Structural and Functional Disruption of Mitochondrial Homeostasis by the Environmental Toxicant Vinyl Chloride

by

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Vinyl chloride (VC) is an environmental toxicant that directly causes liver injury at high concentrations. However, lower concentrations (< OSHA limits), which are not overtly hepatotoxic, enhance injury caused by high-fat diet (HFD), at least in part, via mitochondrial dysfunction and endoplasmic reticulum (ER) stress. Mitochondria and the ER closely interact via mitochondria-associated membranes (MAMs). Alterations in these contact sites are associated with mitochondrial dysfunction and ER stress. The purpose of this study was to investigate the impact of VC exposure on the damage, function and interaction (via MAMs) of these organelles. C57Bl/6J mice, fed HFD, or low-fat control diet (LFD), were exposed to VC (<1 ppm), or room air for 6 hrs/d, 5 d/wk for up to 12 wks. Alpha mouse liver 12 (AML12) cells were exposed to the VC metabolite, chloroacetaldehyde (CAA) for 20 hrs at 37°C. VC exposure modified mitochondrial morphology by increasing mitochondrial area, independent of diet while mitochondrial DNA content was unaffected. VC also decreased levels of key MAM complex proteins. CAA exposure increased mitochondrial size and altered the proximity between the ER and mitochondria in AML12 cells. Taken together, VC and CAA altered mitochondrial structure and organelle interactions. These stress responses may be causal in VC-mediated toxicity and sensitization. These data highlight that current safety restrictions may be insufficient to address VC-induced hepatotoxicity in humans.

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List of Abbreviations

AML12	Alpha Mouse Liver 12
ATSDR	Agency for toxic substance disease registry
BMI	Body mass index
CAA	Chloroacetaldehyde
CAE	Chloroacetate esterase
CE	Chloroethanol
CEO	Chloroethylene oxide
CYP2E1	Cytochrome P450 Enzyme
Disc-1	Disrupted-in-schizophrenia 1
DMEM	Dulbecco's Modified Eagle's Medium
Drp1	Dynamin related protein-1
EM	Electron microscopy
ER	Endoplasmic reticulum
ETC	Electron transport chain
GAPDH	Glyceraldehyde 3-phospohate dehydrogenase
Grp75	Glucose-regulated protein 75
HFD	High fat diet
Ip3r-1	Inositol 1 ,4, 5-trisphophate receptor 1
LFD	Low fat diet
MAM	Mitochondrial-associated membrane
Mfn2	Mitofusion-2

NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
Opa1	Optic Atrophy 1
OSHA	Occupational safety and health administration
OXPHOS	Oxidative phosphorylation
PCE	Tetrachloroethylene
PFA	Paraformaldehyde
PLA	Proximity Ligation Assay
PVC	Polyvinyl Chloride
ROS	Reactive oxygen species
Serca	Sarcoendoplasmic reticulum Ca^{2+} transport ATPase
SS-31	Szeto-Schiller-31
TASH	Toxicant-associated steatohepatitis
TCE	Trichloroethylene
VC	Vinyl chloride
VOC	Volatile Organic Compounds
WHO	World Health Organization

Dedication

I would like to dedicate this thesis to the educators I've had throughout my life; especially Dr. Bruno Hagenbuch. Your mentorship and guidance have led me to be where I am today.

I would also like to thank my parents, Claire Capaldo and Terry Schnegelberger. I would not be the person or scientist I am today without your continued love, support, and motivation.

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1.0 Background and rationale for the study

1.1 Obesity and fatty liver disease

According to the World Health Organization (WHO) the prevalence of obesity has almost tripled since 1975 and there are over 650 million obese individuals worldwide. In the United States, 34.2% of the adult population is overweight (BMI≥ 25) and 33.8% are obese (BMI≥ 30).[1] Obesity is associated with detrimental health effects, including cardiovascular disease, type 2 diabetes, and metabolic syndrome.[2] The major hepatic manifestation of metabolic syndrome is non-alcoholic fatty liver disease (NAFLD) and is as such closely correlated with incidence of obesity.

NAFLD is a spectrum of liver diseases ranging from steatosis, to inflammation, to fibrosis and cirrhosis.[3] The first stage in the development of NAFLD is characterized by lipid accumulation in the liver (steatosis).[4, 5] Although eating a diet high in fatty acids is a major player in developing NAFLD,[6] other risk-factors can promote the progression of NAFLD to more severe forms of liver injury such as steatohepatitis and cirrhosis. Such modifying risk factors include genetics, comorbidities, and xenobiotics.[7]

The idea of multiple 'hits' in liver disease has been well-established.[8, 9] We propose that low-dose vinyl chloride (VC) may serve as a risk-modifying factor in the progression of steatohepatitis. Indeed, data by our laboratory demonstrate that VC, at concentrations that are not hepatotoxic per se, exacerbates liver damage in animals fed high-fat diet (HFD).[10, 11] Importantly, VC has recently been shown to be a risk factor for liver disease, independent from other causes.[12]

1.2 Vinyl chloride

VC is a volatile organic compound (VOC) and can be formed as a degradation product from chlorinated chemicals such as trichloroethylene (TCE) and tetrachloroethylene (PCE).[13, 14] VC is used in industry to create the polymer polyvinyl chloride (PVC). Occupational exposure to VC occurs in facilities that produce PVC and has affected more than 80,000 American chemical workers.[15, 16] The annual VC production has been estimated at 27 million metric tons.[17] Therefore, VOCs are found in significant concentrations in the ambient air surrounding manufacturing complexes.[14] There is a risk for widespread VC exposure, not only under occupational circumstances but also to the general population. The main environmental exposure risk occurs from contaminated groundwater in areas surrounding production facilities and superfund locations.[14] VC is present in landfills and in natural gas fracking fluids that can leak into groundwater wells in close proximity.[18] For example, up to 1,000,000 individuals (military, civilian personnel and their families) at Camp Lejeune alone have been exposed to VC.[13] Owing to its widespread presence in EPA superfund sites, its usage in industry, and its known potential human risk, VC is ranked #4 on the Agency for toxic substance disease registry (ATSDR) Hazardous Substance Priority List.[19]

VC can be ingested through contaminated ground water; however, it is primarily inhaled. VC is rapidly absorbed and widely distributed within the body. The main site for VC metabolism is the liver, as a consequence, the liver is sensitive to VC exposure. The liver is responsible for the removal of toxic compounds from the body in order to protect other organs from exposure.[20] Within the liver, VC is primarily metabolized by the cytochrome P450 enzyme, CYP2E1. This enzyme catalyzes the oxidation of VC to 2-chloroethylene oxide (CEO), a highly reactive epoxide. CEO is rearranged to form a reactive aldehyde species, 2-chloroacetaldehyde (CAA). VC can also

be oxidized to form 2-chloroethanol (CE), which is metabolized to CAA via alcohol dehydrogenase. CAA then enters into a subsequent reaction catalyzed by aldehyde dehydrogenase to form chloroacetic acid (Figure 1). VC intermediates are detoxified via conjugation with glutathione and excreted as thioacetic acid in the urine.[21, 22] VC metabolites are electrophilic and attack nucleophilic biomolecules such as lipids, nucleic acids, and proteins.[22-25] Work from the Beier laboratory has shown that VC metabolites sensitize hepatocytes to injury from a second stimulus. Furthermore, sub-hepatoxic doses of CE altered hepatic metabolism and enhanced damage when combined with another factor *in vivo*.[26, 27] These pathologic changes were mediated, at least in part, via CAA's direct toxicity to the mitochondria via decreasing mitochondrial respiration, depletion of cellular ATP levels, and depolarization of the mitochondrial membrane potential.[28]

1.3 Vinyl chloride and hepatic injury

High occupational exposure to VC causes toxicant-associated steatohepatitis (TASH) in human subjects.[29-31] TASH is recognized as a unique hepatic disorder with its own etiology and pathophysiology.[29] Although this direct hepatotoxic effect of VC has been identified, the mechanism(s) by which low concentrations of VC enhance hepatotoxicity, have not been determined.

1.4 Vinyl chloride and mitochondrial function

Mitochondria are essential to overall function and health of all cell types. They respond dynamically to stress signals, nutrient availability and are responsible for many functions within the cell, including redox signaling, calcium homeostasis, programmed cell death, and energy metabolism through oxidative phosphorylation (OXPHOS). If any of these functions are disrupted, cellular processes can become dysfunctional, resulting in biochemical and physiological stress and ultimately lead to cell death. Therefore, maintenance and regulation of mitochondrial homeostasis is crucial to overall health of an organism.

It is known that ingestion of a HFD causes mitochondrial metabolic disruption. Mitochondria play key roles in hepatic (mal)adaptation to NAFLD; however, the underlying mechanisms are incompletely understood. Mitochondrial-driven alterations in substrate supply, metabolism, and cell death have been consistently identified as likely players. Indeed, mitochondrial function and morphology are known to be altered both in experimental NAFLD and in humans presenting with NAFLD and non-alcoholic steatohepatitis (NASH).[32, 33]

Mitochondria are a significant source of endogenous reactive oxygen species (ROS), as electron leakage occurs during normal oxidative respiration.[34, 35] Mitochondrial-generated ROS are involved in physiological signaling cascades regulating various cellular functions.[34, 35] However, overproduction of ROS and the resulting oxidative stress are known to play a central role in the pathogenesis of many diseases, i.e. NAFLD.[36,37] Moreover, our lab has shown that VC enhances NAFLD-induced oxidative stress.[11, 26] This result suggests that one of the mechanisms by which VC exerts damage is by increasing ROS production, resulting in disruption of normal cellular funciton and sensitizes the hepatocytes to further damage.

1.5 Mitochondrial membrane potential

Work from the Beier laboratory demonstrated that independent of diet, VC exposure significantly decreases mitochondrial respiration and maximum respiratory capacity in mitochondria.[11] One mechanism by which oxidative phosphorylation is controlled is via the mitochondrial membrane potential (MMP), which is generated and maintained by the proton pumps of the electron transport chain (ETC) (complexes I, III and IV).[38] The MMP and proton gradient form an electrical gradient, which together, form the transmembrane potential of hydrogen ions used by ATP synthase to form ATP.[38] Previous work by our group has demonstrated that *in vitro* exposure to VC metabolites renders hepatocytes more sensitive to cell death.[26] Similarly, VC exposure *in vivo* decreases mitochondrial membrane potential and sensitizes hepatocytes to *ex vivo* cytotoxic stimuli resulting in cell death. We propose that these effects contribute, at least in part, to the overall phenotype.

1.6 Mitochondrial-associated membranes

Recent work suggests that stress to mitochondria and the ER is not distinct, but rather that mitochondrial/ER crosstalk is critically-involved in normal and altered function in both organelles.[39-41] Mitochondria and the ER physically interact via specialized contact sites called mitochondria-associated membranes (MAMs).[42-45] Importantly, MAMs house key components that impact cellular and organelle function by regulating and controlling mitochondrial function, ER stress signaling and autophagy,[40] making them sensitive targets. It has been demonstrated that ER-mitochondria interactions are decreased in obese mice,[39] leading to ER-mitochondria

miscommunication. Therefore, a major goal of this work is to determine the role VC exposure on MAMs.

1.7 Statement of goals

There are many known risk factors for liver disease progression including nutrient overload and toxicant exposure that need to be considered when studying disease development. Due to VC being a common industry toxicant and the high prevelance of obesity, these two factors need to be considered simultaneously. We aim to demonstrate that low-level VC exposure induces morphological and functional changes to the mitochondria and therefore lead to an exacerbation of NAFLD. Therefore, the purpose of this thesis is to further identify the underlying mechanisms.

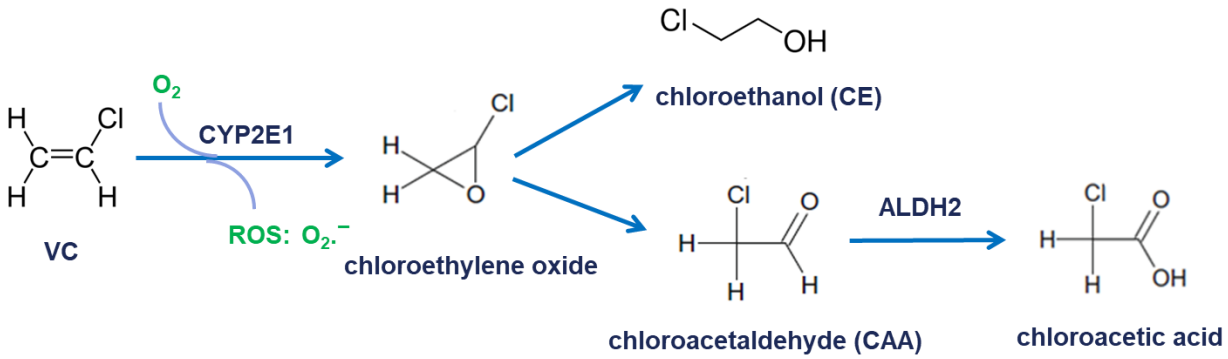

Figure 1: Schematic illustration of VC metabolism in the liver.

2.0 Overall aim of the thesis

The overall aim of this thesis is to address the hypothesis that low- level VC exposure (sub-OSHA level) is sufficient to exacerbate mitochondrial dysfunction by altering mitochondrial morphology and disrupting mitochondria-ER communication in HFD induced liver disease. Recent data from this lab have determined that sub-hepatotoxic concentrations of VC/VC metabolites can induce mitochondrial dysfunction by depleting cellular ATP levels and decreasing oxygen consumption rate in the mitochondria. Furthermore, VC/VC metabolites increase ER stress. Therefore, the effect of sub-OSHA exposure to VC via inhalation on mitochondrial/ ER communication via MAMs was assessed. Mitochondrial structure and dynamics are crucial for whole cell homeostasis. As discussed in the Background, VC/VC metabolites have been shown to disrupt mitochondrial function and respiration. However, the effect on mitochondrial structure and integrity has yet to be determined. Thus, this will be addressed in this thesis. Taken together, the goal of this thesis is to provide further insight into sub-OSHA exposure of VC and its effect on mitochondrial function and integrity.

3.0 Introduction

The current obesity epidemic in the United States is prevalent with over 1/3 of the population being considered obese (BMI > 30 mg/km^2).[46] There are several pathologies associated with obesity, including metabolic syndrome with the primary hepatic manifestation of metabolic syndrome being non-alcoholic fatty liver disease (NAFLD). The progression and severity of liver disease can be enhanced by other factors, such as environmental toxins/toxicants, which are only beginning to be understood as an important factor in contributing to the progression of liver disease, and the underlying mechanisms.[8] Vinyl chloride (VC) is a potent, ubiquitous compound in which individuals can be exposed to environmentally or occupationally.[47] VC is found as a degradation product of other chlorinated chemicals and is direct hepatotoxicant at high concentrations.[13, 48 29, 49, 50] While most studies focus solely on the effect of VC exposure on human health alone, our lab takes into consideration the interaction of low-dose VC with risk-modifying factors, such as over nutrition.

Our recent studies suggest that obesity and hepatic steatosis increase susceptibility to VC, making them vulnerable to worse hepatic pathology.[10, 26, 27, 51] Since NAFLD is prevalent in the developed world,[52] our lab is focused on defining the interactions between a high-fat diet (HFD) and VC exposure. Our lab has documented that sub-OSHA levels of VC inhalation cause oxidative stress and mitochondrial dysfunction leading to disruptions of hepatic energy metabolism and steatohepatitis.[11] Furthermore, previous data from this group demonstrated that VC exposure leads to endoplasmic reticulum (ER) dilation,[10] which activates ER stress pathways.[53] We hypothesize that ER stress and mitochondrial dysfunction in the interaction of VC and HFD causes the metabolic derangements of the liver in this model, at least in part, via mitochondrial-ER

miscommunication. The purpose of the current study, therefore, was to elucidate the impact of VC exposure on the function and interaction (via mitochondria-associated membranes-MAMs) of these organelles and to further discern the impact of altered MAM proteins have on mitochondrial morphology.

4.0 Materials and Methods

4.1 Animals and Procedures

Six-week-old male C57BL/6J mice from Jackson Laboratory (Bar Harbor, ME) were held in a pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and procedures were approved by the local Institutional Animal Care and Use Committee. Animals were housed in shoebox cages with corncob bedding and were allowed food and water *ad libitum* on a 12-hour light/dark cycle.

4.1.1 Diets

Low fat diet (LFD). 13% calories as fat; Casein 195.0 g/kg, DL-Methionine 3.0 g/kg, Sucrose 120.0 g/kg, Corn Starch 432.89 g/kg, Maltodextrin 100.0 g/kg, Anhydrous Milkfat 37.2 g/kg, Soybean Oil 12.8 g/kg, Cellulose 50 g/kg, Mineral Mix, AIN-76 (170915) 35.0 g/kg, Calcium Carbonate 4.0 g/kg, Vitamin Mix, Teklad (40060) 10.0 g/kg, Ethoxyquin, antioxidant 0.01 g/kg; (Envigo Teklad Diets, Madison, WI).

High fat diet (HFD). 42% calories as fat; Casein 195.0 g/kg, DL-Methionine 3.0 g/kg, Sucrose 341.31 g/kg, Corn Starch 75.0 g/kg, Maltodextrin 75.0 g/kg, Anhydrous Milkfat 210.0 g/kg, Cholesterol 1.5 g/kg, Cellulose 50.0 g/kg, Mineral Mix, AIN-76 (170915) 35.0 g/kg, Calcium Carbonate 4.0 g/kg, Vitamin Mix, Teklad (40060) 10.0 g/kg, Ethoxyquin, antioxidant 0.04 g/kg; (Envigo Teklad Diets, Madison, WI).

4.1.2 Chronic model of VC exposure

The *in vivo* results generated in this study were used from preexisting samples. The exposure paradigm used was modified from Drew et al.[54] Mice were chronically exposed to VC at 0.85 ± 0.1 ppm, or room air, for 6 hours per day, 5 days per week, for a maximum of 12 weeks.[54] Mice were exposed in a state-of-the-art 2-tiered inhalation chamber system capable of performing simultaneous exposures with up to 100 mice at one time (50 mice/tier) housed at the University of Louisville, in the Clinical Translational Research Building barrier facility designed with extensive housing capacity, card-coded entry, and separate HVAC system to maintain barrier, temperature and humidity condition.[54] Mice were allowed food and water *ad libitum* the entire course of the study. Mice were fed low fat diet or high fat diet (Envigo, Teklad Diets, Madison, WI). Body weight for each animal was measured once per week and food consumption was monitored and recorded twice per week. Animals were euthanized at 6 and 12 weeks of exposure.

4.1.3 Key chemicals and resources

VC obtained from Kin-Tek (La Marque, TX) was validated by the Kentucky Institute for the Environment and Sustainable Development of the University of Louisville and was stored at -20°C in the barrier facility when not in use. The VC concentration in the inhalation chamber was measured by gas chromatography/mass spectrometry (GC/MS) in full scan mode according to the EPA method TO-15, using a quadrupole GD (HP 6890) with a HP 5973 Mass Selective Detector. Grab air samples from the inhalation chamber were collected as the air exited the chamber into pre-evacuated six-liter Silcosteel canisters.

4.1.4 Animal sacrifice, tissue collection, and storage

At the time of sacrifice, 4 hour fasted animals were anesthetized with ketamine/xylazine (100/15 mg/kg, i.p.). Blood was collected from the inferior vena cava just prior to sacrifice by exsanguination and citrated plasma was stored at -80°C for further analysis. Portions of liver tissue were snap-frozen in liquid nitrogen, embedded in frozen specimen medium (Sakura Finetek, Torrance, CA), or were fixed in 10% neutral buffered formalin for subsequent sectioning and mounting on microscope slides.

4.2 Cell Culture

AML12 cells (ATCC # CRL-2254, American Type Culture Collection, Manassas, Virginia) were maintained in Dulbecco's modified Eagle's medium (DMEM) with phenol red, supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, 10 µg/ml streptomycin, 10µg/ml insulin, 40ng/ml dexamethasone. Cells were grown in 25-cm^2 cell culture flasks at 37°C with 5% CO_2 in a humidified incubator.

4.2.1 Mitochondrial Reconstruction

For determination of mitochondrial volume changes, mitochondrial reconstruction was performed as previously described.[55] AML12 cells in 8-well chamber slides were pre-incubated for 1 hr with Szeto-Schiller (SS)-31, followed by an incubation with chloroacetaldehyde (CAA) for 20 hrs in the presence or absence of SS-31. Cells were fixed in 4% paraformaldehyde (PFA).

Fixed cells were stained using anti-TOM20 (Invitrogen, PA5-52843) to label the mitochondria. Cells were co-stained with DAPI (nuclei). Confocal z-stacks were collected using 60X (1.49NA) optic on a Nikon A1 equipped with GASP detectors and NIS Elements software (Nikon Inc., Melville NY). The confocal datasets were deconvoluted using 3D Landweber capabilities of Nikon Elements (Nikon Inc., Melville, NY) and then surface rendering and calculation of mitochondrial volume were collected.

4.2.2 Proximity Ligation Assay (PLA)

PLA was performed as described previously.[56, 57] AML12 cells were fixed in 4% PFA. Samples were incubated with anti-Tom20 (Invitrogen, PA5-52843) primary antibody at room temperature for 1 hr then incubated with anti-Calnexin (Invitrogen, MA3-027) primary antibody at 4°C overnight. Secondary antibodies conjugated with oligonucleotides were added to the reaction and incubated at 37°C for 1 hr. Ligation solution, consisting of two oligonucleotides and ligase, was added and incubated at 37°C for 45 min. In this assay, the oligonucleotides hybridize to the two PLA probes on the secondary antibodies and join to a closed loop if they are within 40 nm of each other. Amplification solution, consisting of nucleotides and fluorescently labeled oligonucleotides, was added together with polymerase and incubated at 37°C for 1 hr and 40 min. The proximity ligated signal is visible as a distinct fluorescent spot and confocal images were collected using 60X (1.49NA) optic on a Nikon A1 equipped with GASP detectors and NIS Elements software (Nikon Inc., Melville NY). The confocal datasets were deconvoluted using 3D Landweber capabilities of Nikon Elements (Nikon Inc., Melville, NY). Then deconvoluted confocal images were analyzed using NIS elements (Duolink; Sigma Aldrich).

4.3 Electron Microscopy (EM)

EM analysis was performed as previously described.[58] Briefly, liver tissues were fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4), followed by 1% OsO_4. After dehydration, thin sections were stained with uranyl acetate and lead citrate for observation under a JEM 1011CX electron microscope (JEOL). Images were acquired digitally at the University of Kansas Medical Center.

4.4 Immunoblots

Liver samples were homogenized in buffer containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO) as previously described.[59] Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was followed by Western blotting. Primary antibodies against MFN2 (ab56889), Disc-1 (ab192258), Calnexin (ab22595) (1:1,000, Abcam, Cambridge, MA), OPA1 (80471), GRP75 (3593), DRP1 (8570), SERCA (4388), glyceraldehyde 3-phospohate dehydrogenase (GAPDH) (5174s) (1:1,000, Cell Signaling Technologies, Danvers, MA), and IP3R-1 (1:1,000, Invitrogen, PA1-901) were used. Results were visualized using the iBright 1500 imager (Thermo Fisher Scientific, Waltham, MA). Densitometric analysis was performed using iBright analysis software (Thermo Fisher Scientific, Waltham, MA).

4.5 Statistical Analysis

Power analysis was used to calculate the number of animals required for the experiments. Based on previous studies and preliminary data, we estimated that we needed a minimum of five animals per group to compare the primary endpoint (i.e., levels of organ injury) in HFD and VC-exposed mice to get an 85% power for detecting a difference of at least 20% with $P < 0.05$ between experimental groups. Results are reported as means ± SEM (n = 4-12). Analysis of variance with Bonferroni's post-hoc test (for parametric data) or Mann–Whitney Rank Sum test (for nonparametric data) were used for the determination of statistical significance among treatment groups, as appropriate. $P < 0.05$ was selected before the study as the level of significance.

5.0 Results

5.1 In vivo studies

5.1.1 VC induces enlarged mitochondria

Our lab has previously shown that liver injury caused by HFD can be enhanced by sub-hepatotoxic concentrations of VC or its metabolites, in part, by dysregulating hepatic energy metabolism and decreasing respiration.[10, 27, 51] Altered mitochondrial integrity has been shown to cause an overall decrease in respiration.[60] It is now known that mitochondrial integrity not only encompasses functional but also structural attributes.[61] Analysis of EM pictures demonstrated that overall area of the mitochondria was significantly increased by VC alone (Figure 2B), while remaining spherical (length/width ratio, Figures 2B and 2C). Moreover, VC alone significantly increased the length/width ratio of the mitochondria (Figure 2B). In line with previous reports,[62] morphometric analysis of these mitochondria showed that HFD caused an elongation of the mitochondria. Furthermore, HFD+VC dramatically altered this morphology resulting in strongly enlarged, spherical mitochondria (Figure 2A). Interestingly, the ratio of mitochondrial to nuclear DNA (mtDNA: nuDNA) was unaffected (Figure 2D).

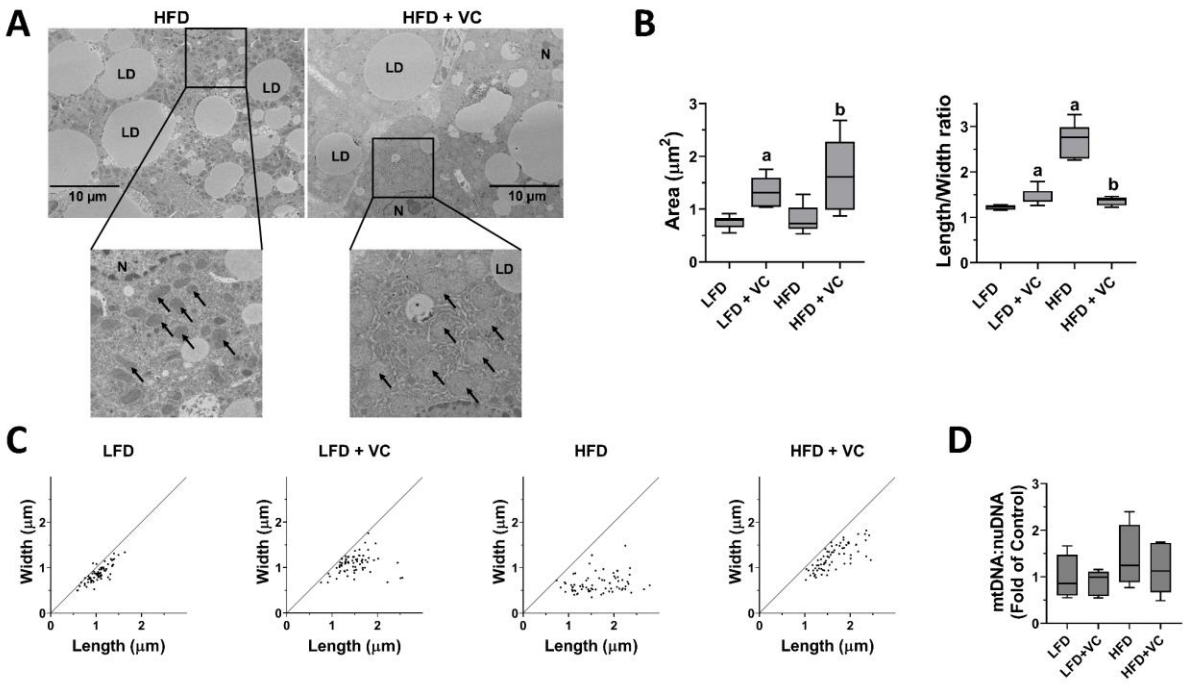

Figure 2: Effect of VC on mitochondrial morphology. A: Representative EM photomicrographs depict elongated organelles in the HFD group and enlarged mitochondria (width, length >1 mm) in the HFD+VC group. Arrow denotes mitochondria, LD denotes lipid droplet, and N denotes nucleus. B: Total mitochondrial area (μm^2) and mitochondrial length/width ratio are shown. C: Distribution of the size of 70 mitochondria/group is shown. D: Hepatic mRNA expression of mitochondrial DNA is shown as fold of control compared to LFD control animals at the 6-week time point. [a], $p<0.05$ compared to LFD control; [b], $p<0.05$ compared to absence of VC. Samples size per group n =8-10.

5.1.2 VC alters protein levels of mitochondrial-associated membranes (MAMs)

The mitochondria and ER physically interact with each other through MAMs (Figure 3A). MAMs, are key to cell survival or death through the transfer of calcium, proteins, and lipids.[63] Our lab has shown that VC induces ER stress and causes mitochondrial dysfunction.[11] We hypothesize that VC causes this effect, in part, by disrupting proper communication between these organelles via MAM proteins. To investigate if VC impacts ER-mitochondria interactions through altering protein levels of MAMs, whole liver lysates and mitochondrial extracts were used for Western blot analyses. The expression of Calnexin, Ip3R1, Serca, and Disc-1 (all localized on the ER), Grp75 (localized on the ER and mitochondria), as well as Opa1, Mfn2 and Drp1 (all localized on the mitochondria) were analyzed. These proteins are involved in Ca^{2+} transport, protein assembly, mitochondrial fission and fusion, and integration of ER-mitochondrial communication, ER homeostasis and autophagy. In isolated mitochondrial extracts, VC alone did not significantly change the protein expression. However, HFD increased MAM protein expression in these samples (Figure 3C). VC exposure blunted HFD induced expression of these markers. In whole liver lysates, VC alone significantly increased Mfn2 expression, required for mitochondrial fusion (Figure 3B). These data support our hypothesis that VC exposure alters mitochondrial morphology.

MAMs are important for mitochondrial dynamics by marking sites of mitochondrial fission and fusion; that is the joining and separating of the organelle.[64] The balance of mitochondrial fission and fusion is imperative for regulating mitochondrial bioenergetics and removing damage during periods of internal stress.[65, 66] We hypothesize that the HFD and VC interaction further disrupts mitochondrial fission and fusion processes by increasing fusion and decreasing fission,

resulting in more changes in mitochondrial morphology (i.e. enlarged mitochondria) and more damaged mitochondria.

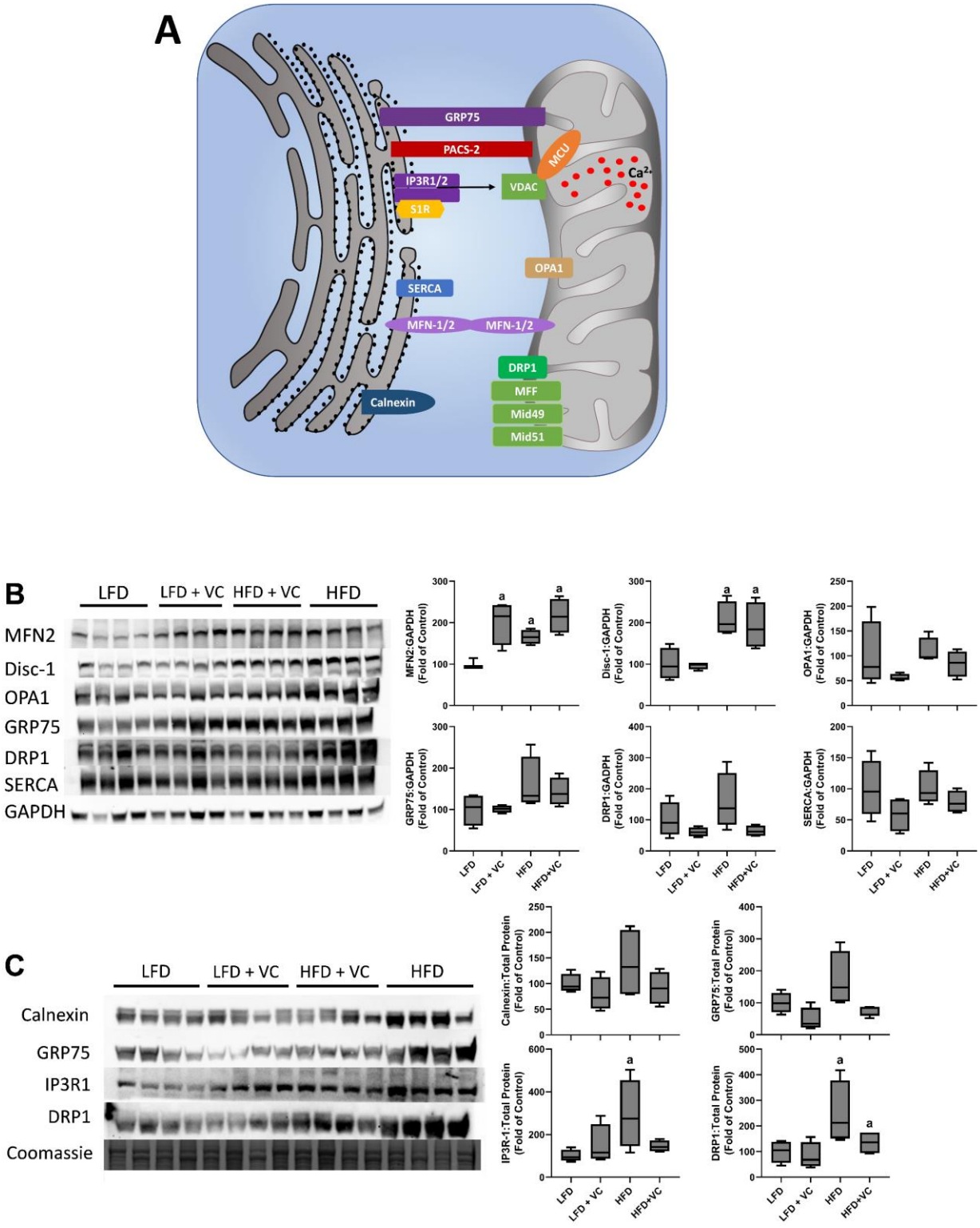

Figure 3: VC alters MAMs protein levels. A: Schematic illustration of functional and structural MAM proteins. B: Representative Western blots and densitometric analysis for whole liver lysates of Mfn2, Disc-1, Opa1, Grp75, Drp1, and Serca are shown and C: Western blots of crude mitochondrial extracts of Calnexin, Grp75, IP3R-1, and Drp1 are shown. [a], $p<0.05$ compared to LFD control; [b], $p<0.05$ compared to absence of VC. Samples size per group n =4.

5.2 In vitro studies

5.2.1 Chloroacetaldehyde (CAA) increases mitochondrial size

Mitochondria are highly dynamic and functionally versatile organelles that fuse and fragment continuously. The morphology of mitochondria (fragmented and extensive fusion) has been shown to strongly influence mitochondrial function.[67] To determine if VC/VC metabolite exposure causes morphologic changes, mitochondrial reconstruction was performed. AML12 cells were incubated with CAA (0μM- 20μM) for 20 hrs. Exposure to CAA significantly increased mitochondrial volume at low concentrations (≤ 2.5 μM) up to 10 μM of CAA in a concentration-dependent manner (Figure 4B). The mitochondrial phospholipid, cardiolipin, plays a central role in many mitochondrial processes, including mitochondrial morphology and dynamics.[68-70] SS-31 is a mitochondrial specific peptide that has been shown to selectively bind to cardiolipin on the inner mitochondrial membrane. We hypothesize that VC/VC metabolites destabilize cardiolipin leading to altered mitochondrial morphology and that stabilization of cardiolipin with SS-31 treatment will attenuate the increase in mitochondrial volume caused by CAA exposure. AML12 cells were pre-treated with 1μM of SS-31 for 1 hr, then cells were exposed to CAA ± 1μM of SS-31 for 20 hrs and mitochondrial reconstruction was performed. Increasing concentrations of CAA increased mitochondrial volume in a dose dependent manner. Addition of SS-31 mitigated this effect at lower concentrations, but did not compensate past 10μM (Figure 4B).

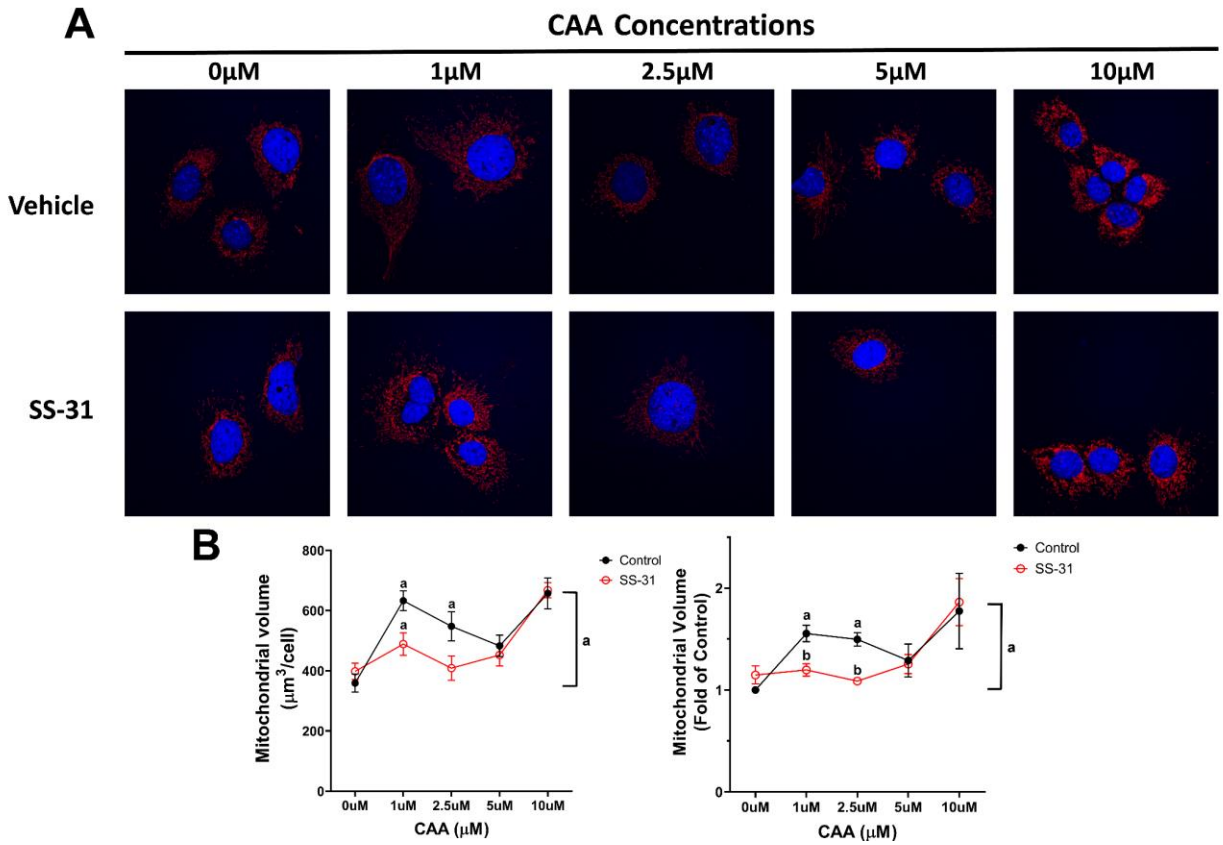

Figure 4: Effect of CAA on mitochondrial volume. AML12 cells were grown on 8-well chamber slides and incubated with 0µM, 1µM, 2.5µM, 5µM, and 10µM of CAA ± 1µM of SS-31 for 20 hrs. A: Confocal microscopy images of mitochondria (red) from AML12 cells. B: Images were deconvoluted to better enable resolution of individual mitochondria. Mitochondrial volume was quantified by multiplying the number of voxels by the voxel volume and expressed as fold of control and as μm^3/ cell. [a], $p<0.05$ compared with absence of CAA. [b], $p<0.05$ compared with absence of SS-31. n =3.

5.2.2 CAA alters the proximity between the ER and mitochondria

The mitochondria and ER are both essential to the cell and proper communication of these organelles requires close proximity. Dysfunction of either organelle, or a change in proximity, can disrupt metabolite transport, lipid metabolism, and induce apoptosis.[41] Data from our lab illustrated that VC/VC metabolites changed MAM protein expression (Figure 3), increased mitochondrial size (Figure 2) and volume (Figure 4), and induced ER stress *in vivo*.[11] In order to determine if the VC metabolite, CAA, affects the distance between the ER and mitochondria, AML12 cells were incubated with 0µM- 10µM of CAA and a proximity ligation assay (PLA) was performed. Tom20 antibody was used to label the mitochondria and Calnexin antibody was used to label the ER. AML12 incubated with 2.5µM of CAA significantly decreased the distance between the ER and mitochondria as imaged by using confocal microscopy and measured by analyzing the overall number of puncta per cell (Figure 5B). However, as the CAA concentration increased the distance did not differ compared to control group.

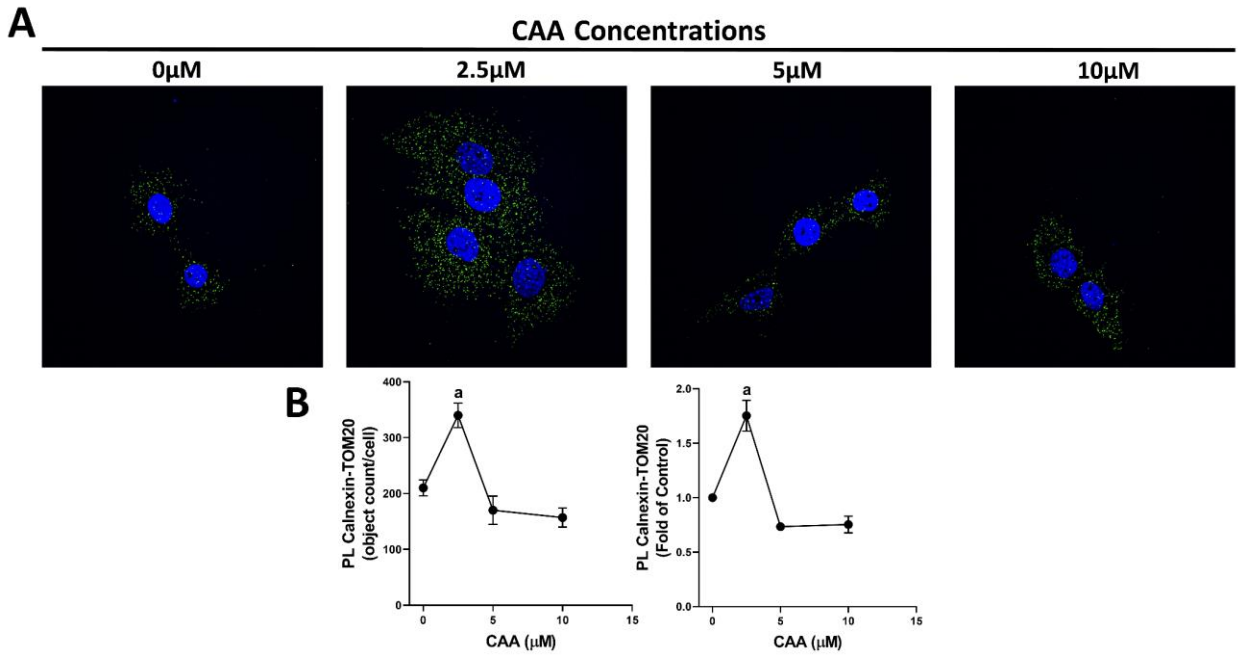

Figure 5: CAA alters the distance between the mitochondria and ER. AML12 cells were grown on coverslips and incubated with 0µM, 2.5µM, 5µM, and 10µM, of CAA for 20 hrs. A: Proximity ligation assay showing interaction between mitochondria (TOM20) and ER (Calnexin), indicated by green puncta. B: Quantification of the mitochondrial-ER proximity ligation signal expressed as fold of control and object count per cell. [a], $p<0.05$ compared with absence of CAA. n =3.

6.0 Discussion

Occupational exposure levels to VC have been regulated since the 1970's. While these regulations lessen concerns about direct hepatotoxicity of high occupational exposure to VC, we have demonstrated that lower exposure levels of VC and metabolites that are not overtly toxic, per se, can augment liver injury caused by another insult.[10, 26, 27] Although low VC exposure alone did not cause overt pathology, it damaged mitochondria, leading to decreased oxygen consumption rates (OCR) in hepatocytes.[10] Mitochondria have been identified to play key roles in hepatic (mal)adaptation to NAFLD. Indeed, mitochondrial function and morphology are known to be altered both in experimental NAFLD and in humans presenting with NAFLD and NASH.[32, 33] Although the underlying mechanisms are incompletely understood, mitochondrial-driven alterations in substrate supply, metabolism, and cell death have been consistently identified as likely players. We hypothesize that VC-induced mitochondrial damage may impede the liver's ability to appropriately adapt to the biochemical stresses caused by experimental NAFLD and thereby exacerbate damage. The findings of the current study support this hypothesis. We have demonstrated here that VC causes significant architectural changes to the mitochondria, even in the absence of HFD (Figure 2). This may, in part, be mediated by changes in organelle interaction (Figure 3). The major goal of this thesis was too further decern the mechanistic impact on the ER and mitochondria following VC-induced liver injury.

Recent studies have indicated that altered mitochondrial morphology may be a key mechanism in mitochondria-mediated cellular injury. Mitochondrial morphology and functionality are strictly correlated, and mitochondrial dynamics are constantly adjusting mitochondrial shape to maintain homeostasis.[71] However, these responses can also be dysfunctional, and drive

pathogenesis. Changes in mitochondrial morphology caused by different insults (e.g., fatty diets or alcohol), are mediated, at least in part, by mitochondrial remodeling, including elongation or overall enlargement of the mitochondria.[61, 62] Enlarged mitochondria can be caused by fusion (i.e. mitochondrial hypertrophy), or by mitochondrial swelling.[72, 73] Mitochondrial hypertrophy is associated with normal cristae, normal matrix density and normal oxidative phosphorylation, whereas mitochondrial swelling is associated with abnormal cristae, irregular matrix density and uncoupled oxidative phosphorylation. In the current model, VC exposure significantly changed mitochondrial shape (Figure 2) towards a more spherical morphology, suggesting swelling more than hypertrophy. Furthermore, VC metabolite CAA significantly increased mitochondrial volume in AML12 cells (Figure 4), also suggesting an enlargement of the mitochondria. However, additional experiments are needed to determine whether these results are due to mitochondrial swelling or hypertrophy.

The mitochondrial structural changes observed under these conditions correlated with altered function. We have demonstrated that liver injury enhanced by VC is associated with VC-induced mitochondrial dysfunction, leading to increases in oxidative stress and energy dysmetabolism, which was demonstrated by significantly decreasing the oxygen consumption rate.[10] One of the possible mechanisms of VC-induced changes to mitochondrial respiration is via decreased protein expression of electron transport chain complexes. Moreover, mtDNA content, which is known to be increased in experimental NAFLD, was not altered following VC exposure (Figure 2D). These data suggest, that VC induces post-translational modifications, which results in mitochondrial dysfunction rather than directly impacting the mtDNA copy number.

Mitochondrial quality and abundance affect the cell's bioenergetic capacity and overall resistance to stress.[74] Mitochondrial damage has also been linked to the induction of ER stress,

which can indirectly affect cellular function. In particular, the ER regulates fundamental metabolites (e.g., lipids) and messengers (e.g., Ca^{2+}) that control mitochondrial function and the fate of the cell, suggesting mitochondrial stress is closely associated with ER stress.[42] Mitochondria and the ER also physically interact via specialized contact sites called mitochondria-associated membranes (MAMs).[42-45] It has been demonstrated that ER-mitochondria interactions are decreased during obesity (i.e., in *ob/ob* mice),[39] leading to ER-mitochondria miscommunication. VC exposure has previously been shown to cause ER stress and mitochondrial dysfunction.[11, 51, 75, 76] Here, we show in mitochondrial extracts that VC exposure decreases expression of proteins involved in ER-mitochondria interaction, including ER-associated proteins (Figure 3B and 3C), suggesting a decrease in physical contact and resulting in potential miscommunication, ER stress, and mitochondrial dysfunction. However, 2.5 μM of CAA decreased the proximity between the mitochondria and ER (Figure 5). Increasing concentrations of CAA, including 5μM and 10μM, showed no significant difference when compared to control. These results could be explained by the increase in mitochondrial volume seen in CAA-exposed cells.

Taken together, our results support the hypothesis that low-level (sub-OSHA limit) VC exposure via inhalation enhances liver injury caused by HFD, mechanistically involving structurally and functionally dysregulated mitochondria and their interactions with the ER. Importantly, these data raise concerns about potential synergistic effects of fatty diets and exposure to VC to enhanced liver injury. Thus, the health implications of this co-exposure for humans need to be considered. It also indicates that current safety restrictions may be insufficient to account for other environmental factors that can influence hepatotoxicity.

7.0 Strengths and Weaknesses

7.1 Strengths

There are many strengths of the work presented in this thesis. This work utilized a well-established animal model that closely mimics human exposure to VC and concomitant exposure to a high fat diet. With this animal model, a chronic administration of sub-OSHA concentrations of VC was used. The *in vivo* results showed here illustrate the importance for continued research using relevant exposure concentrations to better understand the impact of environmental toxicants have on underlying liver disease. The work presented here builds on previous data and further supports the hypothesis that VC exacerbates liver injury induced by HFD by causing mitochondrial dysfunction. This research further demonstrates VC-induced mitochondrial dysfunction by altering mitochondrial architecture and disrupting communication of the mitochondria with other organelles. This work utilized both *in vitro* and *in vivo* models to illustrate that VC/VC metabolite exposure induces architectural changes to the mitochondria which, in part, leads to mitochondrial dysfunction.

7.2 Weaknesses

This research builds on previous work illustrating VC-enhanced liver injury in order to further determine the mechanism of VC induced hepatotoxicity. The experiments described here in which mitochondrial protein are analyzed used only crude mitochondrial extracts. Therefore, it

is important to note that isolating pure mitochondria may yield different results. Furthermore, isolation of the ER would also be beneficial to fully discern VC impact on MAMs. While the *in vivo* parts of this thesis used an animal model that is comparable to human exposure, the *in vitro* data would need to be recapitulated in the *in vivo* model or in primary hepatocytes in order for the results to be more comparable to human studies. Lastly, the higher concentrations of CAA may be increasing the distance between these organelles and the PLA fails to provide that information. The PLA has a limitation that it can only detect within a 40nm and will not detect any changes in proximity beyond that distance. Furthermore, while CAA alters the proximity, future experiments will need to be performed to explore the mechanistic impact of this alteration.

8.0 Future Directions

While the experiments in this thesis aided in our understanding of chronic, low-level VC-induced liver injury, it has also led to new research questions which need to be addressed in future studies. Some of these questions are addressed below.

8.1 Does VC exacerbate mitochondrial dysfunction via dysregulation of cardiolipin?

This thesis demonstrated that VC exposure alters mitochondrial morphology causing enlarged, spherical mitochondria. Morphometric analysis of these mitochondria demonstrated that the overall area of the mitochondria was increased by VC, independent of diet. Previous data showed that VC also caused mitochondrial dysfunction.[11] The mitochondrial phospholipid, cardiolipin, has been shown to have a central role in several mitochondrial processes, including respiration, energy production, apoptosis, morphology, and stability.[69, 70, 77-80] It has also been shown to play a key role in mitochondrial dynamics (fission and fusion), which drive organelle morphology and size.[80-82] Based on the previous data and the observations of this thesis, it would be of interest to determine if the VC-induced mitochondrial dysfunction and changes in mitochondrial morphology are caused, at least in part, by dysregulation of cardiolipin as destabilized cardiolipin has been shown to cause instability of the membrane and increase mitochondrial size.[83]

8.2 Will prevention of altered mitochondrial morphology protect against VC-induces hepatotoxicity?

Results from this thesis illustrate that VC-induced mitochondrial morphological changes may be a driving factor in liver injury progression. Therefore, it would be of interest to determine if preventive measures could be taken to mitigate injury progression in this model. There are several mitochondrial specific small molecules being studied due to their ability to prevent injury. One of these molecules is SS-31, which is a water-soluble peptide, which directly targets the mitochondrial membrane.[84, 85] It can access the inner mitochondrial membrane and selectively bind to cardiolipin. The interaction of SS-31 and cardiolipin has been shown to reduce ROS production while also stabilizing the ETC supercomplexes and increases respiratory function through oxidative phosphorylation.[84, 85] Therefore, it would be of interest to determine if the hepatotoxicity induced by the combination of VC and HFD can be attenuated by this molecule.

9.0 Summary and Conclusions

Taken together, this thesis supports the hypothesis that low-level (sub-OSHA-limit) chronic exposure to VC via inhalation enhances liver injury caused by HFD, mechanistically involving structural, functional and dynamically dysregulated mitochondria and their interactions with other organelles. Furthermore, this thesis illustrates that VC metabolite, CAA, induced architectural changes to the mitochondria. This research provides potential mechanisms by which injury can be exacerbated following VC/VC metabolite exposure. Importantly, these data raise concerns about potential for overlap between fatty diets and exposure to VC and the health implications of this co-exposure for humans. It also emphasizes that current safety restrictions may be insufficient to account for other factors that can influence hepatotoxicity.

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