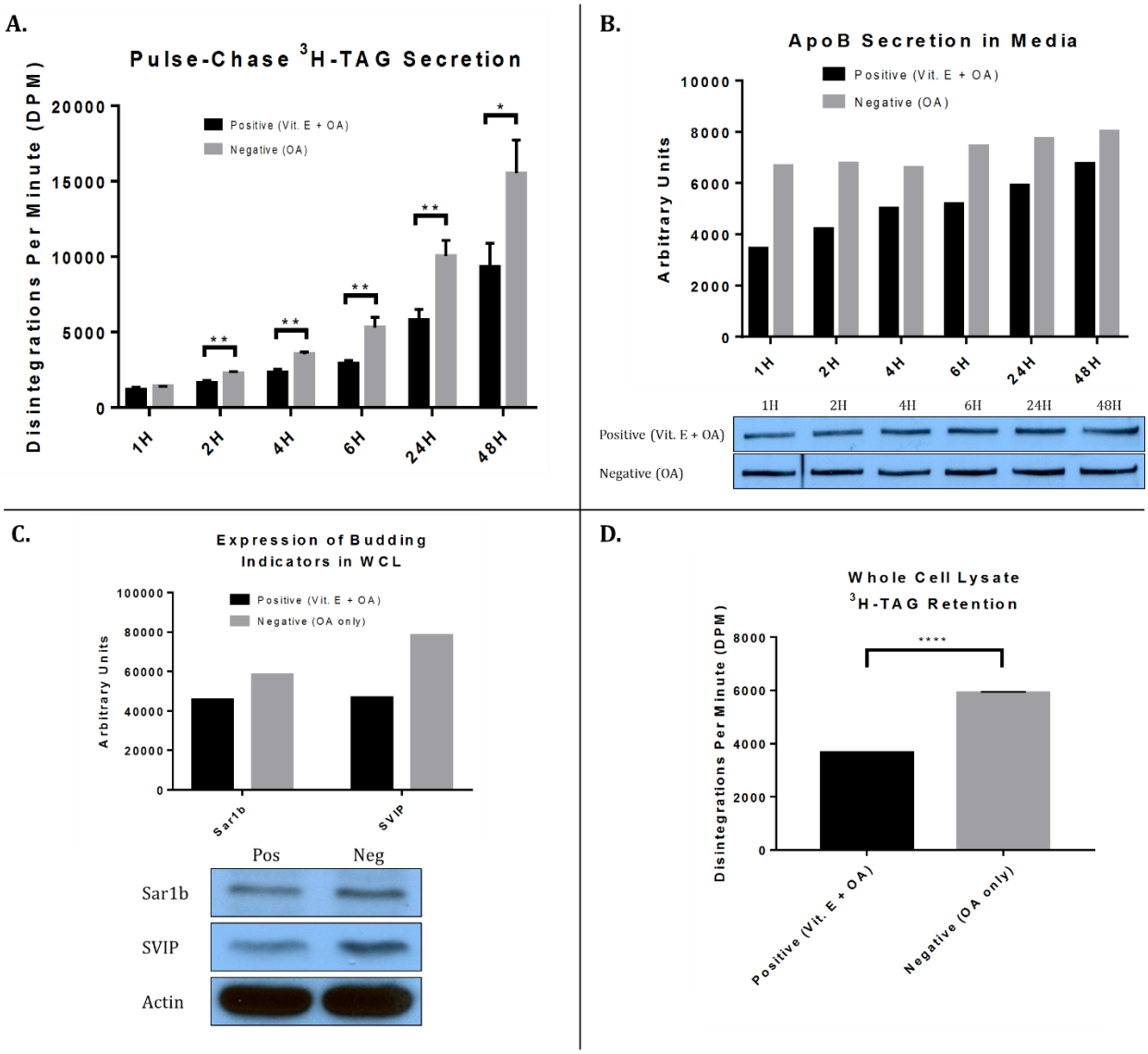

Figure 2: Effects of Vitamin E on VLDL Secretion and Hepatocellular Lipid Retention

Vitamin E Decreased In Vitro VLDL Secretion and Reduced Intracellular Tag Retention

A pulse-chase experiment was performed in McARH-7777 cells as described. Briefly, cells were treated either with 100 μM alpha-tocopherol and 75 μM OA-BSA (Positive) or only 75 μM OA-BSA (Negative) with 2 μCi ^{3}H-OA per well for one hour, then washed and

placed in chase media for 48 hours with media samples collected intermittently. Cells were lysed after 48 hours, and both WCL and media samples were tested via LSC and immunoblotted for key VTV budding proteins. Measurement of ^{3}H-TAG secretion from McARH-7777 cells into chase media revealed vitamin E-treated cells secreted less ^{3}H-TAG, reaching a maximum difference of 45.3% at 6 hours and maintaining a 42.4% and 40.0% difference at 24 and 48 hours, respectively (Figure 2A). A corresponding decrease in ApoB100 secretion into the media was detected via immunoblotting against media fractions, indicating a reduction in VLDL particles in the media (Figure 2B). VTV budding markers Sar1b and SVIP were decreased 48 hours post-incubation, correlating the observed reduction in VLDL secretion (Figures 2A and 2B) with reduced ER-to-Golgi transport of VLDL, the rate-limiting step of VLDL secretion (Figure 2C). WCL of treated cells showed a 40% reduction in intracellular ^{3}H-TAG retention 48 hours after treatment (Figure 2D). (Discovery determined using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with Q = 1%. Each row was analyzed individually, without assuming a consistent SD.)

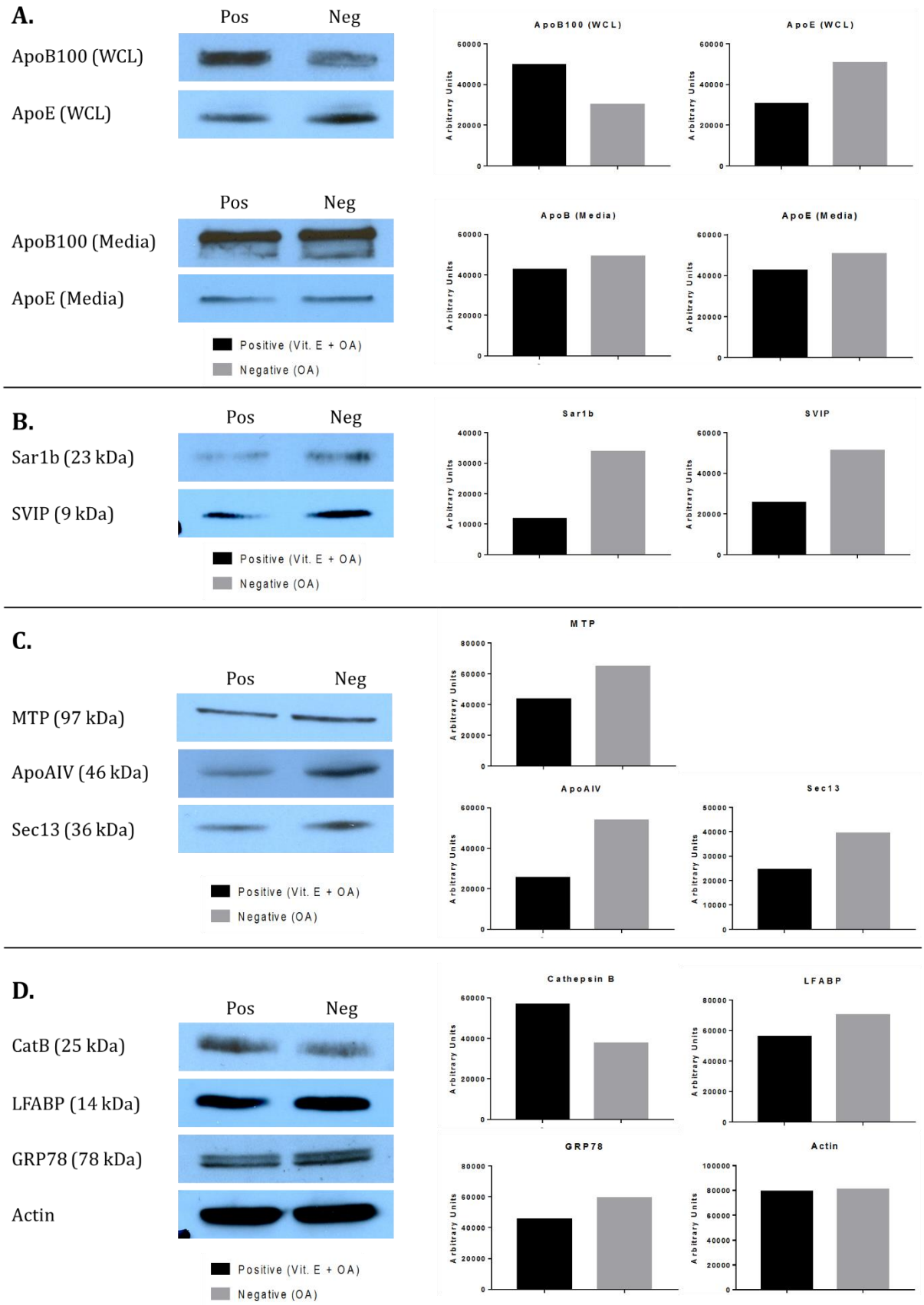

Figure 3: Vitamin E Affected Expression of Markers for Key Steps in VLDL Biosynthesis

Vitamin E Disrupted the ER-to-Golgi Transport of VLDL

HepG2 cells were treated with alpha-tocopherol and BSA-OA (Positive) or only BSA-OA (Negative) for one hour as described, then lysed and immunoblotted. Media samples exhibited a reduction in ApoB and ApoE secretion, demonstrating reduced VLDL secretion as seen in Figure 2. However, WCLs demonstrated an increase in ApoB retention and decrease in ApoE expression, indicating pre-Golgi disruption of VLDL biogenesis (Figure 3A). Sar1B and SVIP, initiators for VTV budding and VLDL-specific cargo selection, were sharply reduced following treatment with alpha-tocopherol, indicating the similar effects seen after 48 hours (Figure 2C) occur soon after alpha-tocopherol introduction (Figure 3B). Markers for three sequential stages in VLDL synthesis and VTV budding are shown, indicating the steps of particle lipidation (MTP), apolipoprotein addition (ApoAIV), and VTV vesicle COPII cage formation (Sec13) are all reduced in response to alpha-tocopherol treatment (Figure 3C). Metabolic markers for other stages in VLDL biosynthesis reveal: diminished exogenous lipid import, as evidenced by the reduction in expression of FFA transfer protein LFABP; increased expression of Cathepsin B, the protease that is responsible for cleavage of and which is normally expressed in opposition to LFABP; and reduced ERAD of ER-level VLDL, shown by the reduction in the ApoB-specific E3 ubiquitin ligase GRP78 (Figure 3D).

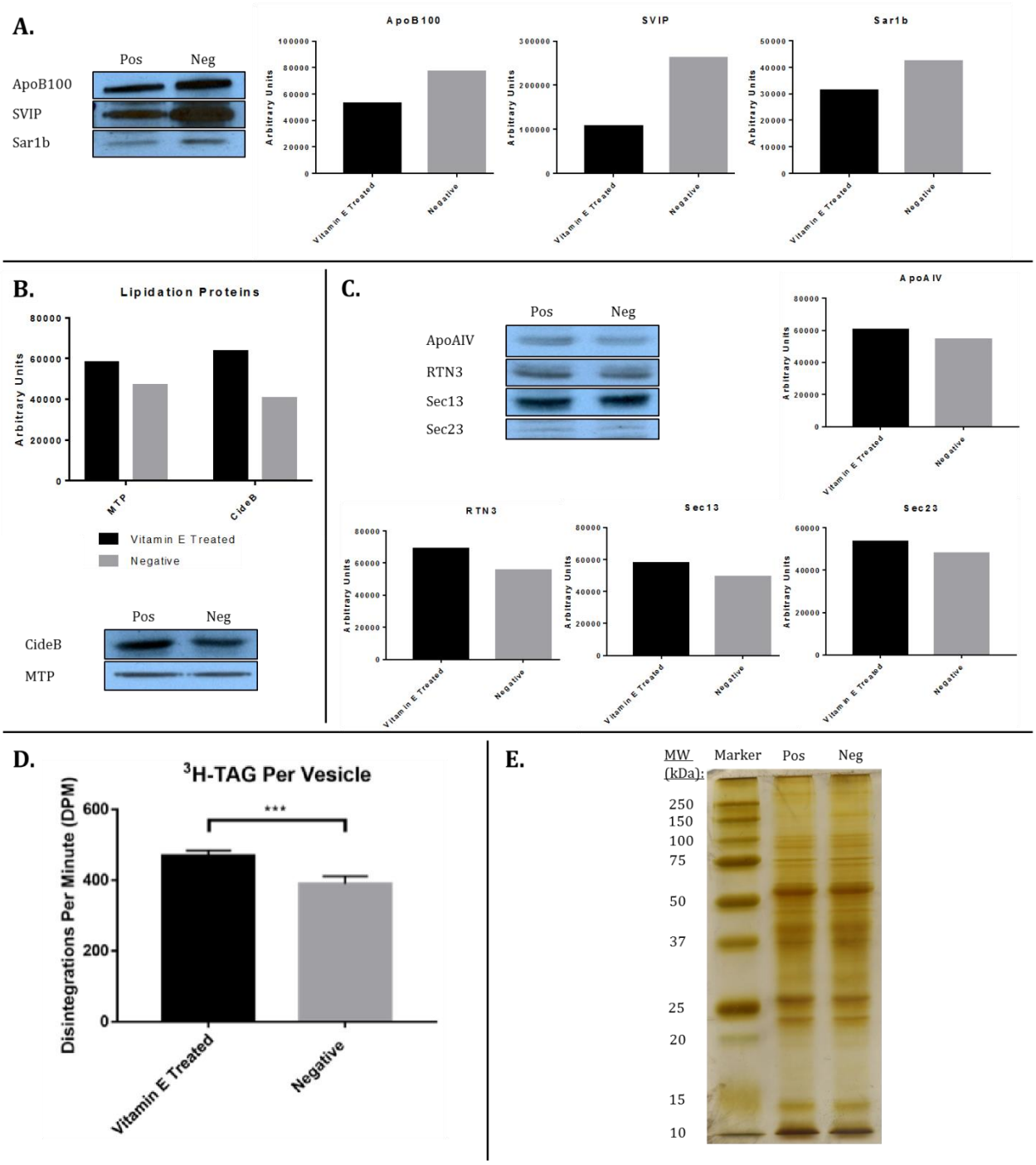

Figure 4: Vitamin E Induced VTV-Specific Effects in Isolated Murine Cytoplasm and ER

Vitamin E Treatment Produced VTVs That Were More Lipidated and Larger in Size

An in vitro VTV budding assay was performed as described. Briefly, primary hepatocytes were isolated from Sprague-Dawley outbred rats, charged with ^{3}H-OA, then ER and cytosol were isolated via centrifugation. Cytosol was incubated with vitamin E for one hour, then added to the ER fraction to induce the VTV budding reaction. Vesicles were isolated, immunoblotted, and measured for TAG content. Figure 4A demonstrates fewer vesicles were produced from the vitamin E-treated samples: enrichment of ApoB100, the principle marker of the VTV, with one molecule added per vesicle; as well as VTV budding markers SVIP and Sar1b were reduced, further corroborating the reduction in VTV particle production seen in Figures 2 and 3. However, Figure 4B shows an increase in lipidation markers MTP and CideB, which indicates the alpha-tocopherol-treated VLDL particles are more lipidated than the control. Peripheral VLDL particle protein ApoAIV is increased in the positive VTVs, as well as enrichment of the VTV membrane protein RTN3 and COPII components Sec13 and Sec23 (Figure 4C). Together, these results indicate an increase in VLDL particle and VTV vesicle size. DPM counts for vitamin E-treated VTVs were increased 21% when equalized by total protein (50 μg) (Figure 4D). While DPM counts from the top three fractions of vitamin E-treated VTV samples were slightly reduced (data not shown) owing to the decrease in total vesicles produced, the increase in ^{3}H-TAG per vesicle combined with increased enrichment of MTP, CideB, ApoAIV, RTN3, Sec13, and Sec23 (Figures 4B and 4C) reveal that, while fewer VTVs were produced following alpha-tocopherol treatment (Figure 4A), those VTVs were more lipidated and larger in size. (E) A gel that was run in parallel with the gel used for immunostaining was silver stained and

used as a loading control (Figure 4E). (Discovery determined using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with Q = 1%. Each row was analyzed individually, without assuming a consistent SD.)

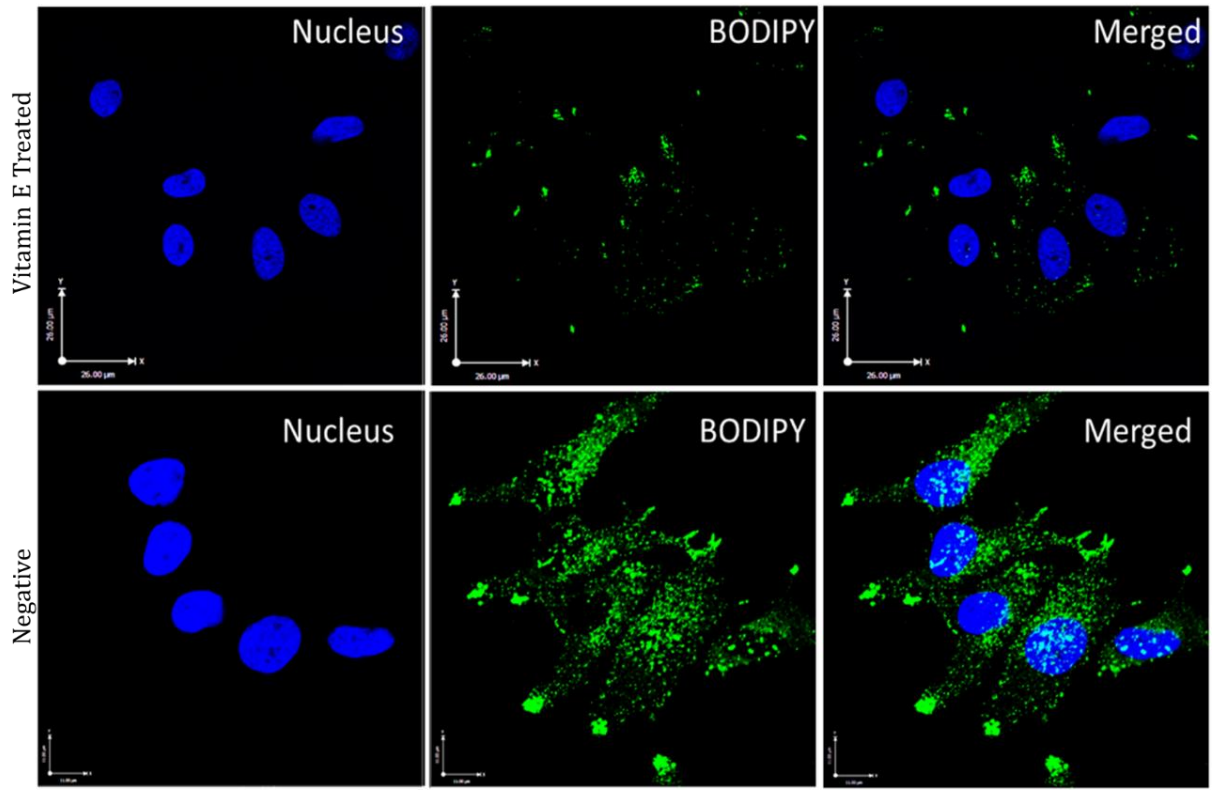

Figure 5: Vitamin E Induced Changes to McARH-7777 Cell Lipid Droplet Staining and Morphology

Vitamin E Reduced Lipid Droplet Staining in McARH-7777 Cells

Confocal imaging was performed following a similar treatment to the pulse-chase experiments described previously. Briefly, McARH-7777 cells were plated on collagen cover slips and underwent a one-hour incubation with vitamin E and BSA-OA (Positive) or BSA-OA alone (Negative), followed by a 48-hour rest period. The cells were then washed, fixed with paraformaldehyde, and stained with DAPI nuclear stain and BODIPY lipid

droplet stain. Vitamin E-treated cells demonstrated a significant reduction in lipid droplet staining, with many of the perinuclear droplets disappearing altogether (Figure 5).

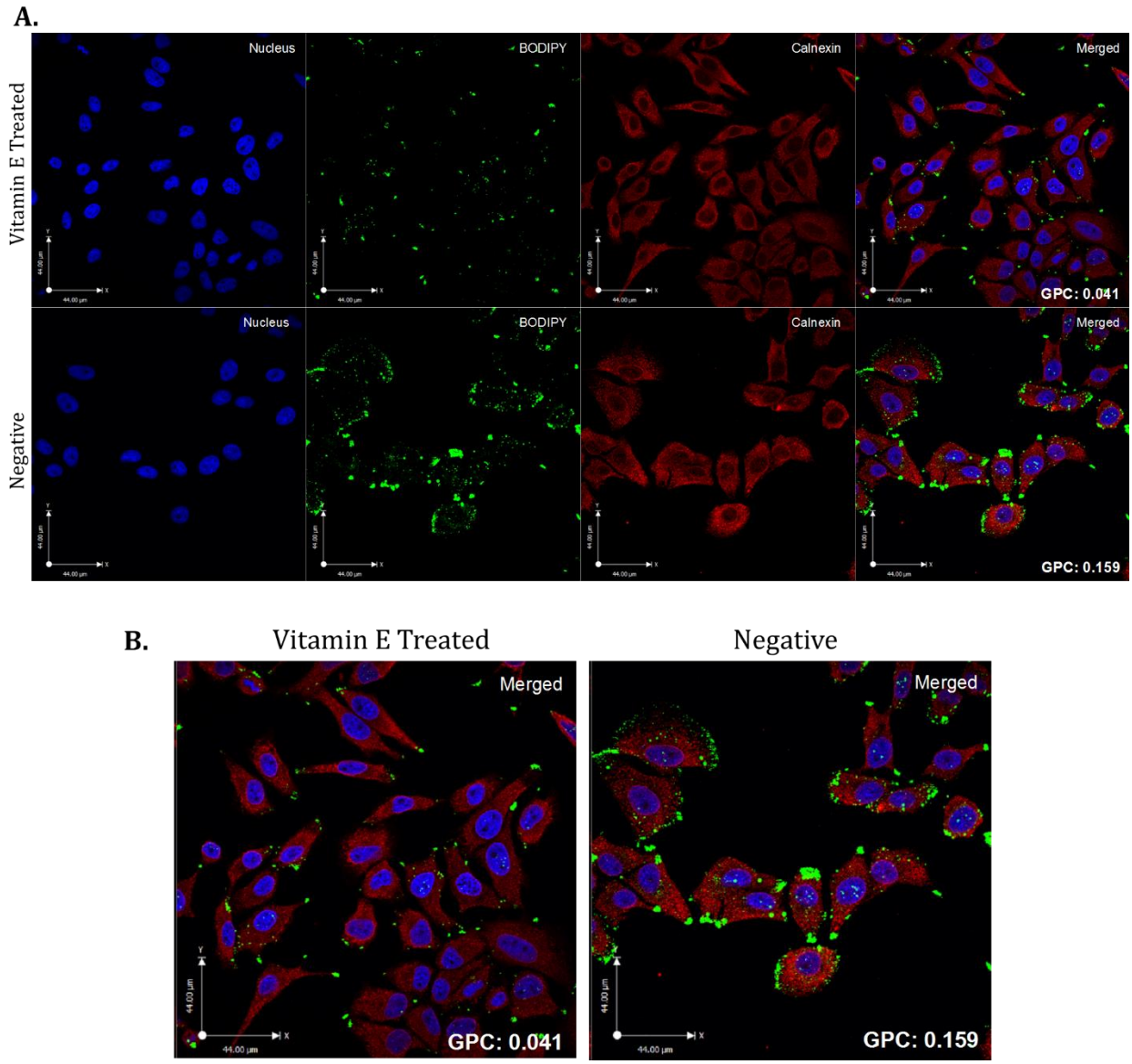

Figure 6: Vitamin E Reduced ER-Associated Lipid Droplet Localization

Fewer Lipid Droplets Were Associated with the ER Following Vitamin E Treatment

In order to investigate changes in the morphology of ER-associated lipid droplets, McARH-7777 cells were incubated as described previously with alpha-tocopherol and BSA-OA (Positive) or BSA-OA alone (Negative), then fixed 48 hours later and stained with DAPI nuclear stain, BODIPY lipid droplet stain, and anti-Calnexin-conjugated Texas Red ER stain. Vitamin E-treated cells displayed decreased BODIPY staining, with the droplets that remain being held closer to the periphery of the cells (Figure 6A). Fewer lipid droplets can be seen associated with the ER network in vitamin E-treated cells, with a 75% lower Global Pearson's Coefficient (GPC) than in the negative cells (Figure 6B).

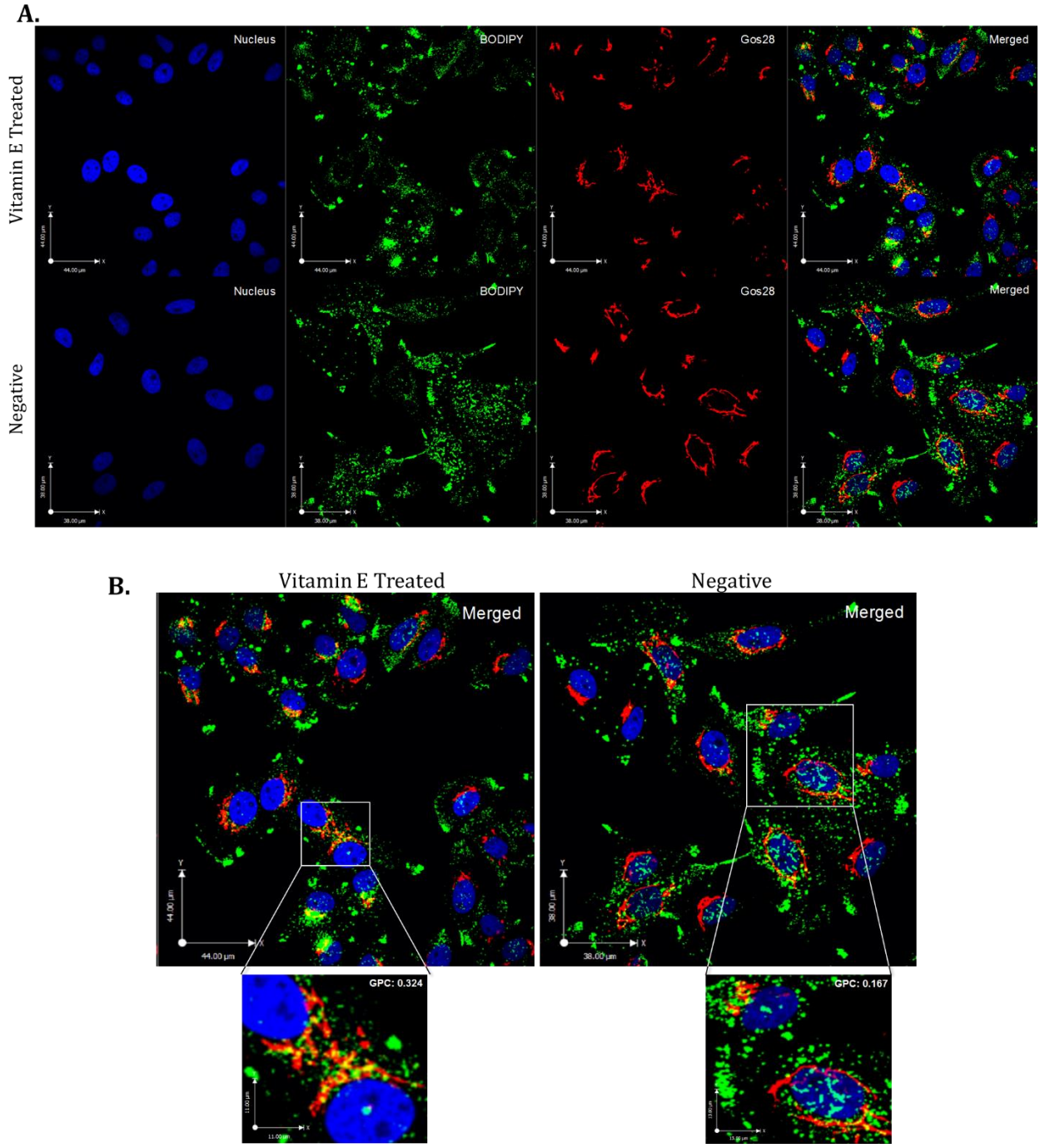

Figure 7: Golgi-Level Lipid Droplet Co-Localization was Affected by Vitamin E Incubation

Vitamin E Incubation Increased Lipid Droplet Co-Localization with the Golgi

Finally, to identify Golgi-level changes to lipid droplet localization, McARH-7777 cells were treated with vitamin E and prepared as described above, then stained with DAPI nuclear stain, BODIPY lipid droplet stain, and anti-Gos28-conjugated Texas Red Golgi stain. Once again, vitamin E-treated cells displayed reduced lipid droplet content (Figure 7A), but with greater co-localization of lipid droplets with the Golgi stain Gos28 (Figure 7B), indicating a possible disruption of post-Golgi VTV trafficking.

CHAPTER 5: DISCUSSION

Recent trials involving vitamin E supplementation in both human patients and mouse models of NAFLD and NASH have demonstrated a remarkable clearance of steatosis and subsequent reversal of inflammation and other disease markers.[9,12] However, similar studies on the direct effects of vitamin E on VLDL synthesis and secretion have yielded mixed results. And because of the difficulty in solubilizing alpha-tocopherol in vitro, the molecular mechanisms by which vitamin E affects these processes remain to be elucidated. For these reasons, we investigated in the present study the impact of vitamin E on VLDL synthesis, intracellular trafficking, secretion, and cellular lipid retention in hepatic cells.

To study this, we first performed a series of pulse-chase experiments in McARH-7777 rat hepatoma cells using a one-hour incubation with alpha-tocopherol and ^{3}H-OA (see methods) followed by a 48-hour chase period. Measurement of ^{3}H-TAG and ApoB100 secretion in media fractions via LSC and immunoblotting, respectively, revealed a sustained decrease in VLDL secretion of over 40% from the sixth through the 48th hour (Figures 2A & 2B). Western blotting of these WCLs revealed a corresponding decrease in VTV budding markers Sar1b and SVIP, indicating that the rate-limiting step of ER-to-Golgi VLDL transport was being affected by the alpha-tocopherol (Figure 2C). We were also able to identify a 40% reduction in ^{3}H-TAG retention in these WCLs 48 hours post-treatment (Figure 2D), correlating with similar results seen in vivo by other groups and verifying that alpha-tocopherol was introduced successfully to the cells.

Based on the observed reduction in VLDL secretion, we next decided to investigate changes in expression of markers for each step in VLDL synthesis and secretion immediately following alpha-tocopherol treatment. Cells were incubated either with alpha-tocopherol and BSA-OA (Positive) or BSA-OA alone (Negative) as described and lysed immediately following the one-hour incubation. As observed in the pulse-chase experiment, there was a reduction in secretion of VLDL markers ApoB and ApoE in the media (Figure 3A). This indicates a reduction in quantity of VLDL particles secreted, due to the fact that each VLDL particle contains one ApoB molecule. The presence of ApoE in the media (Figure 3A) also indicates that these particles underwent post-ER maturation, as this apolipoprotein is added at the Golgi level. However, immunostaining of WCLs revealed surprising results: while ApoE expression was reduced as expected, indicating fewer VLDL particles were able to reach the Golgi; ApoB expression was increased, implying a disruption in a pre-Golgi step in the process of VLDL synthesis that resulted in the retention of ApoB within the lysate (Figure 3A).

To narrow down the point of disruption, we examined the expression of markers for various steps within the VLDL biosynthetic pathway. Budding initiators Sar1b and SVIP, which are responsible both for the initiation of VTV vesicle formation and for VLDL-specific cargo selection, were decreased both one hour (Figure 3B) and 48 hours (Figure 2C) post-incubation, indicating a reduction in VTV quantity and subsequent ER-to-Golgi trafficking. Next, we investigated the expression of MTP and ApoAIV, markers for the distinct pre-budding processes of lipidation and ER-level maturation, respectively. Immunostaining revealed reduced expression for both MTP and ApoAIV (Figure 3C), indicating a decreased

rate of successful VLDL synthesis within the ER. The COPII vesicle component Sec13 was also reduced (Figure 3C), further corroborating the reduction in VTV generation implied by the observed reduction in Sar1b and SVIP expression (Figure 3B, Figure 2C). Signs of reduced exogenous FFA uptake were also seen, as evidenced by a reduction in LFABP expression and corresponding increase in the expression of its protease Cathepsin B (Figure 3D).

Given this apparent disruption of early-stage VLDL synthesis, we investigated whether ER-associated degradation (ERAD) was responsible for the reduction in VLDL synthesis. ERAD of VLDL is mediated by the E3 ubiquitin ligase and global regulator of the unfolded protein response GRP78, whose protein-level expression correlates with the rate of ERAD within the ER.[37] However, we were surprised to find that the expression of GRP78 was slightly reduced (Figure 3D), indicating that ERAD is likely not responsible for the observed reduction in VLDL synthesis. While the mechanism by which the cells are eliminating this ApoB remains unknown, the impact of alpha-tocopherol on other means of lipid catabolism, such as autophagy, should be investigated in future work.

To determine the effects of alpha-tocopherol at the VTV level, we next performed an in vitro VTV budding assay, which involved incubation of isolated rat primary hepatocyte cytosol with vitamin E, which was subsequently removed from the vitamin E and incubated with ER also isolated from rat primary hepatocytes. This generated a VTV budding reaction that approximated the physiological trafficking of vitamin E while avoiding direct introduction to the ER. As observed in previous results (Figures 2 and 3), VTV budding and VLDL particle quantity was reduced as shown by the reduction in ApoB100, SVIP, and

Sar1b (Figure 4A). However, we were surprised to see an increase via western staining of the enrichment of MTP and CideB in these vesicles (Figure 4B), which indicates an increase in lipidation of the VLDL particles. This was confirmed via LSC, which demonstrated a 21% increase in DPM counts in the vitamin E-treated vesicles (Figure 4D). Markers for the size of the particle and vesicle were similarly increased: ApoAIV, which is added in the ER before the VLDL particle is loaded into the VTV; RTN3, which is an outer ER membrane protein that is enriched in the VTV and participates in cargo selection; and Sec13/23, which help form the COPII coat of the VTV (Figure 4C). Because this assay is performed with isolated ER and cytoplasm, it is insulated from changes that may also be occurring at the transcriptional level as in the whole-cell experiments, as well as from prenyltransferases that reside in the mitochondria which may similarly mediate secondary effects at the cellular level. Overall, this experiment demonstrates that vitamin E can produce an effect directly on the cytoplasm of these cells, which in turn can affect ER-level processes such as VLDL assembly, VTV formation, and vesicular protein enrichment without requiring changes at the transcriptional level.

Next, confocal imaging was performed in order to verify the reduction in intracellular TAG and investigate morphological changes resulting from vitamin E treatment. McARH-7777 cells were seeded onto collagen cover slips and incubated with vitamin E and BSA-OA for one hour in a similar manner as in the pulse-chase experiments. Following a 48-hour rest period, cells were fixed with paraformaldehyde and stained with combinations of DAPI nuclear stain, BODIPY lipid droplet stain, anti-Calnexin-conjugated Texas Red ER stain, and/or anti-Gos28-conjugated Texas Red Golgi stain. In all cases, cells

demonstrated a reduction in lipid droplet retention (Figures 5, 6, and 7), verifying the diminished TAG retention observed in the pulse-chase experiments (Figure 2D). Much of this reduction in intracellular TAG was the result of a reduction in ER-associated lipid droplets (Figure 6). Intriguingly, an increase in Golgi-level lipid droplet localization was also discovered (Figure 7), which indicates that post-Golgi VLDL trafficking may also be affected by vitamin E.

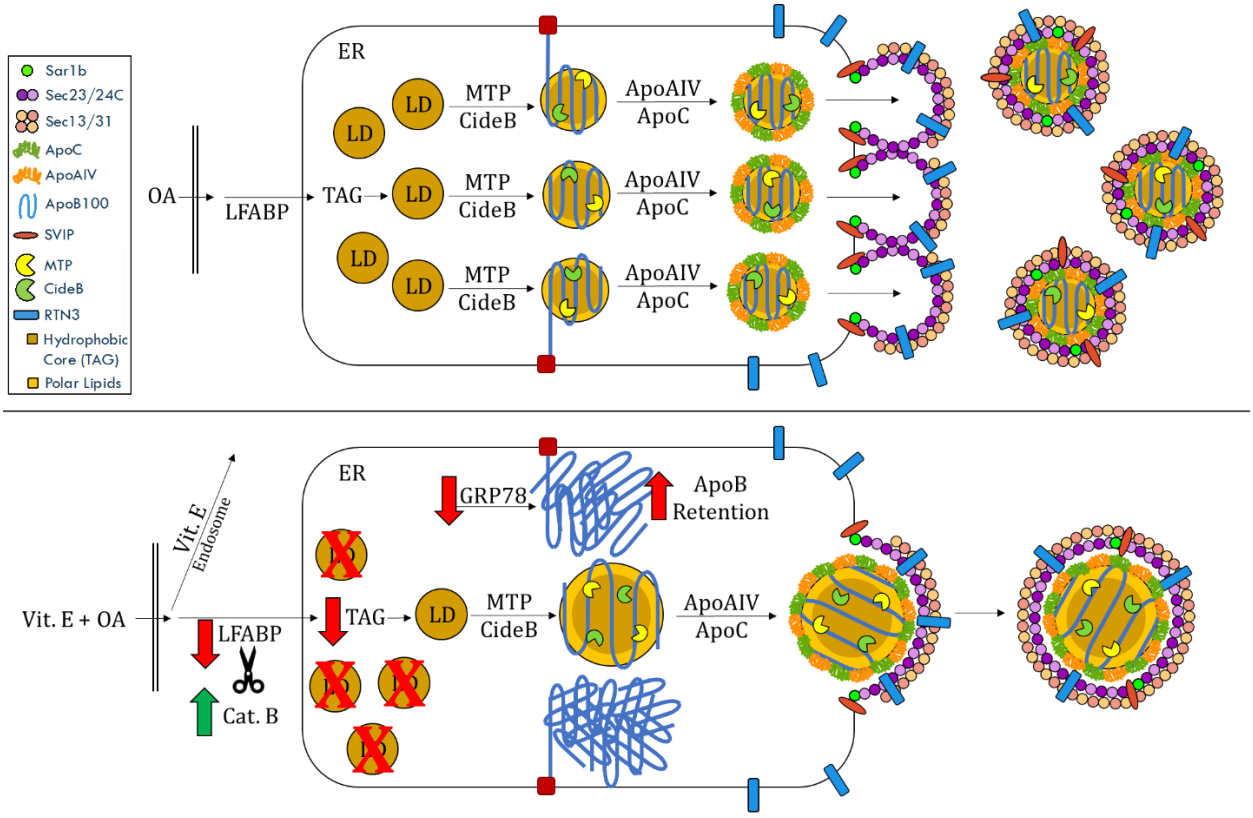

Figure 8: Summary of Vitamin E-Induced ER-Level Changes to VLDL Synthesis and VTV Budding

Summary of Results

Vitamin E treatment of hepatocytes resulted in reduced LFABP-mediated OA import, reduction in GRP78-mediated ERAD, aberrant retention of ApoB100, decreased VTV production, and suppression of VLDL secretion. However, the VTVs that were successfully produced were larger in size and more lipidated than the untreated VTVs. Confocal imaging demonstrated fewer overall lipid droplets were retained within the hepatocytes, and while fewer of these lipid droplets were located at the ER, more were seen at the Golgi level.

CHAPTER 6: CONCLUSION

In conclusion, the results of these experiments show alpha-tocopherol disrupts early VLDL synthesis in vitro, resulting in a decrease in the successful assembly, lipidation, vesicular transport, maturation, and eventual secretion of the VLDL particle. This decreased VLDL secretion is sustained for up to 48 hours post-incubation and occurs in conjunction with an apparent decrease in exogenous lipid uptake as well as a decrease in intracellular lipid retention. While fewer in quantity, the VLDL particles that are produced following alpha-tocopherol treatment also appear to be larger and more lipidated. It is possible that the greater observed utilization of ER-associated lipid droplets, combined with a decrease in FFA import, can largely account for the 40% reduction in cellular TAG observed over the 48-hour span following alpha-tocopherol treatment. However, the role played by other lipid catabolic processes such as autophagy or lipophagy remains unknown and warrant further investigation. Similarly, the apparent retention of lipids within the Golgi that was discovered through confocal imaging hints at a possible impact of vitamin E on post-Golgi VLDL trafficking, and should be investigated in future projects.

In this study, we successfully introduced vitamin E to two different hepatoma cell lines using two distinct methods of solubilization and were able to demonstrate a direct effect on VLDL synthesis, secretion, and trafficking. Furthermore, our in vitro VTV experiment utilized cellular components isolated from primary hepatocytes to demonstrate that some of the VTV-specific effects were not reliant on transcriptional changes but were rather mediated by an unknown cytoplasmic mechanism. The results of

this thesis align with existing in vivo studies which have demonstrated a reduction in hepatocellular lipid retention following vitamin E supplementation, and to our knowledge represents the first time this has been investigated in cell culture. Similarly, we have successfully demonstrated some of the direct mechanistic effects of vitamin E on VLDL synthesis, trafficking, and secretion, which has otherwise yielded conflicting results in vivo. Despite the relatively scant attention paid to vitamin E in molecular research owing to the difficulty of alpha-tocopherol utilization in vitro, this study demonstrates the need for greater understanding of the mechanisms by which vitamin E affects lipid and lipoprotein metabolism, and in the case of untreatable NAFLD/NASH, represents a key area of focus in the search for future treatments of high-burden metabolic diseases.

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