5-2018

Vinyl chloride enhances diet-induced liver injury via metabolic dyshomeostasis : critical role of mitochondria.

Anna L. Lang
University of Louisville

VINYL CHLORIDE ENHANCES DIET-INDUCED LIVER INJURY VIA METABOLIC DYSHOMEOSTASIS: CRITICAL ROLE OF MITOCHONDRIA

By

Anna L. Lang

B.S. Northern Kentucky University, 2013

M.S. University of Louisville, 2016

A Dissertation

Submitted to the Faculty of the

School of Medicine of the University of Louisville

In Partial Fulfillment of the Requirements for

the Degree of

Doctor of Philosophy in Pharmacology and Toxicology

Department of Pharmacology and Toxicology

University of Louisville

Louisville, KY

May 2018

VINYL CHLORIDE ENHANCES DIET-INDUCED
LIVER INJURY VIA METABOLIC DYSHOMEOSTASIS:
CRITICAL ROLE OF MITOCHONDRIA

By
Anna L. Lang
B.S. Northern Kentucky University, 2013
M.S. University of Louisville, 2016

Dissertation Approved on

04-12-2018

By the following Dissertation Committee:

_____
Juliane Beier, Ph.D.

_____
Gavin Arteel, Ph,D.

_____
Matt Cave, M.D.

_____
John Wise, Ph.D.

_____
Jonathan Freedman, Ph.D.

_____
Russell Prough, Ph.D.

DEDICATION

I would like to dedicate this dissertation to the educators who have inspired me throughout my life. Dr. Christine Curran, your mentorship and enthusiasm for research has led me to where I am today.

I would also like to dedicate this dissertation to my parents, Frank and Linda Lang. I would not be the woman or scientist I am today without your enduring love, encouragement, and support throughout my life.

Also for my dear Sweetie Pie, you were my best friend, I love and miss you.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my mentor, Dr. Juliane Beier, for always believing in my scientific abilities, even when I could not see them and for her constant support and mentorship. I would also like to thank my co-mentor, Dr. Gavin Arteel, for always pushing me to be my best and think critically. Thank you to my committee, Drs. Matt Cave, John Wise, Jonathan Freedman, and Russell Prough, for giving me scientific support throughout my dissertation.

Thank you to my family, my parents and sister, Frank and Linda and Clare Lang for always supporting me in all that I do, I love you. To my loving boyfriend, Bradley, for keeping me grounded and supporting me throughout this journey. Lastly, to my friends who have kept me sane over the past year. Cierra, Shanice, Brenna, Kim, Regina, Doug, Zimple, Christine: thank you for being there when I needed you. Your love and support has been tremendous, thank you.

ABSTRACT

VINYL CHLORIDE ENHANCES DIET-INDUCED
LIVER INJURY VIA METABOLIC DYSHOMEOSTASIS:
CRITICAL ROLE OF MITOCHONDRIA

Anna L. Lang

April 12, 2018

Background. Vinyl chloride (VC) is an environmental toxicant and has been shown to be directly hepatotoxic at high exposures. However, recent studies suggest low-level toxicant exposure can cause subtle changes to the liver. Given the high prevalence of non-alcoholic fatty liver disease (NAFLD) in the United States, it is important to determine the impact of low-level toxicant exposure on the progression of underlying liver injury when combined with other factors. Therefore, the overarching goal of this dissertation was to develop a model of VC co-exposure with high-fat diet (HFD) and to determine the mechanisms by which VC contributes to the development of liver injury. **Methods.** Mice were fed a low fat diet (LFD) or high fat diet (HFD) and exposed to sub-OSHA levels of VC (0.85 ± 0.1 ppm) or room air 6 hours per day, 5 days per week, for either 6, 8, or 12 weeks. Metabolic phenotyping, biochemical and histological assessment of liver injury, indices for oxidative and endoplasmic reticulum (ER) stress, and

mitochondrial function were examined for each time point. **Results.** Chapter III of this dissertation describes a VC inhalation model in which co-exposure to a HFD significantly enhances liver injury associated with NAFLD. Specifically, mice exposed to both VC and HFD had significantly enhanced indices of liver injury, inflammation, and cell death. In Chapter IV, metabolic dyshomeostasis is evaluated as a mechanism by which VC exposure sensitizes the liver to secondary insults. Indeed, mice exposed to VC alone had significant alterations in glucose homeostasis in addition to enhanced oxidative and ER stress. Finally, Chapter V examines the effect of VC exposure on mitochondrial integrity and function. Importantly, VC's detrimental effect on mitochondria is specific to enzymes involved in oxidative phosphorylation, rather than general mito-toxic action. **Discussion.** In conclusion, the work presented in this dissertation has shown that sub-OHSA levels of VC exposure are sufficient to enhance underlying liver injury. Moreover, VC exposure can cause metabolic disruption even in combination with a LFD. VC directly targets complexes of oxidative respiration which sensitize hepatocytes to subsequent injury and cell death.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

A. Background and rationale for this study

1. Liver function as a target for toxicity

The liver has several important metabolic and physiologic functions. It is the primary site of dietary nutrient conversion, storage, and supply for other organs and also facilitates the removal of toxic compounds from the body. The central location of the liver enables it to function as both a biochemical and physical filter in order to protect other organs from exposure to potentially toxic compounds. By virtue of its function, the liver is often a target of toxicant exposure and injury. Liver function can be influenced by several factors such as alcohol consumption, genetics, and diet composition. The main hepatic cell type that functions to maintain nutritional homeostasis via synthesis and secretion of major carbohydrate and lipid molecules are hepatocytes. Hepatocytes constitute approximately 80% of the liver by mass and express specific enzymes that allow for detoxification and nutrient metabolism (1).

In addition to hepatocytes there are also several other non-parenchymal cell types in the liver which are vital to its overall health and function such as Kupffer cells (resident hepatic macrophages) and hepatic stellate cells. These

cells play a pivotal role in contributing to local inflammation, recruitment of other immune cells, and formation of extracellular matrix proteins, all of which contribute to liver disease and injury progression (2).

The hepatic portal vein receives blood and compounds directly from the intestines and ensures that nutrient breakdown occurs and harmful chemicals are removed prior to reaching system circulation. As such, hepatocytes are equipped with various enzymes that are capable of metabolizing and detoxifying chemicals to mitigate such potentially damaging compounds. The most diverse and prominent class of enzymes are the mixed function oxidases, also known as the cytochrome P450 family of enzymes. These enzymes are able to metabolize a variety of drugs, chemical compounds, and toxicants (3). However, because CYPs often execute Phase I metabolic reactions, the end result is commonly activation of the compound to form reactive intermediates or metabolites, which may cause more harm than the parent compound. For example, reactive metabolites can form protein or DNA adducts, subsequently inciting a local inflammatory response. Therefore, hepatocytes are often the initial and primary target of damage caused by chemical and/or toxicant exposure. However, hepatocytes have the capacity to regenerate in order to prevent such injuries and preserve the health and function of the entire organ. Although this protective mechanism serves as a defense against damage, the regenerative capacity of the liver is limited. If the damage is too severe or if the injury is chronic, hepatic cell death and irreversible injury may occur.

2. VOCs: prevalence and hepatotoxicity

Volatile organic compounds (VOCs) are chemicals that easily vaporize. Ambient levels are often higher indoors than outdoors, and concentrations can be significantly affected by how often windows are opened and the location of the building or residence (i.e., proximity to industrial and traffic pollution) (4-6). Additionally, VOCs are often ingredients in common household products such as paints, varnishes, cleaning supplies, degreasing agents, gasoline, and dry-cleaned clothing (7). As such, the typical route for human exposure is via inhalation or dermal absorption (7). However, VOCs are also common ground water contaminants as they are present at many waste disposal sites and are the most common class of chemical found at National Priority List (NPL) Superfund sites. A substantial number of compounds on the 'ATSDR hazard substance priority list' represent VOCs, with many in the top 50 chemicals (8). Although there have been several instances of mass exposure to VOCs, one of the more studied cases is from the Camp Lejeune military base in North Carolina. Up to 1 million people, including military personnel and their families, were exposed to a mixture of VOCs (TCE, PCE, benzene, and VC) via contaminated drinking water in the 1980s (9). A retrospective mortality cohort study demonstrated increased mortality and deaths from cancer, including liver, in personnel exposed at Camp Lejeune compared to a non-contaminated military base (10). However, other health effects and long-term outcomes are still being evaluated.

Although researching these compounds in the context of occupational exposures (i.e., acute, high doses) has been invaluable for characterizing

hepatotoxicity and developing risk assessment models, the field has begun to shift away from this exposure model.

3. Vinyl chloride

Vinyl chloride (VC) is a common VOC and is ranked #4 on the ATSDR Substance Priority List with a global annual production estimated at 27 million tons and a global capacity of 40 million tons (11). It is colorless when liquefied under pressure and at high volumes has sweet-smelling odor. VC is a chemical monomer that is used in the commercial production of polyvinyl chloride (PVC) polymer resins or plastics. VC has been used commercially in the United States for over 70 years and has been characterized as a Group A human carcinogen by the EPA and as a Group 1 known human carcinogen by IARC. One of the major methods of VC production is by subjecting 1, 2-dichloroethylene to extreme pressure and temperature, a process known as thermal cracking, which results in VC synthesis. PVC has a wide range of uses in the production of pipes, pipe fittings, windows, doors, flooring, and plastic products with both residential and commercial applications (12).

VC was first recognized as a direct hepatotoxicant and carcinogen in 1974 by Drs. Creech and Johnson (13;14). Several VC factory workers at the B.F. Goodrich facility in Louisville, KY presented with a rare form of liver cancer, hepatic angiosarcoma. These workers were occupationally exposed to VC at extremely high levels up to 1,000 ppm. Due to the extreme rarity (fewer than 200 cases diagnosed annually worldwide (15)) of this cancer and the clustering of diagnoses, VC was recognized as the causative agent. Indeed, other reports also

suggest a direct correlation between occupational exposures to VC and development of this hepatic cancer (16). In 1975, the Occupational Safety and Health Administration (OSHA) evaluated the safety guidelines for VC exposure and established a guideline and exposure threshold for occupational exposure to VC. The current OSHA threshold for VC exposure is a time-weighted average of no more than 1 ppm over an 8-hour work day, and no more than 5 ppm in a 15 minute period (OSHA Vinyl Chloride Standard 29 CFR 1910.1017)(17).

The main route of VC exposure is via inhalation. VC absorption into the circulation is rapid, has a wide distribution, and it acts systemically. The half-life of VC is only a few hours. Given the high reactivity of its metabolic intermediates (see below) it is unlikely that VC gas directly damages tissue. The main site of the enzymes that metabolize VC are located in the liver, as a consequence, the liver is exquisitely sensitive to VC exposure. As mentioned earlier in this Chapter (Section 1), this allows the liver to counteract any harmful effects that might be caused by such chemicals to prevent them from entering systemic circulation. VC is metabolized in a very similar manner as ethanol. A major pathway of VC metabolism is via the cytochrome P450 enzyme, CYP2E1. CYP2E1 catalyzes a Phase I reaction in which VC is oxidized to the highly reactive epoxide, 2-chloroethylene oxide. This can then be conjugated to glutathione in a Phase II reaction and subsequently excreted. 2-chloroethylene oxide (CEO) can also spontaneously rearrange to form a reactive aldehyde species, 2-chloroacetaldehyde (CAA). Another pathway of VC metabolism is its oxidation to 2-chloroethanol (CE). CE is also metabolized into 2-chloroacetaldehyde (CAA)

via alcohol dehydrogenase. CAA, from either metabolic pathways, then enters into a subsequent reaction catalyzed by aldehyde dehydrogenase to form chloroacetic acid. These intermediates are detoxified via glutathione conjugation and excreted in the urine, commonly as thioacetic acid (18;19). Further, sentinel studies performed during the 1970s and 1980s provided essential insight into the mechanisms by which VC is metabolized. From these studies it has been shown that chloroethylene oxide and chloroacetaldehyde are the major reactive intermediates formed through VC metabolism. Both are highly electrophilic and as such are able to attach nucleophilic biomolecules such as lipids, DNA, RNA, and proteins. Chloroethylene oxide has been shown to be a potent mutagen, targeting DNA, while chloroacetaldehyde is more specific forming protein adducts (19-22).

Historically, research regarding VC hepatotoxicity has focused on its mutagenicity and carcinogenicity. These studies have employed acute exposure paradigms and high levels of VC exposure (23;24). More recently, Swenberg et al., has shown that VC exposures at concentrations ranging from 10-1,100 ppm are sufficient to give rise to harmful DNA adducts (25). However, little work has been done on lower exposure levels and the potential for overlap with other risk factors for liver disease.

Recently, work from the Beier laboratory has focused on the reactive metabolic VC intermediates, CE and CAA. Indeed, data from this work have shown that exposure to these metabolites sensitize hepatocytes to injury from a secondary stimulus. These studies show that sub-hepatotoxic doses of CE are

sufficient to alter hepatic metabolism and enhance damage when combined with another factor *in vivo* (26;27). Additionally, CAA has been shown to be directly toxic to mitochondria (28).

4. Natural history of liver disease

Liver disease is not one pathology; rather, it is classified as a spectrum of disorders that range in severity from relatively benign to end-stage liver disease (29). Typically, the first stage of liver disease is identified by an accumulation of lipids within hepatocytes, termed steatosis. The exact mechanism which causes the influx of lipids can be variable; however, the end result is unbalanced lipid and mitochondrial homeostasis. If this metabolic disruption is continued chronically, it can cause an inflammatory response or steatohepatitis. Steatohepatitis is characterized as persistent lipid accumulation within the liver in addition to active inflammation (hepatitis). If the damaging factor is not mitigated, injury is progressive and can develop into more severe phenotypes such as fibrosis and cirrhosis, characterized as end-stage liver disease. Fibrosis and cirrhosis are characterized by an accumulation of extracellular matrix proteins, regenerative nodules, and scar tissue. These may ultimately lead to the development of hepatocellular carcinoma.

Interestingly, not all patients who have liver disease will develop severe liver injury. Several studies have now established that the development of liver disease is not attributable to solely one factor (30;31). Rather, liver disease progression can be influenced by a variety of factors ranging from genetics to diet composition and nutrient intake and exists as a ‘multi-hit’ paradigm of

development. As such, multiple factors must be taken into consideration when studying liver disease progression. Although the progressive nature of liver disease is consistent regardless of the source of injury, several unique etiologies have been characterized. These include alcoholic (ALD), non-alcoholic (NAFLD), and toxicant-associated (TAFLD) fatty liver diseases (32).

The liver is not a static organ and is able to respond dynamically to stressors and injury. It is now well-characterized that the liver can be sensitized to damage by a secondary agent, causing more damage than either factor individually. This paradigm allows a broader scope for research and introduces the potential for interactions with other to influence and enhance underlying liver injury. Liver damage occurs in a 'multiple-hit"' paradigm, in which chronicity and a combination of genetic, environmental, and intracellular events determine disease progression (33).

5. Environmental liver disease: TASH

Toxicant-associated fatty liver disease and steatohepatitis (TAFLD, TASH, respectively) have recently been characterized by the Cave laboratory. TASH is unique from the other forms of liver disease in that patients who present with pathologies do not have the classic risk factors associated with liver disease such as alcohol consumption or being overweight. In 2010, Cave et al., analyzed several biopsies and serum from highly exposed VC chemical plant workers that were collected subsequent to the hepatic angiosarcoma diagnoses in order to better understand the health effects in this unique population. Patients did not have the typical serum elevation of liver enzymes (i.e. transaminases) that are

clinically associated with liver injury. However, upon histologic evaluation of these samples, pathologic indices of liver injury including steatosis, steatohepatitis, and fibrosis were elevated. In fact, of the 25 subjects examined, 80% had steatohepatitis as evidenced via histology and 55% of that subset had fibrosis (34). Importantly, this study concluded that the liver injury present was contributable to their occupational exposures to VC and not to another factor. Indeed, other studies have noted development of severe liver injury in industrial chemical workers (35;36).

Since OSHA set stricter exposure guidelines for industrial exposure to VC, the opportunity for such high, acute exposures are significantly decreased. However, little research has been conducted on the effect of this low-level VC exposure over an extended timeline in either the occupational or environmental context. Moreover, there is little known regarding low level VC exposure and how it may act to enhance underlying liver injury, such as that caused by obesity.

6. Obesity and NAFLD

Obesity is growing health concern both in the United States and globally and is caused by excessive caloric intake and overconsumption of dietary fats and characterized by abdominal fat accumulation. According to the World Health Organization (WHO), worldwide obesity has nearly tripled since 1975 (37). In 2016, it was estimated that 1.9 billion adults were overweight (BMI>25 kg/m^2) worldwide with 650 million being classified as obese (BMI>30 kg/m^2)(37). In the United States alone, more than 2/3 (70.2%) of adults are considered overweight

or obese (38) causing a substantial burden with the medical cost associated with obesity-related health concerns being 147 billion dollars in 2008 (39).

Obesity has many associated detrimental health effects including cardiovascular disease, type 2 diabetes, and metabolic syndrome. Metabolic syndrome is a collective term for obesity-associated malignancies such as insulin resistance, hypertension, and dyslipidemia (40). NAFLD is the hepatic manifestation of metabolic syndrome and as such it is closely correlated with incidence of obesity. Given the high prevalence of overweight and obesity globally, it is not surprising that the worldwide burden of NAFLD substantial. Younossi et al., has recently performed a meta-analyses yielding that 25% of the global population has underlying NAFLD (41;42). As mentioned above, not all patients diagnosed with NAFLD will go on to develop non-alcoholic steatohepatitis (NASH)(43;44). This is a critical fact to consider when studying liver disease risk, as other risk-modifying factors may be involved in disease progression to more severe pathologies.

There are several risk-factors associated with NAFLD severity including genetic variations in key genes (45). One of the most well-known single nucleotide polymorphisms is in the *Pnpla3* gene which is involved in hepatic lipid processing. Populations which express a specific variant of this gene are more susceptible to develop NAFLD (46;47). However, other comorbidities such as alcohol consumption and environmental toxicant exposures may also influence risk for disease. As such, it is critical that multiple factors are considered when studying liver disease. In that regard, the field of hepatology has begun to

evaluate a multi-factor approach for NAFLD and ALD influence on disease progression (48).

One of the research goals of the Beier laboratory is to study the effects of chronic, low-level VC exposure on liver health and the potential interactions with underlying liver injury, such as early stages of NAFLD. As previously mentioned, studies from this laboratory have established that VC metabolites enhance severity of liver injury when combined with another factor such as an inflammatory stimuli (LPS, (26)) or high-fat diet feeding (HFD, (27)). The field of environmental toxicology has already begun to shift towards a more integrated approach on studying how toxicants may act to enhance pre-existing organ injury.

7. Statement of goals

As discussed throughout this Chapter, the development of liver disease is a complex and incompletely understood process. Although there are known risk factors for progression of liver disease, in order to better simulate human disease, multiple risk factors must be taken into consideration when studying liver disease development. As discussed in Sections 5 and 6, both toxicant exposure and NAFLD are major contributors for liver disease. Additionally, as mentioned in Section 3, although acute toxicity for VC has been studied, there is a knowledge gap regarding the effects of low-level, chronic VC exposure. Given the high prevalence of obesity and the occurrence of significant risk for exposure to VC from both Superfund and industrial sites, there is a high likelihood of

exposure overlap. Therefore, the purpose of this dissertation will be to prove the hypothesis that low-level VC exposure interacts and enhances NAFLD and to investigate the mechanisms by which VC induces hepatocyte susceptibility to injury. These goals will be discussed in detail in the following section.

B. Aims and Proposals

1. Develop and characterize an animal model of NAFLD and low-level VC exposure and determine if VC enhances NAFLD

This dissertation proposes that low-level VC exposure can interact with underlying liver injury to enhance damage. This will provide novel insight into environmentally relevant levels of VC exposure in conjunction with ingestion of a diet rich in fatty acids (high fat diet, HFD). Previous work has used VC metabolites as a surrogate for VC exposure instead of the parent VC gas. Therefore, the goals if the first Aim of this dissertation are to, 1.) Develop and characterize a model for concomitant low-level VC exposure via inhalation and HFD feeding to better mimic human exposure paradigms, 2.) Evaluate indices of liver injury and inflammation to determine if VC exposure enhances NAFLD-induced liver injury, and 3.) Determine the major cell death pathways involved in this model of injury, which will provide mechanistic insight into how VC contributes to liver damage.

2. Determine mechanisms by which VC enhances NAFLD via metabolic dysregulation and oxidative stress

As mentioned previously, Anders et al ., has demonstrated that VC metabolites exacerbate liver injury both via inflammatory and metabolic pathways

(26;27). Intriguingly, work from those same studies reveal that metabolite exposure alone is sufficient to alter metabolic homeostasis *in vivo* by causing alterations of key pathways involved in energy and nutrient metabolism. Additionally, CE was found to enhance hepatic endoplasmic reticulum (ER) and oxidative stress. Therefore, the goals of this aim are to build upon these previous observations and to evaluate their role in a more relevant mouse model. Accordingly, the goals of Aim 2 of this dissertation are to, 1.) Determine if sub-OSHA concentrations of VC exposure alone, absent any other stimulus, are sufficient to alter hepatic metabolism and, 2.) Evaluate if ER and oxidative stress play a role in the enhancement of liver injury observed with the combination of VC and a HFD.

3. Determine the effect of VC exposure on mitochondrial integrity and function

Mitochondria are vital to the health and function of all cell types. They are critical producers of cellular energy, key regulators of cellular energy metabolism, and are important modulators of cell death pathways. If any of their functions are perturbed, cellular processes can become deranged and have biochemical and physiological consequences. Indeed, mitochondrial dysfunction is known to play a part in development of NAFLD and several other types of liver injury. The Beier lab has investigated mitochondrial damage with VC metabolite exposure, specifically chloroacetaldhyde (CAA), *in vitro*, both in cultured and primary hepatocyte models. These studies revealed that CAA decreases mitochondrial respiration, decreases cellular ATP levels, and depolarizes mitochondrial membrane potential in a concentration dependent manner (26). However, it is

unknown how sub-OSHA levels of VC exposure via inhalation affect mitochondrial dynamics *in vivo*. As such, the goals for Aim 3 of this dissertation are to, 1.) Evaluate the effect of VC and diet on mitochondrial respiration *in vivo*, and 2.) Determine the mechanisms by which VC affects mitochondrial respiration and energy production in hepatocytes.

Overall aim of this dissertation

The overall aim of this dissertation is to address the unifying hypothesis that low-level VC exposure may be sufficient to enhance underlying liver disease. As discussed above, high-level exposures to VC have been characterized, but the effect of lower, chronic exposures have not been studied, nor has low-level VC exposure been studied in the context of experimental NAFLD. Therefore, this question will be addressed in Aim 1. Additionally, recent data from this group have determined that sub-hepatotoxic concentrations of VC metabolites can influence hepatic metabolism and induction of ER stress. The effect of sub-OSHA exposures to VC via inhalation on hepatic metabolism and stress responses would be informative to establish. Therefore, this will be the major goal of Aim 2. Finally, mitochondrial dynamics are crucial for whole cell homeostasis. VC metabolites and other environmental toxicants are known to disrupt mitochondrial function and respiration. However, the effect of direct exposure to VC via inhalation, with and without the co-exposure of high fat diet, on mitochondrial integrity and respiration has not been studied. Aim 3 will serve to address this question. Taken together, this dissertation will provide novel

insight into sub-OSHA exposures of VC and its impact on liver health when combined with underlying liver injury caused by diet.

CHAPER II

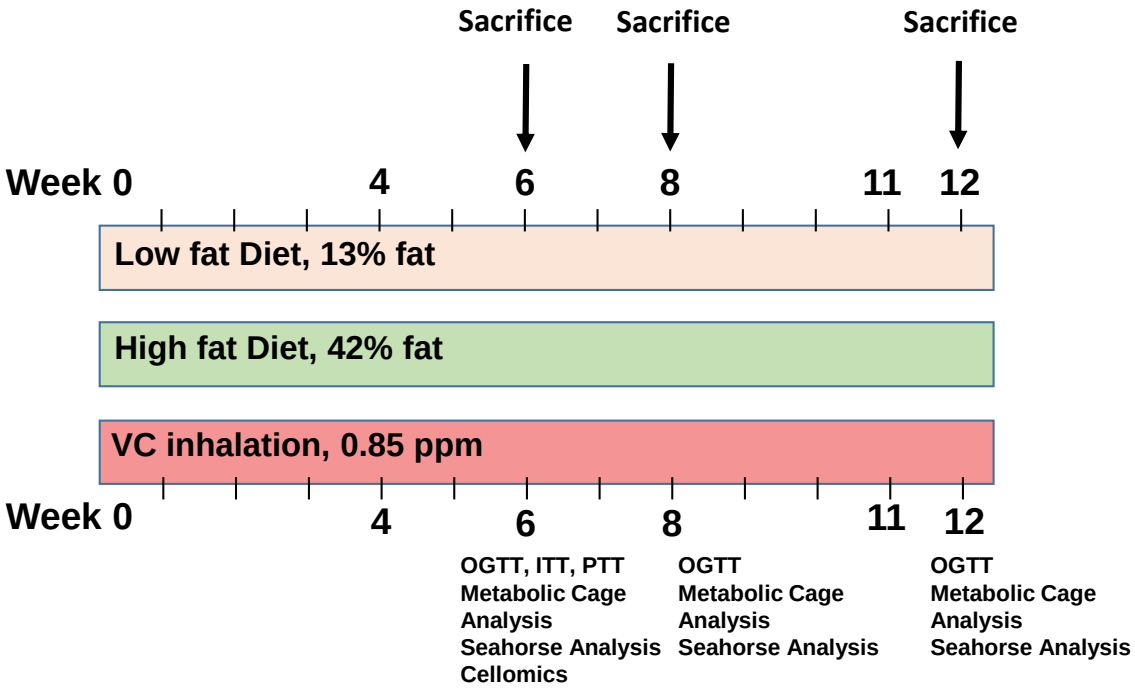
EXPERIMENTAL PROCEDURES

A. Animals and Treatments

Six week old, male C57BL/6J mice from Jackson Laboratory (Bar Harbor, ME) were housed in a pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and procedures were approved by the University of Louisville's 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.

1. Chronic model of VC exposure

The exposure paradigm used was modified from Drew et al. 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 (49). 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 UofL CTRB barrier facility designed with extensive housing capacity, card-coded entry, and separate HVAC system to maintain barrier, temperature and humidity conditions (49).



Scheme 2.1. Model of chronic VC co-exposure with HFD feeding. Mice were fed either a low fat control diet (LFD) or a high fat diet (HFD) and were exposed to vinyl chloride for up to 12 weeks. Animals were euthanized at 6, 8, and 12 weeks of exposure.

Mice were allowed food and water *ad libitum* the entire course of the study. Mice were fed LFD or HFD (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, 8, and 12 weeks of exposure (Scheme 2.1).

2. 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.

3. Key chemical 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. 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).

5. Metabolic Phenotyping

Oxygen consumption rates, carbon dioxide production rates, respiratory exchange ratios, food and water consumption an activity (sum of ambulatory and fine movements) of animals were measured using a physiologic/metabolic cage system (TSE Phenomaster System, Bad Homberg, Germany) during the dark cycle for each of the 3 time points. Body composition was measured using dual-energy x-ray absorptiometry (DEXA) mouse densitometer (PIXImus2, Lunar, Madison, WI) at 6, 8, and 12 weeks of exposure (Scheme 2.1).

Pyruvate tolerance test (1g/kg, PTT) and insulin tolerance test (0.75 U/kg, ITT) were performed at 6 weeks of exposure and oral glucose tolerance test (2g/kg, OGTT) was performed at 6 and 11 weeks. Animals were fasted overnight prior to the PTT and for 6 hours prior for both the ITT and OGTT. Blood was sampled from the tail vein and blood glucose measurements were taken using an AccuCheck Aviva Plus glucometer and test strips (Roche, Indianapolis, IN). Measurements were taken at 0, 15, 30, 60, 90, and 120 minutes after administration of each compound.

Hepatic mitochondria were isolated as previously described (50). Briefly, crude mitochondria were isolated by differential centrifugation using a Beckman Coulter Avanti Ultracentrifuge (J26xPI, rotor: JA 25.50). Liver homogenates were centrifuged two times at 700*g* for 5 minutes at 4°C, supernatants were retained and the pellet (unbroken cells and nuclei) was discarded. After the second spin, the supernatant was collected and centrifuged at 9,000*g* for 10 minutes at 4°C. The resulting supernatant (cytosolic fraction) was discarded and the pellet (crude mitochondria) was collected and centrifuged two times at 10,000*g* for 10 minutes at 4°C. After the final spin, the crude mitochondrial pellet was resuspended in 2mL of mitochondrial resuspension buffer (250mM mannitol, 5mM HEPES, 0.5mM EGTA). Lowry protein determination assay was performed on all samples and samples were plated on XF96 culture plates at 15µg/µl. Pyruvate/malate/ADP (Complex I substrates; 5mM/2.5mM/1mM), oligomycin (Complex IV inhibitor; 1µg/mL), FCCP (mitochondrial uncoupler; 4µM), and succinate/rotenone (Complex II substrates; 10mM/1µM) were administered.

Oxygen consumptions rates were subsequently measured with a Seahorse XF96 extracellular flux analyzer (Agilent, Santa Clara, CA).

B. Histology

1. General morphology

Formalin fixed, paraffin embedded liver tissue was sectioned at 5 μM and mounted on charged glass slides. Sections were deparaffinized with Citrosolv (Fisher Scientific, Waltham, MA) and rehydrated through addition of graded ethanol solutions. Sections were then stained with hematoxylin and eosin (H&E). After staining, samples were dehydrated through graded ethanol, washed in Citrisolv and mounted with Permount (Fisher, Waltham, MA).

2. Neutrophil accumulation

Neutrophil accumulation in liver tissue was assessed using chloroacetate esterase (CAE) staining. Formalin fixed, paraffin embedded liver tissue was sectioned at 5 μM and mounted on charged glass slides. Sections were deparaffinized with Citrisolv (Fisher, Waltham, MA) and rehydrated through addition of graded solutions of ethanol. Sections were incubated in a solution of napthol AS-D chloroacetate (1mg/mL) in N,N-dimethylformamide, with a 4% sodium nitrite and 4% new fuschin (Sigma - Aldrich, St. Louis, MO). The AS-D is enzymatically hydrolyzed by chloroacetate esterase in neutrophils, freeing the napthol compound. Napthol then combines with the freshly formed diazonium salt, resulting in bright pink deposits at the site of the enzymatic activity, which can then be visualized and quantified.

3. Oxidative Stress

Lipid peroxidation adducts were visualized by 4-hydroxynonenal (4-HNE) staining. Liver sections (5 μM) were deparaffinized with Citrsolv (Fisher, Waltham, MA) and rehydrated through graded ethanol. To prevent background binding, sections were incubated in a peroxidase blocking buffer for 5 minutes (Dako North America Inc, Carpinteria, CA). Slides were washed and incubated with 4-HNE rabbit anti-mouse primary antibody (1:500; Alpha Diagnostics, San Antonio, TX) for 30 minutes at room temperature. Slides were washed again and secondary antibody was applied for 15 minutes at room temperature. Slides were washed again and DAB solution was applied for visualization of positive (brown) staining. Slides were then rinsed in water and counterstained with hematoxylin. Sections were dehydrated through graded ethanol and Citrisolv, and mounted with Permount.

Malondialdehyde (MDA) adducts were visualized by a similar immunohistochemical staining procedure. Liver sections (5 μM) were deparaffinized with Citrisolv (Fisher, Waltham, MA) and subsequently rehydrated through graded ethanol. Target retrieval was performed overnight at 72°C. The next day, sections were incubated with a 3% hydrogen peroxide blocking buffer to prevent any endogenous peroxidase activity. Sections were incubated with avidin and biotin blocking reagents for 5 minutes at 30°C (Life Technologies, Frederick, MD) to quench excess tissue biotin. To further minimize non-specific binding, sections were incubated with 10% goat serum for 30 minutes at room temperature. Monoclonal rat anti-mouse primary antibody for MDA (1:500; Alpha

Diagnostics, San Antonio, TX) was applied to the positive section and allowed to incubate for 2 hours at room temperature, while the negative sections received non-antibody containing buffer. Biotinylated anti-rat secondary antibody was applied to both positive and negative sections for 15 minutes at room temperature. Slides were washed and an avidin-HRP solution was applied for 30 minutes at room temperature. Slides were rinsed and DAB solution was added to visualize positive (brown) staining. Slides were washed with water and counterstained with hematoxylin. Sections were dehydrated through graded ethanol, Citrisolv, and mounted with Permount.

4. Macrophages

Hepatic macrophages were visualized via immunohistochemistry. Liver sections (5 μM) were deparaffinized with Citrisolv (Fisher, Waltham, MA) and subsequently rehydrated through graded ethanol. Target retrieval was performed overnight at 72°C. The next day, sections were incubated with a 3% hydrogen peroxide blocking buffer to prevent any endogenous peroxidase activity. Sections were incubated with avidin and biotin blocking reagents for 5 minutes at 30°C (Life Technologies, Frederick, MD) to quench excess tissue biotin. To further minimize non-specific binding, sections were incubated with 10% goat serum for 30 minutes at room temperature. Monoclonal rat anti-mouse primary antibody for F4/80 (1:50; Abcam, Cambridge, MA) was applied to the positive section and allowed to incubate for 2 hours at room temperature, while the negative sections received non-antibody containing buffer. Biotinylated anti-rat secondary antibody was applied to both positive and negative sections for 15 minutes at room

temperature. Slides were washed and an avidin-HRP solution was applied for 30 minutes at room temperature. Slides were rinsed and DAB solution was added to visualize positive (brown) staining. Slides were washed with water and counterstained with hematoxylin. Sections were dehydrated through graded ethanol, Citrisolv, and mounted with Permount.

5. Fibrin

Immunofluorescent detection of fibrin accumulation has been described previously (51), and was conducted with minor modifications. Liver sections (5 μM) were deparaffinized with Citrisolv (Fisher, Waltham, MA) and rehydrated through graded ethanol. Autofluorescence of paraffin-embedded tissue was quenched with 70% ethanol containing 0.25% NH_4OH for 1 hour at room temperature during deparaffinization. Proteolytic digestion was performed by incubating tissue sections in 0.03% Pronase E (Sigma-Aldrich, St. Louis, MO) for 10 minutes at 37°C, sections were then incubated in sodium borohydride (10 mg/mL) for 40 minutes at room temperature for additional autofluorescence quenching. To minimize non-specific antibody binding, sections were incubated in 10% goat serum in PBS for 30 minutes at room temperature. Sections were then incubated with rabbit polyclonal anti-fibrinogen (Agilent Technologies A0080, Santa Clara, CA) in blocking buffer (1:1000) overnight at 4°C. The next day, sections were washed in PBS and incubated with AlexaFluor 488 goat anti-rabbit secondary antibody (1:500; Life Technologies, Carlsbad, CA) in blocking buffer for 3 hours at room temperature. After washing, slides were mounted with VECTASHIELD Antifade Mounting Medium with DAPI (Vector Labs, Burlingame,

CA). Slides were visualized with a Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan) using Metamorph software (Molecular Devices, Sunnyvale, CA).

6. Apoptosis

Apoptosis was detected via terminal deoxynucleotide transferase biotin-dUTP nick end labeling (TUNEL; Millipore, Billerica, MA). Briefly, formalin fixed, paraffin embedded liver tissue was sectioned at 5 μM and mounted on charged glass slides. Sections were deparaffinized with Citrisolv (Fisher, Waltham, MA) and rehydrated through graded ethanol. Tissue samples were processed according to the TUNEL kit protocol.

7. Metabolism

Neutral lipid accumulation was detected via Oil Red-O (ORO) staining. Liver tissue was embedded in Tissue-Plus optimal Cutting Temperature (OCT) embedding medium (Sakura Finetek, Torrance, CA) by freezing in liquid nitrogen. After embedding, samples were brought to -20°C and sectioned at 10 μM on a Leica cryostat. Sections were then stained with Oil Red-O solution (Sigma-Aldrich, St. Louis, MO), washed, and counterstained with hematoxylin for 45 seconds. Samples were mounted with Permount (Fisher, Waltham, MA). Hepatic glycogen reserves were visualized as a dark purple color using Periodic-Acid Schiff (PAS) staining. Formalin fixed, paraffin embedded liver tissue was sectioned at 5 μM and mounted on charged glass slides. Sections were deparaffinized with Citrisolv (Fisher, Waltham, MA) and rehydrated through

graded ethanol. Sections were incubated in 0.5% Periodic Acid solution for 5 minutes. Samples were counterstained with hematoxylin for 45 seconds, washed in water, dehydrated through graded ethanol, washed in Citrisolv, and then mounted with Permount.

8. Histology quantification

Quantification of CAE staining was performed using Image J software in which CAE positive (bright pink) cells were counted and analyzed as positive cells out of 1,000 hepatocytes. Similarly, TUNEL positive (brown) cells, both hepatocytes and non-parenchymal cells (NPCs) were counted and are expressed as positive cells out of 1,000 hepatocytes.

Image analysis was performed to quantify areas of positive staining for fibrin, F4/80, 4-HNE, MDA, ORO, and PAS stains. Metamorph software (Molecular Devices, San Jose, CA) was used to analyze positive staining based on setting unique color thresholds for each stain examined. Positive area is expressed as a percent of total microscope field area.

C. Clinical Chemistry

1. Biochemical analyses

Plasma activity levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined spectrophotometrically using standard kits (Thermo Fisher Scientific, Waltham, MA). Plasma levels of lactate and β-hydroxybutyrate were determined using standard kits (Abcam, Cambridge, MA and Cayman Chemical, Ann Arbor, MI, respectively). Liver tissue levels of

Thiobarbituric Acid Reactive Substances (TBARS) were determined using a commercially available kit (R&D Systems, Minneapolis, MN).

Hepatic lipids were extracted as described previously (52;53). Briefly, mouse liver (25-50 mg) was pulverized in a liquid-nitrogen cooler mortar and pestle. Pulverized liver tissue was incubated in a methanol:chloroform (2:1) mixture on ice for 30 minutes. After the incubation, extracts were centrifuged at 900*g* for 10 minutes at 15°C. After centrifugation, lipids were contained within the chloroform layer and were removed with a glass syringe. Extracts were dried using nitrogen gas. The resulting pellets were resuspended in 5% fatty acid free bovine serum albumin and kept at -80°C. The lipid extracts were then analyzed for levels of triglycerides (TG), cholesterol, and free fatty acids (FFA) using commercially available kits (Thermo Scientific, Waltham, MA and Roche Diagnostics, Indianapolis, IN, respectively).

2. Luminex

Protein levels of IL-6, PAI-1, TNF-α, MCP-1, Resistin, Leptin, and Insulin were detected in citrated plasma using a commercially available Milliplex® mouse adipokine panel (Millipore, Billerica, MA). The plate was read using a Luminex 100 plate reader and Exponent software. The assay was performed according to the manufacturer's recommendation. Plasma samples were analyzed undiluted on a clear bottomed black 96 well plate. Antibody-immobilized beads were added to each sample and incubated at 4°C overnight with gentle shaking. Detection antibody was added to wells and incubated for 1 hour with subsequent addition of streptavidin-phycoerythrin. Quantitative analysis was

performed using the Luminex 100 IS. Data capture and analysis were performed using the Luminex Xponent software.

3. ELISA

Plasma thrombin-antithrombin (TAT) complex levels were detected in citrated plasma using a commercially available ELISA kit (Abcam, Cambridge, MA) as described previously (51;54).

D. Primary hepatocyte isolation

Mouse hepatocytes were isolated and separated from anesthetized mice via collagenase perfusion from each treatment group at 6 weeks of exposure as previously described (55;56). Briefly, livers were perfused through the inferior vena cava (IVC) at a constant rate of 5mL/minute at 37°C. First, livers were perfused with an EGTA buffer for 6 minutes, followed by subsequent perfusion with collagenase buffer (0.36mg/mL) for 10 minutes. Livers were removed and immediately minced in Waymouth's medium and the gallbladder was removed. The resulting suspension was filtered a sterile cell strainer (70μM; Corning Life Sciences, Tewksbury, MA). The suspension was centrifuged at 50*g* for 5 minutes at 4°C. Cells were washed two times with HBSS (Life Technologies, Grand Island, NY) followed by centrifugation at 50*g* for 5 minutes at 4°C. After the final spin, cell were resuspended in Waymouth's media and viability was determined via Trypan blue exclusion.

E. Quantitative cell analysis

Immediately following isolation, primary mouse hepatocytes were plated (10,000 viable cells/well) on collagen I coated 96 well plates (Life Technologies, Grand Island, NY). Cells were allowed to incubate for 90 minutes in Waymouth's media supplemented with FBS and insulin transferrin solution (ITS) (Gemini Bio products, Life Technologies, Grand Island, NY). Cells were then incubated with media containing chloroacetaldehyde (CAA, Sigma-Aldrich, St. Louis, MO) from 0 to 400 μM for one hour. CAA-containing media was removed and one hour prior to the commencement of measurements media containing: Hoechst 33342 (12.5 μM; nuclear stain), tetramethylrhodamine (TMRM; 20 nM, mitochondrial membrane potential), and quinolinium, 1,1'-[1,3-propanediylbis[(dimethyliminio)-3,1-propanediyl]]bis[4-[3-(3-methyl-2(3*H*)-benzothiazolylidene)-1-propen-1-yl]-,iodide (1:4) (TOTO-3, 1μM, cell death) dyes were added to the wells (Invitrogen, Carlsbad, CA) . Following the one hour incubation with the dyes, the plate was placed into the Cellomics Array Scan VTI HCS reader and relative fluorescence units (RFU) were analyzed, as described previously (57).

F. RNA isolation and quantitative reverse-transcription polymerase chain reaction

Total RNA was extracted from liver tissue by a guanidinium thiocyanate-based method (RNA STAT-60, Tel-Test, Inc, Friendswood, TX). RNA concentrations were determined spectrophotometrically and 1 μg of total RNA was reverse transcribed using a kit (Quanta Biosciences, Beverly, MA). Real-time qRT-PCR was performed using a Quant Studio 3 real time PCR system (Thermo Fisher Scientific, Grand Island, NY).

The hepatic mRNA expression of select genes was detected by qRT-PCR, which is routine for the Beier laboratory. Primers and probes for *Cpt1a1, Dgat2, Gsk3β, Pparα, Pck1, G6pase, Pgc1α, Sirt1, Atf4, Chop, Hsp90, Tfam, Fis1, Drp1,* and *Cox4* were ordered as commercially available kits (Thermo Fisher Scientific, Grand Island, NY). The comparative CT method was used to determine fold changes in mRNA expression compared to an endogenous reference gene (18S). This method determines the amount of target gene normalized to an endogenous reference and relative to a calibrator ($2^{\Delta\Delta CT}$).

G. Immunoblots

Liver samples were homogenized in RIPA buffer, containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO). Protein was quantified by Lowry assay using bovine serum albumin as a positive control. Liver homogenates or mitochondrial protein isolates (2.5 µg/µL, 1 µg/µL respectively) were loaded on to Bolt™ 4-12% Bis-Tris Plus gels (Invitrogen, Carlsbad, CA), followed by electrophoresis and Western blotting onto PVDF membranes (Immobilon-P, Millipore, Billerica, MA). Primary antibodies against GAPDH (1:2,000; Santa Cruz Biotechnology, Dallas, TX), cleaved caspase-3, pro-caspase-3, cytochrome *c*, phospho-Stat3S727, phospho-Stat3Y705, total Stat3, IP3-R1 (1:1,000; Cell Signaling Technologies, Danvers, MA), Chop (1:1,000; Thermo Scientific, Waltham, MA), and OXPHOS antibody cocktail (1:500, Abcam, Cambridge, MA) were used. Blots were developed onto blue sensitive autoradiography film (MidSci, St. Louis, MO) using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL).

Densitometric analysis was performed using UN-SCAN-IT gel software (Silk Scientific Inc., Orem, UT).

H. 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.

I. Statistical analyses

Power analysis was used to calculate the number of animals required for the experiments. Results are reported as means ± standard error (SEM, n=4-12) and were analyzed using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA). Unless otherwise specified, one-way or two-way ANOVA with Bonferroni's post-hoc test (for parametric data) or Mann-Whitney Rank Sum test (for nonparametric data) were used for determination of statistical significance among treatment groups, as appropriate. A *p* value less than 0.05 was selected before the study and the level of significance.

CHAPTER III

VINYL CHLORIDE ENHANCES DIET-INDUCED LIVER INJURY IN MICE

A. Introduction

Vinyl chloride (VC) is a volatile organic compound and organochlorine toxicant. VC is known to cause direct hepatic damage at high, occupational exposures (34). As mentioned in Chapter I, OSHA enforced stricter exposure guidelines for occupational VC exposure in 1975. Although this prevents acute exposure in the industrial setting, there are still opportunities for chronic, low-level exposure via environmental contamination and exposure. VC is ranked #4 on the CDC's Agency for Toxic Substances and Disease Registry (ATSDR) Substance Priority List (59). This list ranks chemicals based on not only toxic potency, but also human risk for exposure. Clearly, due to its top five ranking, VC remains a relevant toxicant when evaluating human environmental pollutant risk. VC is ubiquitous at many EPA designated Superfund sites across the United States, as both a direct contaminant and as a degradation product of trichloroethylene (TCE) and perchloroethylene (PCE) (60). Many of these Superfund sites are in close proximity to residential areas, causing concern for

exposure. Additionally, factories that produce polyvinyl chloride (PVC) plastic products utilize VC monomer and also emit VC gas as a waste product into the environment. Residential areas surrounding both manufacturing and Superfund sites are susceptible to VC migrating through soil into home foundations where it readily volatilizes to enter showers, basements, and living spaces in which these vapors recirculate and are inhaled (61;62).Therefore, understanding the effects of VC toxicity on human health is imperative.

The obesity epidemic is a prevalent and concerning health issue in the United States with over 1/3 (35.6%) of the adult population considered obese (BMI > 30 mg/km^2) (39). Indeed, in a recent analysis by Flegal et al., current trends depict that obesity rates are still rising and are predicting to continue to rise in the coming years (63). Previous studies in the Beier laboratory have demonstrated enhanced liver injury in mice with exposure to HFD and CE, a VC metabolite. There are several pathologies associated with obesity including cardiovascular disease, metabolic syndrome, and type 2 diabetes. The major hepatic manifestation of metabolic syndrome is non-alcoholic fatty liver disease (NAFLD). It is now well-known that underlying liver injury, such as NAFLD, can influence progression and severity of liver disease development. Due to the fact that the average BMI of the American population has increased dramatically since the OSHA regulation was implemented (37), it is crucial to re-evaluate the impact of low-level VC exposure in the context of underlying liver injury.

VC concentrations below the current OSHA limit are relevant not only for industrial workers, but also for environmental exposures for residents living in

proximity to both Superfund and PVC-manufacturing facilities. Therefore, the goal of this aim was to develop an animal model relevant to human VC exposure to more closely mimic liver injury and risk for disease progression observed.

B. Experimental Procedures

1. Animals and treatments

6 week old, male C57Bl/6J mice were exposed to sub-OSHA concentrations of VC via inhalation or room air for 6 hours per day, 5 days per week, for up to 12 weeks as described in Chapter II, Section A.1. Mice were fed either a low fat diet (LFD) or high fat diet (HFD) as detailed in Chapter II, Section A.4. Upon sacrifice, tissues were collected as described in Chapter II, Section A.2.

2. Metabolic phenotype

Metabolic phenotype was described via metabolic cage analyses, DEXAscan analysis, and monitoring of body weight and food consumption was done as described in Chapter II, Section A.5.

3. Biochemical analyses

Plasma transaminase levels were analyzed as described in Chapter II, Section C.1. Plasma protein levels of inflammatory cytokines were analyzed as described in Chapter II, Section C.2.

4. ELISA

To quantify thrombin-anti thrombin (TAT) complexes in all experimental groups, a TAT ELISA was performed as described in Chapter II, Section C.3.

5. Histology

Liver sections were stained with hematoxylin and eosin for general morphology as described in Chapter II, Section B.1. Visualization of neutrophils and macrophages was achieved by CAE and F4/80 staining as described in Chapter II, Section B.2, B.4. Apoptosis was detected via TUNEL staining as described in Chapter II, Section B.6. Detection of fibrin via immunofluorescence was performed as described in Chapter II, Section B.5.

6. Immunoblots

Whole liver lysates were examined via Western blot for cleaved caspase-3, pro-caspase-3, and GAPDH, and mitochondrial lysates were examined for cytochrome C via Western blot as described in Chapter II, Section G.

7. Statistical analyses

Results are reported as means ± standard error (SEM, n=4-12) and were analyzed using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA). Unless otherwise specified, one-way or two-way ANOVA with Bonferroni's post-hoc test (for parametric data) or Mann-Whitney Rank Sum test (for nonparametric data) were used for determination of statistical significance among treatment groups, as appropriate. A *p* value less than 0.05 was selected before the study as the level of significance. [a], $p<0.05$ compared to the absence of HFD; [b], $p<0.05$ compared to the absence of VC.

C. Results

1. Vinyl chloride does not alter diet-induced body mass composition

To assess animal condition and whole animal metabolism in this model, body weight and food consumption were measured weekly. Figure 3.1A shows body weight gain for the 12-week experiment for all groups. Animals exposed to LFD + VC did not gain more body weight compared to LFD controls. Although there was a diet effect on body weight with HFD, the addition of VC did not significantly enhance this effect. Body mass composition analysis at the 12 week time point revealed that there were no significant alterations in either lean or fat mass distribution with VC exposure (Figure 3.1B). Independent of diet or VC exposure, food consumption did not change between groups across the 12-week experiment (Figure 3.1A). Liver to body weight ratios were similar for LFD ± VC animals. HFD significantly increased liver to body weight ratios at both 8 and 12 weeks. However, VC did not further enhance this effect (Figure 3.1A).

In order to evaluate systemic metabolism, mice were placed in metabolic chambers for 12 hours in which food and water intake, oxygen consumption (V_{O2}), carbon dioxide production (V_{CO2}), and physical activity measurements for individual mice were obtained. Food and water intake were similar in both HFD and HFD + VC treated animals at 12 weeks of exposure (Figure 3.1C). However, VC significantly decreased V_{O2} and V_{CO2} values (Figure 3.1C). Interestingly, the respiratory exchange ratio (RER) did not change compared to HFD control animals, suggesting an equal decrease in both oxygen consumption and carbon

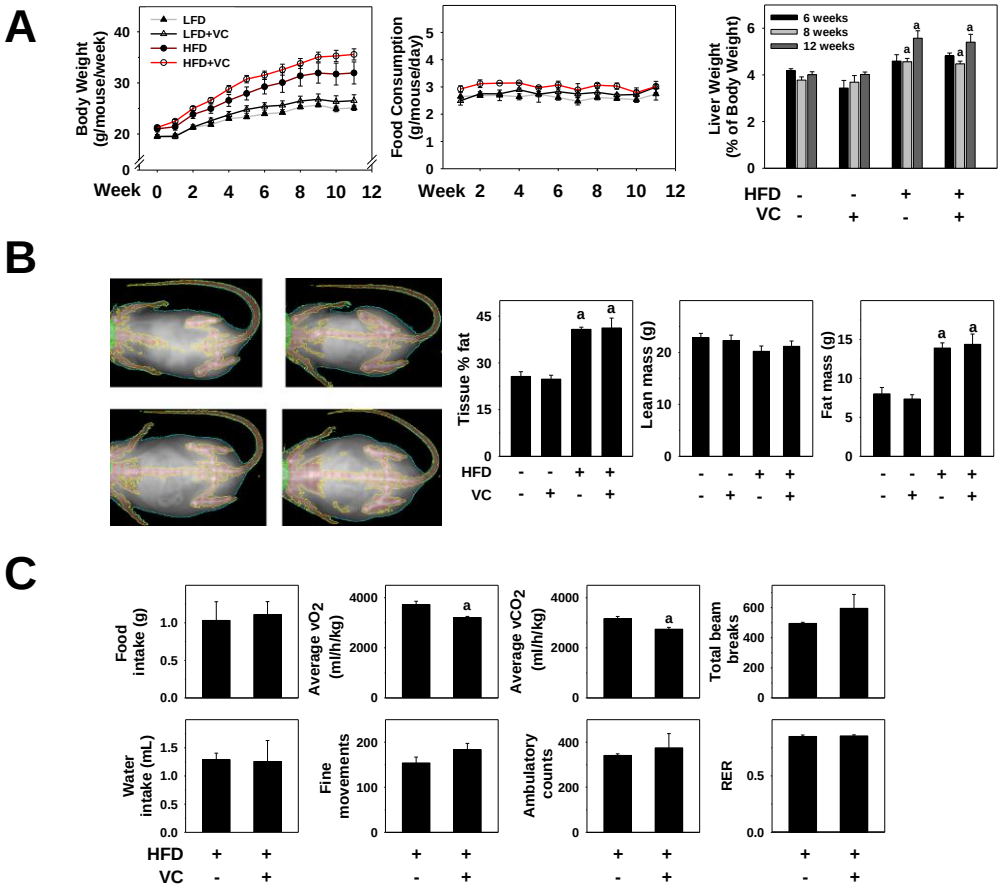


Figure 3.1. VC does not alter body mass composition. A: Body weights were measured once per week and are depicted for the 12-week exposure period. Food consumption was measured twice/week for the 12-week exposure period. Liver weight to body weight ratios were calculated for each group for each time-point. B: DEXAscan analysis was performed for all treatment groups at the 12-week time point. Representative photos are shown. Body mass is graphed as body fat in percent of total tissue, lean body mass and fat mass in grams. C: Representative parameters of metabolic function are depicted for mice exposed to HFD ± VC. [a], $p<0.05$ compared to LFD or HFD control.

dioxide production and no shift in macromolecule source for energy expenditure. While a decrease of RER may be caused by a decrease in physical activity, VC did not alter physical activity levels (assessed by fine movement, ambulatory counts, and total beam breaks) of the mice (Figure 3.2C).

2. Vinyl chloride enhances HFD-induced liver injury

Recently, the Beier laboratory has demonstrated enhanced liver injury with exposure to a VC metabolite (chloroethanol, CE), and HFD feeding (27). Here, using a more relevant model of VC exposure, histological and biochemical indices of liver damage were assessed. Figure 3.2A depicts representative photomicrographs of liver sections stained with hematoxylin and eosin (H&E) for visualization of general liver morphology and fibrin staining as an index of local tissue injury. Normal morphology and transaminase activity were observed in LFD control animals, and no overt pathologic changes were observed in animals exposed to LFD + VC, nor did they display increases in transaminase activity (Figure 3.2A, B). HFD feeding increased both steatosis, as evidenced by the H&E stain, and fibrin accumulation (Figure 3.2A). Interestingly, VC significantly enhanced indices of damage observed including fibrin accumulation and transaminase activity in mice fed a HFD (Figure 3.2A, B). In order to determine the mechanism of enhanced fibrin deposition in the HFD + VC group, plasma PAI-1 and TAT levels were analyzed (Figure 3.2C). Fibrin accumulation can occur either by increased thrombin generation via the coagulation cascade or

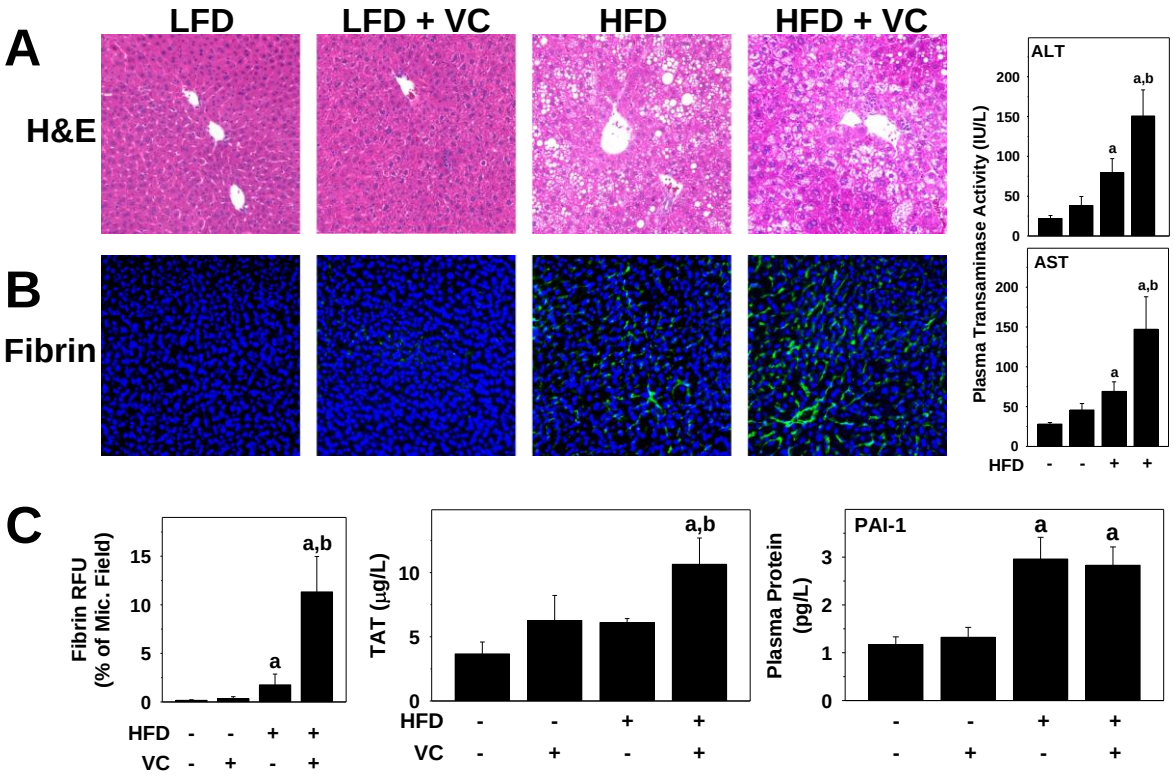


Figure 3.2. VC enhanced diet-induced liver injury. A: Representative photomicrographs of H&E (general morphology, 200x) and plasma transaminase (ALT/AST) levels were determined for the 12-week time point. B: Representative photomicrographs for fibrin immunofluorescence (200x). C: Image analysis for fibrin is graphed as relative fluorescence units (RFU) as percent of microscope field and plasma thrombin-antithrombin (TAT) and protein concentrations of PAI-1 are shown in µg/L and pg/L, respectively. [a], $p<0.05$ compared to LFD control; [b], $p<0.05$ compared to absence of VC.

through inhibition of fibrin degradation via PAI-1 activity. Here, VC exposure had no effect on either TAT or PAI-1 levels in the absence of HFD feeding. Interestingly, while PAI-1 levels increased in a diet-dependent manner, TAT levels were significantly increased in the HFD + VC group, while PAI-1 levels remained unchanged. These data suggest that fibrin accumulation in this model is due to altered coagulation rather than changes in PAI-1 expression.

3. VC enhanced diet-induced neutrophil infiltration

VC enhanced HFD-induced liver injury observed in this model, therefore indices of liver inflammation were assessed to determine if hepatic inflammation is involved in the mechanism of VC-induced damage. Here, histologic markers of neutrophil infiltration (CAE staining), and macrophages (F4/80 immunohistochemistry), were examined for the 12 week time point (Figure 3.3A, B). There were no differences in either neutrophil or macrophage recruitment in animals exposed to LFD ± VC. HFD control animals had significantly more infiltrating neutrophils than their LFD counterparts, however no changes were observed in macrophage recruitment. VC significantly enhanced neutrophil accumulation, indicating more hepatic inflammation and injury compared to HFD control animals. Similar to the HFD group, VC also caused no alterations in macrophage recruitment. Plasma protein concentrations of inflammatory cytokines were also examined (Figure 3.3C). While HFD significantly elevated plasma protein concentrations of MCP-1, this effect was not altered by VC. Plasma protein levels of TNFα and IL-6 were unchanged across all groups.

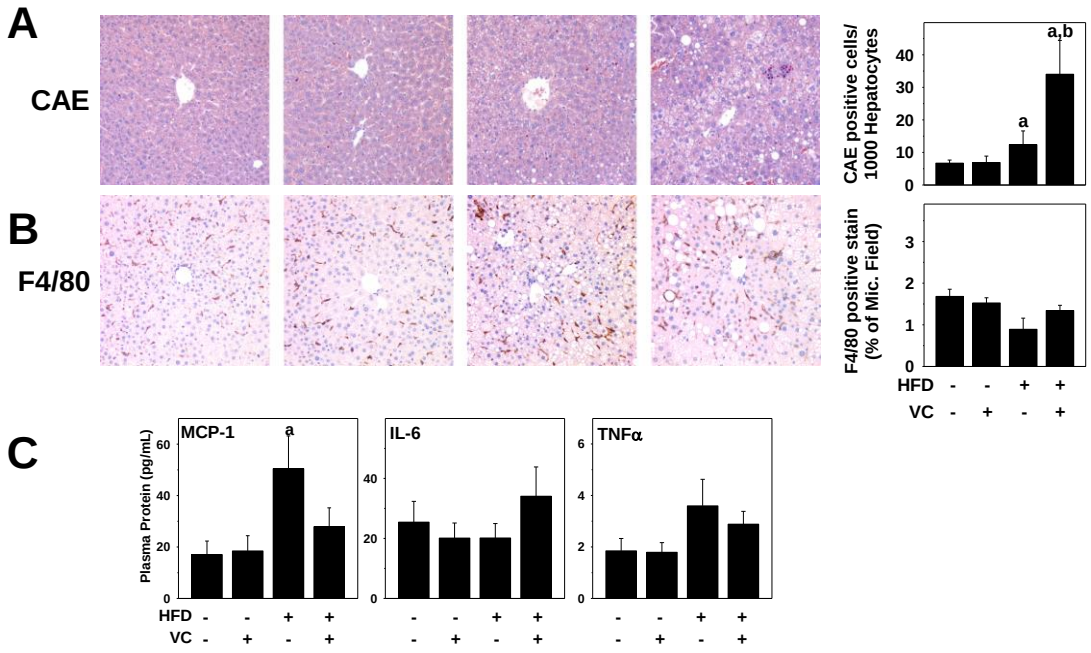


Figure 3.3. VC enhanced diet-induced neutrophil infiltration. A: Representative photomicrographs of CAE (neutrophils, 200x) are shown and CAE-positive cells were counted and graphed as positive cells per 1000 hepatocytes. B: Representative photomicrographs of F4/80 (macrophages, 200x) IHC is shown for the 12-week time-point and image analysis for F4/80 is graphed as positive staining as percent of microscope field. C: Plasma protein concentrations of MCP-1, IL-6, and TNFα are shown in pg/mL. [a], $p<0.05$ compared to LFD control; [b], $p<0.05$ compared to absence of VC.

4. Vinyl chloride causes increased NPC apoptosis, but not hepatocyte apoptosis

The mechanism of cell death and cell type specificity is often imperative for determining the mechanism of injury and pathways involved in cellular damage. For example, apoptotic hepatocyte death is characteristic of NAFLD pathogenesis and this effect has been well characterized (64;65). In this study, in order to better understand the increased liver injury and inflammation observed, cell death pathways were investigated. Cleaved caspase-3 protein levels were analyzed via Western blot for whole liver lysates at the 12 week time point (Figure 3.4A). No differences were observed for LFD + VC animals compared to LFD controls. HFD significantly increased the amount of cleaved capase-3 protein levels. Interestingly, HFD + VC had significantly enhanced levels of cleaved caspase-3, indicating enhanced activation of the apoptotic pathway. Additionally, mitochondrial protein isolates from the same treatment groups at 6 weeks of exposure were analyzed for cytochrome *c* via Western blot (Figure 3.4B). Cytochrome *c* release from mitochondria is known to be involved in the activation of apoptotic signaling cascades (66). Here, a significant VC effect observed. Independent of diet, VC significantly decreased cytochrome *c* levels within mitochondria, indicating an increased release into the cytosol, which suggests activation of apoptosis.

However, for a more cell-specific analysis of apoptosis and to determine the levels of apoptosis occurring, TUNEL staining was performed for the 12 week time point. Representative photomicrographs of TUNEL stained liver section

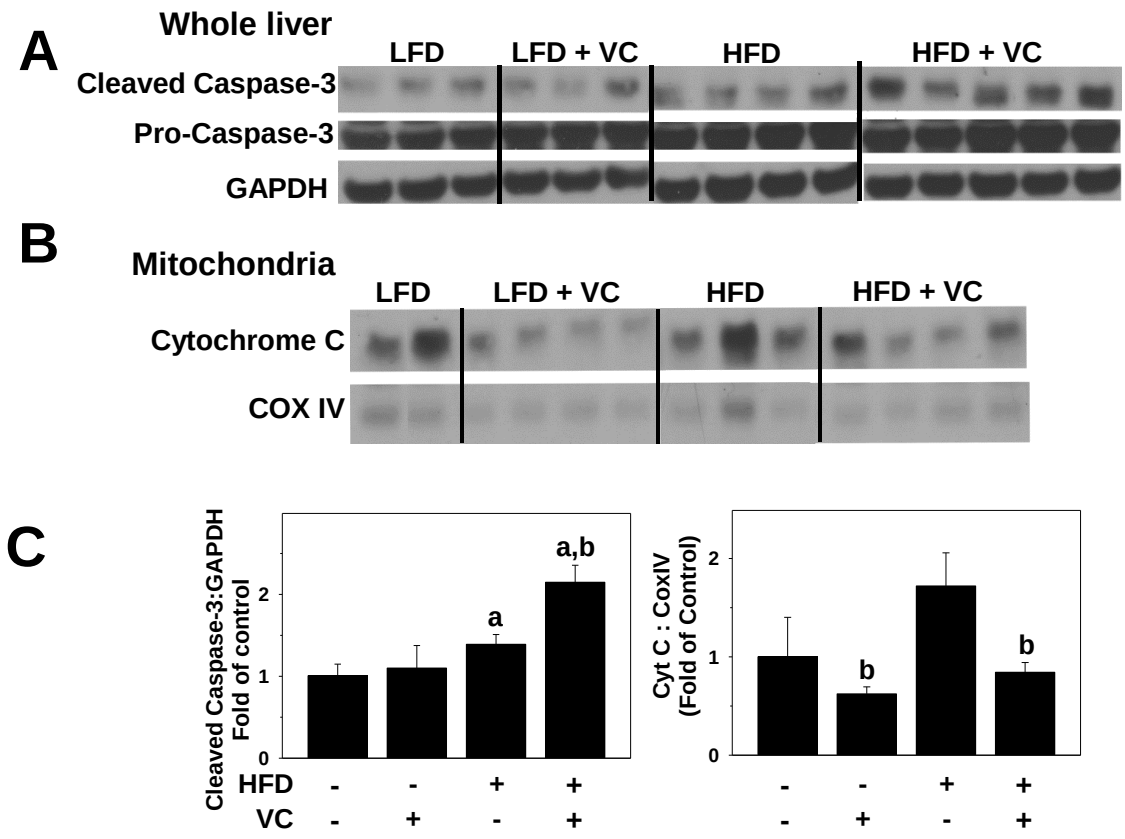


Figure 3.4. Effect of VC on apoptotic signaling. A: Representative Western blots for whole liver Caspase-3 protein are shown. B: Representative Western blots for hepatic mitochondrial Cytochrome *c* protein are shown. C: The corresponding densitometric analyses are shown for both Caspase-3 and Cytochrome *c* are shown as fold of control. [a], $p<0.05$ compared to LFD control; [b], $p<0.05$ compared to absence of VC.

and the corresponding TUNEL-positive cell counts (hepatocytes and NPCs) are shown in Figure 3.5A,B. Animals exposed to LFD ± VC did not exhibit changes in the number of TUNEL-positive cells. Interestingly, HFD feeding did not change the number of apoptotic hepatocytes or NPCs. VC did not change this effect in hepatocytes. It did, however, significantly increase the number of TUNEL-positive NPCs.

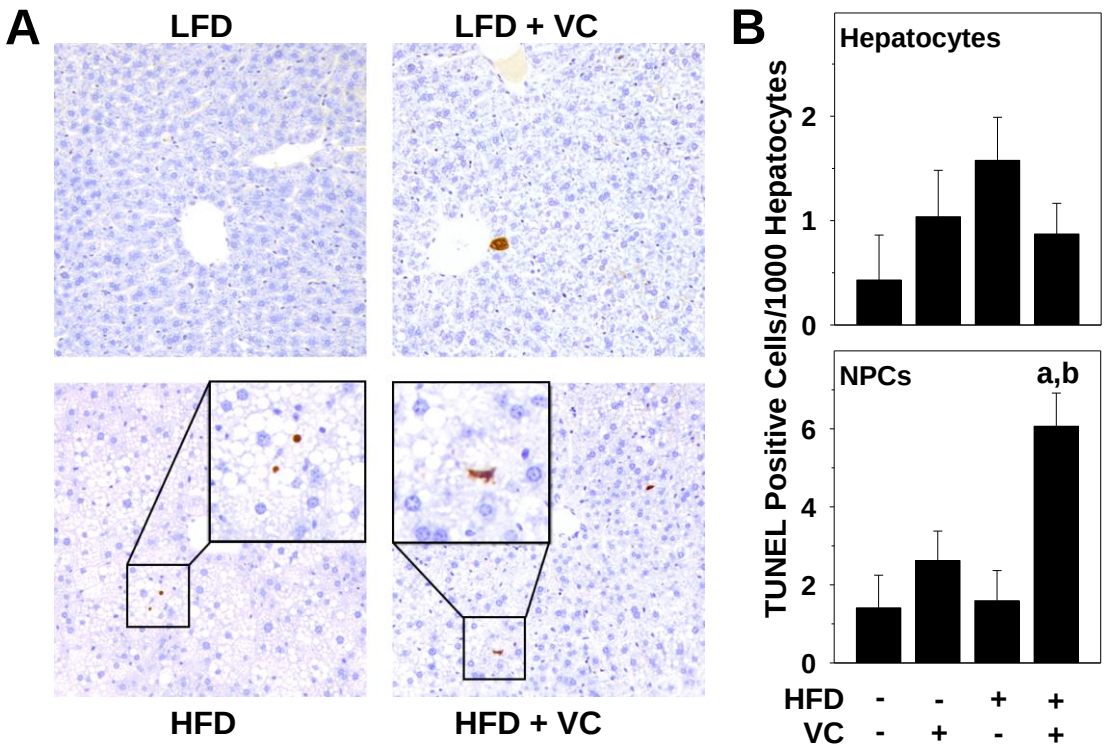


Figure 3.5. VC did not cause hepatocyte apoptosis, but increased NPC apoptosis. A: Representative photomicrographs for TUNEL staining are shown (200x). B: TUNEL-positive hepatocytes and NPCs were counted and TUNEL-positive cells per 1000 hepatocytes are depicted. [a], $p<0.05$ compared to LFD control; [b], $p<0.05$ compared to absence of VC.

D. Discussion

As mentioned in the Introduction, VC exposure at high levels is known to directly cause malignant and nonmalignant liver disease. OSHA enforced stricter VC exposure guidelines in the occupational setting after a series of severe and rare liver angiosarcomas diagnoses occurred at a VC production facility during the 1970's. The new exposure thresholds significantly reduced the risk of acute exposures to high concentrations of VC. However, more recently, the field of environmental toxicology has begun to shift to an 'exposure biology' paradigm. Exposure biology focuses on the impact of moderate/low level, chronic exposures in contrast to high, occupational exposures. The risk for low-level, chronic VC exposure remains a concern both for workers and for residential populations living in close proximity to industrial and Superfund sites. Furthermore, the majority of experimental studies with VC have used extremely high concentrations (>100 ppm) and focused mainly on its carcinogenic effects (23;24). Therefore, the potential impact of lower, chronic exposure remains unknown. The major goal of this study was to develop and characterize a mouse model of chronic, sub-OSHA VC exposure in order to fill the gaps in our understanding regarding the impact of VC exposure at low concentrations.

Exposure biology also acknowledges that underlying disorders and sensitivities may act to modify risk for developing more severe disease phenotypes and as such should be a critical consideration. The pandemic of obesity is quite possibly the most prevalent underlying liver disorder that impacts the American population. More than 2/3 of the United States is at risk for

developing obesity-associated NAFLD, the hepatic manifestation of metabolic syndrome. Several studies have identified potential interactions between environmental chemical exposures and experimental NAFLD (67-70). Although the mechanisms by which this occurs are incompletely understood, most of these environmental chemicals enhance pathologic responses (e.g., inflammation) and act to sensitize hepatocytes to injury. Due to the fact that the American average BMI has increased dramatically since the VC safety thresholds were initially set (37), a second major goal of this study was to determine the impact of VC exposure on liver injury caused by a HFD. The data presented here demonstrate that sub-OSHA levels of VC exposure are sufficient to enhance liver injury caused by a HFD. This raises concerns regarding the current OSHA regulations in place. These results are in line with previous epidemiological studies with VC exposure and comorbidities. For example, Mastrangelo et al. showed that VC workers were at greater risk for developing alcoholic liver disease (71).

First, the effects of VC exposure on overall hepatic inflammation and damage were determined for 12 weeks of exposure. Here, VC exposure alone did not cause any overt toxicity or liver damage in animals fed control diet. (Figure 3.2). Additionally, VC did not affect body weight gain, food consumption, adiposity, or indices of liver injury (liver weight, transaminase levels, and histology) for the LFD group. The combination of HFD feeding and VC exposure significantly enhanced indices of liver damage as assessed by histology and transaminase levels. Interestingly, although VC exacerbated HFD-induced neutrophil recruitment to the liver (Figure 3.3) it did not affect macrophage

recruitment or cytokine levels (Figure 3.3). This effect can be partially explained by the observed increase in NPC apoptosis with the combination of HFD and VC exposure (Figure 3.5).

Neutrophils are acute responders to local injury as part of the innate immune response (72). Additionally, neutrophil accumulation within liver tissue is a hallmark of NAFLD pathogenesis (73). In accordance with the literature, here, HFD feeding caused significant neutrophil recruitment to the liver. Interestingly, enhancement of this effect was observed with HFD feeding and VC exposure in this model, suggesting enhanced local tissue injury or alterations in the hepatic extracellular matrix (ECM), specifically fibrin accumulation (Figure 3.2). Indeed, the combination of HFD and VC caused significant hepatic fibrin accumulation. Fibrin has been shown to facilitate both neutrophil chemotaxis and activation (74). However, neutrophils have also been shown to be critically involved in the breakdown of fibrin ECM. Fibrin ECM accumulation can occur via two pathways; increased deposition or impaired generation (75). A major inhibitor of fibrin degradation is PAI-1. Notably here, although PAI-1 protein levels increased with HFD, this effect was not enhanced by VC. This suggests that the fibrin accumulation observed is due to enhanced deposition via coagulation rather than impaired degradation by PAI-1. Upon further analysis, plasma thrombin anti-thrombin levels were found to be significantly increased with HFD and VC exposure, suggesting that *de novo* fibrin deposition by the coagulation cascade contributes to the local hepatic damage observed under these conditions.

Cave et al. previously showed that occupational VC exposure increased total plasma cytokeratin 18 in humans, but not the caspase-cleaved isoform (34); suggesting an increase in necrosis rather than apoptosis in the liver (76). Indeed, similar results were observed here; although the increase in hepatic caspase-3 cleavage caused by HFD was significantly enhanced by the addition of VC exposure, the number of TUNEL-positive hepatocytes did not change. This suggests that apoptosis does not play a major role in hepatocyte cell death in this model. In contrast, the number of TUNEL-positive NPCs, such as Kupffer cells, significantly increased with the combination of HFD and VC. This significant increase in NPC death could also explain the decrease plasma MCP-1 protein levels with HFD and VC, as Kupffer cells and other cells are able to secrete MCP-1 for recruitment of monocytes to aid in tissue repair (77), and a decrease in their population would result in a lower levels observed in circulation.

Taken together, the results from this study show that sub-OSHA levels of VC exposure are sufficient to exacerbate experimental NAFLD. Although 12 weeks of VC exposure alone does not cause overt livery injury or toxicity, it did cause the liver to be more susceptible to diet-induced damage. Indeed, increased indices of tissue injury, liver damage, and neutrophil recruitment were all observed with the combination of HFD feeding and VC exposure. This study reinforces the need to better mimic human exposure, taking into account multiple factors when evaluating liver disease and pathology. This study also supports the need for further investigation into low-level toxicant exposure and its effect and interaction with the progression of NAFLD.

CHAPTER IV

VINYL CHLORIDE DYSREGULATES HEPATIC GLUCOSE METABOLISM AND ENHANCES OXIDATIVE AND ENDOPLASMIC RETICULUM STRESS

A. Introduction

Vinyl chloride (VC) is a known hepatotoxicant and carcinogen at high exposure levels. The majority of its damaging effects are attributable to the reactive metabolites that are formed upon VC metabolism by the liver. VC metabolism occurs strikingly similar to that of ethanol with the majority of its reactive intermediates being produced by and within hepatocytes themselves. Therefore it is likely that VC may act by many of the same mechanisms to both cause and enhance injury. It is important to consider that the liver may be uniquely susceptible to metabolic disruption and endogenous stress caused by VC exposure. In VC workers exposed to high concentrations, hepatic steatosis or fat accumulation is known to occur. Hsiao et al. performed an ultrasound study of PVC facility workers that were asymptomatic for liver disease. Intriguingly, 38.9% of the workers analyzed presented with hepatic steatosis even though they had a normal mean body mass index (78). This suggests that occupational exposures

to VC are sufficient to alter hepatic lipid metabolism. More recently, Guardiola et al., determined significant changes in the metabolome of highly exposed VC workers who did not develop cancer (79). This work identified alterations in key pathways that regulate energy homeostasis including the AMP-activated protein kinase (AMPK) and Protein kinase B (AKT) signaling pathways. Additionally, Anders et al., observed similar metabolic changes with the administration of a single dose of chloroethanol, CE, in a mouse model (26). The concomitant activation of both mTOR and AMPK, which are typically activated in opposition of one another was observed, indicating dysregulated energy metabolism. Supporting the changes in phosphorylation observed, were histological changes as well. Increased hepatic lipid accumulation accompanied by depleted glycogen suggest altered energy homeostasis in that model.

Importantly, metabolic stress can act in concert with other stressors to cause enhanced cellular damage and potentially cell death. Both oxidative and endoplasmic reticulum (ER) stress are known contributors to NAFLD pathogenesis (80,81,82). Indeed, the VC metabolite 2-chloroethylene oxide is known to cause oxidative DNA damage (12) and its reactive aldehyde species, chloroacetaldehyde (CAA) has been shown to cause protein adducts (26).

The purpose of this study was, therefore, to examine metabolic homeostasis at both early and later time points of low-level VC exposure both alone and in combination with a HFD to determine if disruption in nutrient homeostasis plays a role in VC-induced liver injury. Additionally, this study also sought to provide

mechanistic insight into the roles of ER stress and oxidative stress activation, as they are known pathogenic regulators of NAFLD progression.

Experimental Procedures

1. Animals and treatments

6 week old, male C57Bl/6J mice were exposed to sub-OSHA levels of VC for 6 hours per day, 5 days per week as described in Chapter II, Section A.1, and fed either a low fat diet (LFD) or high fat diet (HFD) as described in Chapter II, Section A.4. Animals were sacrificed at 6 and 12 weeks of exposure and blood and tissue were collected as specified in Chapter II, Section A.2. Whole animal glucose homeostasis was evaluated by OGTT, PTT, and ITT at 6 weeks of exposure as described in Chapter II, Section A.5.

2. Biochemical analyses

Hepatic lipids were extracted from snap frozen liver tissue as described in Chapter II, Section C.1 and levels of triglycerides, free fatty acids, and cholesterol were determined as described in Chapter II, Section C.1. Hepatic TBARS concentrations were measured as detailed in Chapter II, Section C.1.

3. Histology and electron microscopy

Histology for neutral lipids and glycogen accumulation was performed as described in Chapter II, Section B.7 and image analysis was performed as described in Chapter II, Section B.8. Immunohistochemistry for lipid peroxidation adducts (4-HNE and MDA) were performed as described in Chapter II, Section

B.3. Tissue processing and visualization via electron microscopy was performed as explained in Chapter II, Section H.

4. Immunoblots

Whole liver lysates were analyzed via Western blot for CHOP as described in Chapter II, Section G.

5. RNA and real-time RT-PCR

Hepatic mRNA was isolated from fresh tissue and converted to cDNA as described in Chapter II, Section F. Hepatic mRNA expression was then measured via real-time qRT-PCR as explained in Chapter II, Section F.

6. Statistical analyses

Results are reported as means ± standard error (SEM, n=4-12) and were analyzed using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA). Unless otherwise specified, one-way or two-way ANOVA with Bonferroni's post-hoc test (for parametric data) or Mann-Whitney Rank Sum test (for nonparametric data) were used for determination of statistical significance among treatment groups, as appropriate. A *p* value less than 0.05 was selected before the study as the level of significance. [a], $p<0.05$ compared to the absence of HFD; [b], $p<0.05$ compared to the absence of VC.

B. Results

1. Vinyl chloride causes enhanced steatosis in high fat diet-fed animals

Hepatic fat accumulation is a characteristic sign of liver disease and is typically the first pathological response to damage. In order to determine steatosis and assess lipid accumulation in this model, Oil Red-O (ORO) staining for neutral lipids (Figure 4.1A) and hepatic lipid analysis (Figure 4.1B) were performed for the 12 week time point. VC did not cause steatosis or alter hepatic lipid accumulation for triglycerides (TG), cholesterol (Chol), or free fatty acids (FFA) in the absence of HFD. HFD feeding significantly increased the amount of ORO positive staining compared to LFD control animals. Additionally, HFD animals had increased concentrations of hepatic TG, Chol, and FFA. HFD + VC exposed animals had significantly enhanced indices of macrovesicular steatosis such as increased ORO positive staining and TG levels. However, VC did not further increase the indices of microvesicular steatosis as shown by cholesterol and FFA concentrations. However, intriguingly, upon mRNA expression analysis of several genes involved in lipid metabolism, no alterations were observed for any of the treatment groups (Figure 4.2).

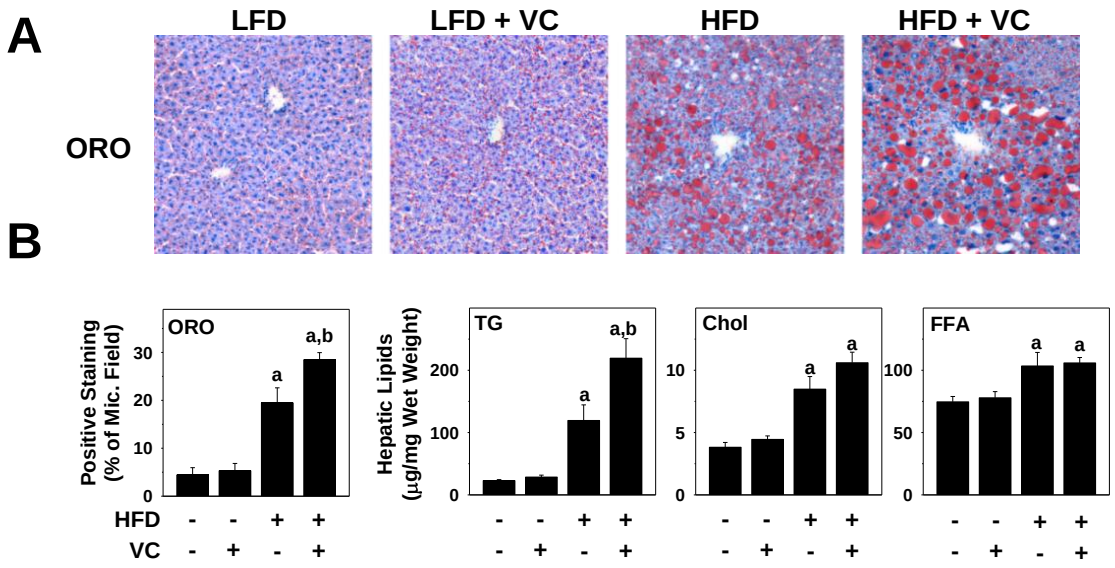


Figure 4.1. VC increased diet-induced steatosis. A: Representative photomicrographs of ORO (neutral lipids, 200x) are shown for the 12 week time point. B: Image analysis of ORO positive staining was performed and results are shown as percent of microscope field. Triglyceride (TG), cholesterol (Chol), and free fatty acid (FFA) levels were measured in hepatic lipid extracts for the 12 week time point.

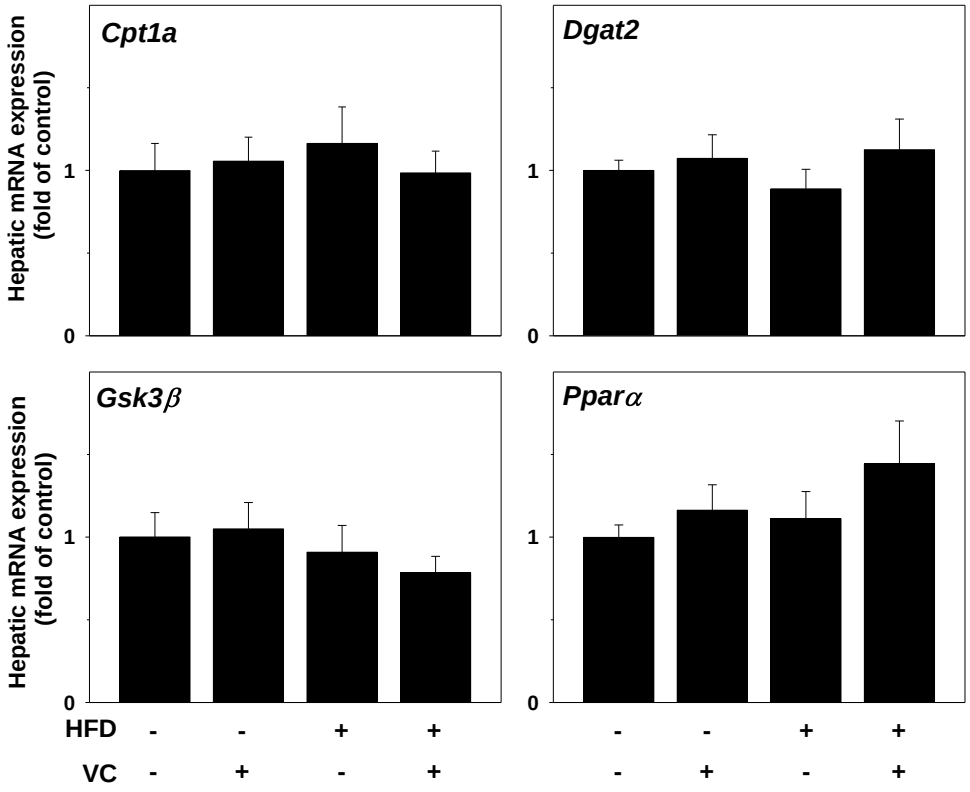


Figure 4.2. VC did not alter mRNA expression of metabolic genes. Hepatic mRNA expression of *Cpt1a1, Dgat2, Gsk3β,* and *Pparα* are represented as fold of control compared to LFD control animals at 12 weeks of exposure.

2. Vinyl chloride exposure is sufficient to alter hepatic glucose metabolism

The Beier laboratory has established that exposure to VC metabolites dysregulate carbohydrate metabolism in the absence of a secondary stimulus (26). Therefore, in this model, indices of both liver specific and whole-body glucose metabolism were analyzed. Hepatic glycogen deposition was assessed by Periodic Acid Schiff (PAS) staining at 12 weeks of exposure (Figure 4.3A). LFD control animals show typical glycogen levels. Interestingly, LFD + VC significantly decreased hepatic glycogen accumulation. A similar trend was observed for HFD ± VC. In order to further determine a mechanism for this decrease in glycogen, mRNA expression of several gluconeogenic regulators were examined at an earlier time point (6 weeks exposure, Figure 4.3B).

Phosphoenolpyruvate carboxykinase (*Pck1*), *Glucose-6-phosphotase* (*G6Pase*), and *Peroxisome proliferator –activated receptor gamma coactivator 1-alpha* (*Pgc1α*) are all critical genes involved in glucose homeostasis. G6Pase catalyzes the reaction that allows glucose to be exported into systemic circulation while PCK1 is responsible for the first committed step of gluconeogenesis, and it therefore crucial for glucose homeostasis (Scheme 4.1). PGC1α is a known positive regulator of gluconeogenesis (83). Notably, VC exposure increased both *Pck1* and *G6Pase* mRNA expression. Additionally, in line with the increased *Pck1* expression, VC significantly increased *Pgc1α* expression compared to LFD control animals. Although significant differences were observed

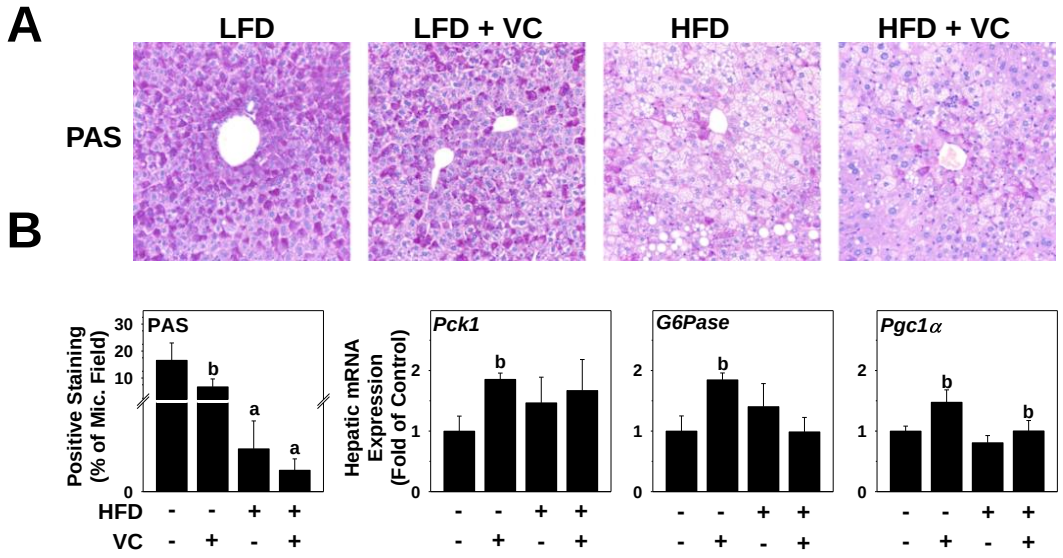
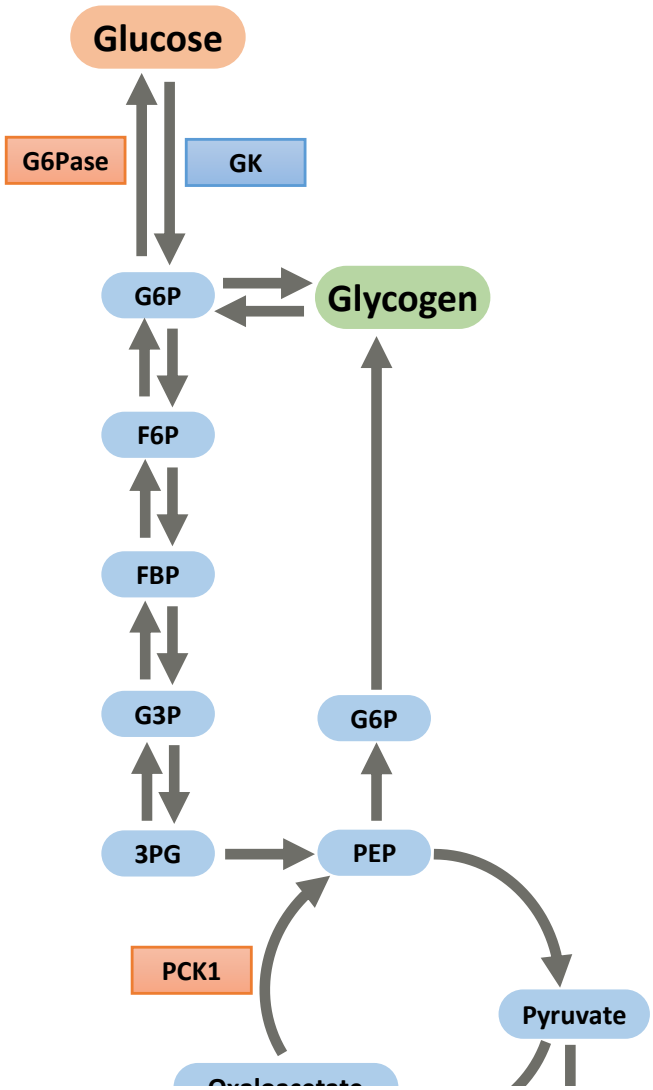


Figure 4.3. VC altered hepatic glucose metabolism. A: Representative photomicrographs for PAS staining are shown for the 12-week time-point (glycogen, 200x). B: Image analysis of PAS positive staining was performed and results are shown as percent of microscope field. Hepatic mRNA expression of *Pck1*, *G6Pase*, and *Pgc1α* are shown as fold change 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.



Scheme 4.1. Hepatic glucose metabolism. Changes in glucose metabolism are represented. Red denotes increased expression or abundance with VC, while green denotes decrease in product levels. Increased expression of glucose-6-phosphotase (G6Pase) and phosphoenolpyruvate kinase (Pck1) are observed in conjunction with decreased glycogen.

for LFD ± VC exposed animals, no significant effect was observed for HFD ± VC exposed animals.

Whole-animal glucose homeostasis was analyzed through oral glucose, insulin, and pyruvate tolerance tests at both 6 and 11 weeks of exposure in order to assess systemic glucose metabolism. Although no significant VC effect was observed at the later time point, differences were seen at 6 weeks (Figure 4.4A). LFD + VC animals did not differ in comparison to LFD control animals in either the OGTT or ITT analyses. However, VC significantly increased glucose levels during the PTT, indicating increased gluconeogenic activity. As expected, HFD significantly decreased glucose tolerance with higher blood glucose levels compared to LFD controls and VC enhanced this effect at the 15 minute time point, indicating more severe glucose intolerance. Additionally, plasma protein levels of several adipokines were analyzed via Luminex (Figure 4.3B) at 6 weeks of exposure. Interestingly, although differences in glucose tolerance were observed at 6 weeks of exposure, no alterations were observed for any of the adipokines examined. However, at 10 weeks of exposure, there are significant differences among the experimental groups. HFD significantly increases leptin and resistin levels compared to LFD controls, however, VC does not enhance this effect. Intriguingly, LFD + VC exposed animals have significantly increased insulin and resistin levels compared to LFD control animals.

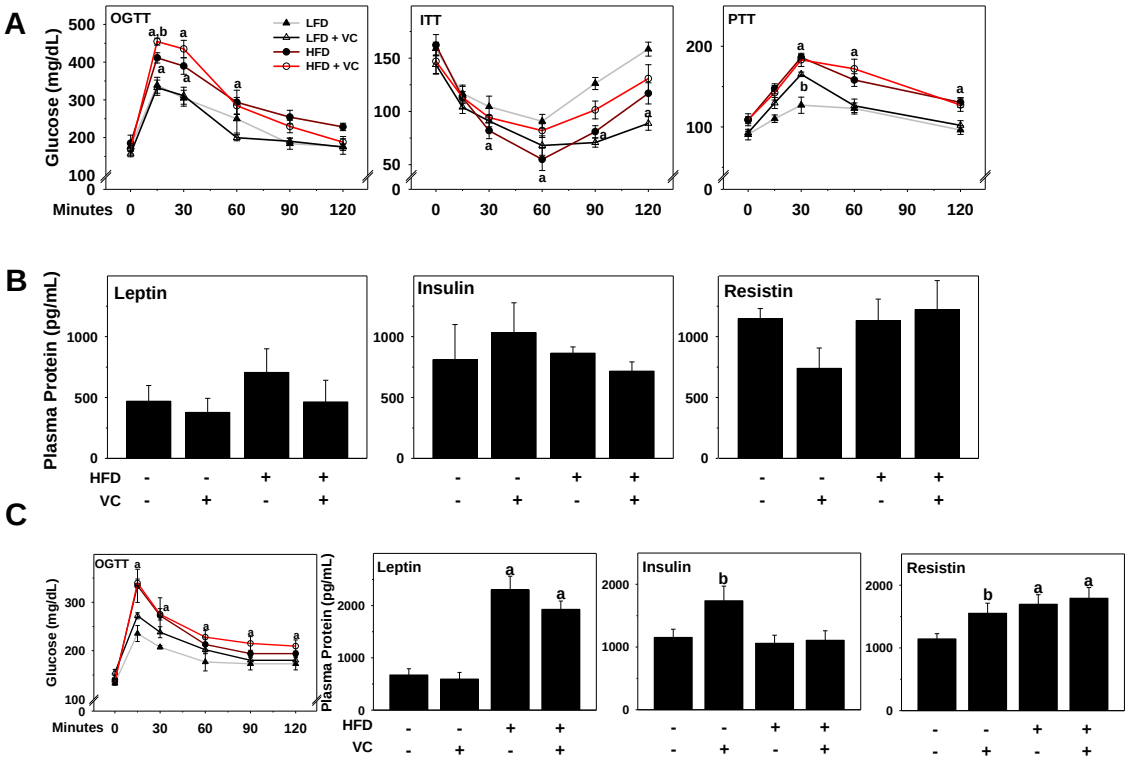


Figure 4.4. VC altered whole-animal glucose homeostasis. A: OGTT, ITT, and PTT were performed at 6 weeks of exposure. B: Plasma protein concentrations of adipokines leptin, insulin, and resistin were determined for the 6 week time point and are represented as pg/mL. C: OGTT was performed at 10 weeks of exposure and plasma protein concentrations of leptin, insulin, and resistin were measured at 12 weeks of exposure and are expressed in pg/mL. [a], $p<0.05$ compared to LFD control; [b], $p<0.05$ compared to absence of VC.

3. Vinyl chloride enhances NAFLD-induced oxidative stress

Oxidative stress plays a critical role in propagating inflammatory and stress responses in multiple disease states and it results from an imbalance of reactive oxygen and reactive nitrogen species (ROS/RNS) and the antioxidant capacity of the cell (84). Oxidative stress is also a known component of NAFLD pathology (85). Additionally, oxidative damage can occur upon covalent adduct formation on major macromolecules such as DNA, lipids, and proteins. Importantly, chloroacetaldehyde has been shown to cause lipid oxidation adducts *in vivo* (26). Here, several lipid peroxidation adduct products were examined immunohistochemically via 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) staining (Figure 4.5A,B). LFD control animals show typical positive staining for both 4-HNE and MDA staining, as some endogenous oxidative stress is present. Interestingly, LFD + VC exposed animals have significantly more positive 4-HNE staining than LFD controls (Figure 4.4A). As expected, HFD feeding increased both 4-HNE and MDA positive staining. VC significantly enhanced both 4-HNE and MDA positive staining compared to HFD control animals, suggesting an enhanced oxidative stress response.

To further evaluate the role of oxidative damage in this model, hepatic levels of TBARS were analyzed from liver tissue (Figure 4.5B). No differences were observed in LFD ± VC animals. HFD significantly increased the amount of TBARS present, however VC did not further enhance this effect.

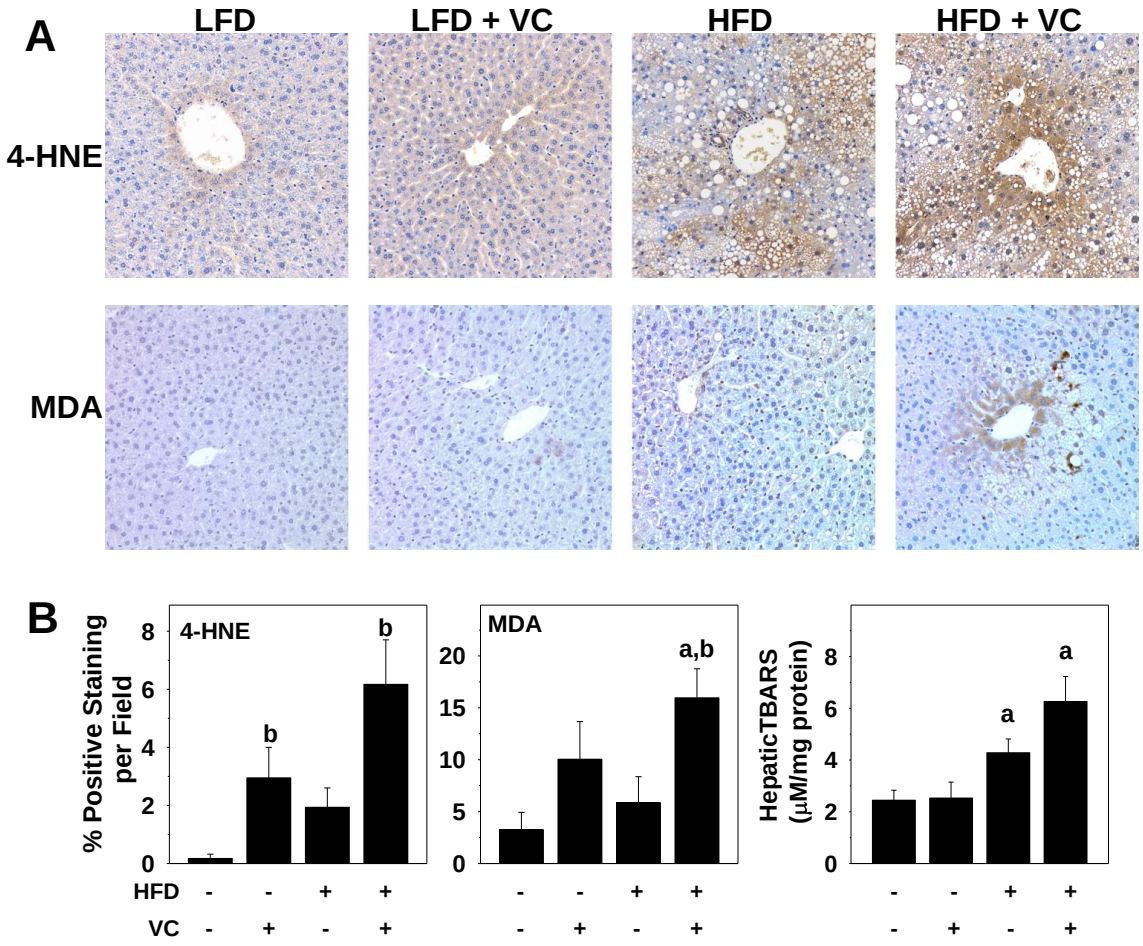


Figure 4.5. Vinyl chloride enhances NAFLD-induced oxidative stress. A: Representative photomicrographs for 4-HNE (oxidative stress, 200x), and MDA (oxidative stress, 200x), are shown at 12 weeks. B: Image analysis for 4-HNE and MDA are graphed as positive staining as percent of microscope field and hepatic TBARS concentration were measured and normalized to protein concentration. [a], $p<0.05$ compared to LFD control; [b], $p<0.05$ compared to absence of VC.

4. Vinyl chloride caused endoplasmic reticulum (ER) stress

The Beier laboratory has identified that ER stress is significantly enhanced by VC metabolite exposure both *in vitro* and *in vivo*. The ER plays a major role in managing intracellular protein homeostasis and its stress activation can cause initiation of inflammatory and apoptotic pathways. Here, several indices for ER stress activation were examined for the 12 week time point. Figure 4.6 depicts transmission electron microscopy images for each of the treatment groups. Both LFD and HFD control animals show normal ER morphology, with tight folds indicative of proper function. Notably, LFD + VC and HFD + VC animals show dilation of the ER lumen, which is indicative of ER stress and an accumulation of misfolded or adducted proteins. As part of the ER membrane, the nuclear membrane was also dilated in these groups, morphologically indicating the activation of the UPR.

Hepatic mRNA expression of several ER stress markers were analyzed for the HFD and HFD + VC experimental groups. *Sirtuin 1* has recently been identified as a negative regulator of ER stress (86). Indeed, here VC significantly decreased *Sirt1* expression compared to HFD controls (Figure 4.7A). Additionally, *Activating transcription factor 4* (*Atf4*), *CCAT-enhancer-binding protein homologous protein* (*Chop*), and *Heat shock protein 90* (*Hsp90*) mRNA expression were examined as markers of ER stress activation. HFD + VC significantly increased expression of all these indices examined. Protein levels of CHOP were analyzed for all experimental groups (Figure 4.7B). VC did not

change relative CHOP levels in the LFD group. Although HFD increased CHOP protein levels, VC did not significantly enhance this effect.

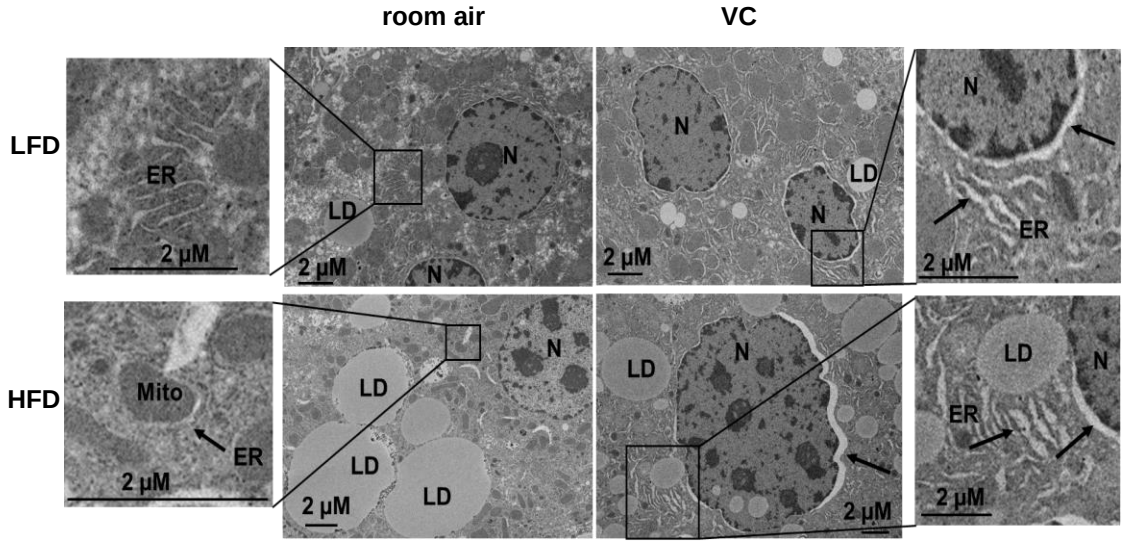


Figure 4.6. VC increased ER dilation independent of diet. Representative EM photomicrographs are shown for all experimental groups at 12 weeks of exposure. Arrows denote dilated ER, including the nuclear membrane as part of the ER membrane. LD = lipid droplet, ER = endoplasmic reticulum, Mito = mitochondria, N = nucleus.

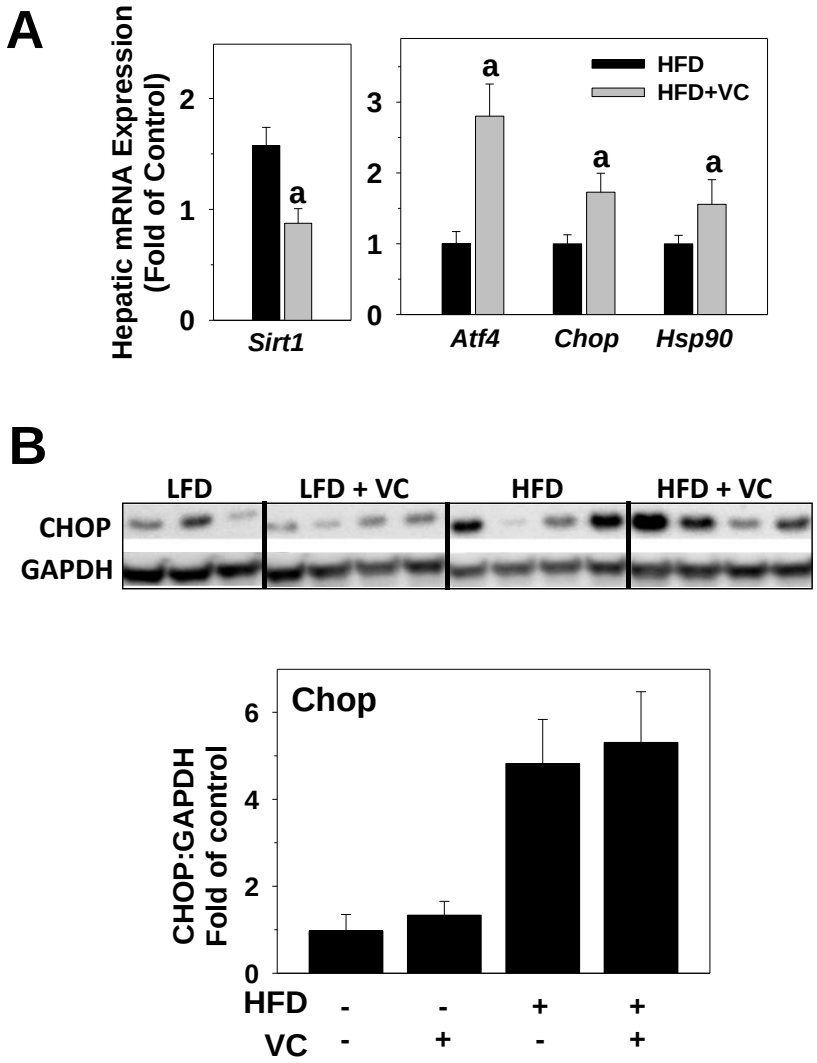


Figure 4.7. VC increased ER stress markers. A: Hepatic mRNA expression of ER stress markers *Sirt1*, *Atf4, Chop,* and *Hsp90* are shown for HFD and HFD + VC at 12 weeks of exposure and are expressed as fold of control compared to HFD control animals. B: Representative Western blot and densitometric analysis of CHOP are shown. [a], $p<0.05$ compared to HFD control.

C. Discussion

As stated in the Introduction, it is known that acute, occupational exposures to VC directly cause hepatic injury and TASH-associated pathologies (34). Additionally, people exposed to VOC (including VC) contaminated drinking water at the Camp Lejeune military base in North Carolina during the 1980's are still presenting with health complications, including liver disease, to this day (87). Therefore, it is imperative to understand the mechanisms by which VC acts to sensitize and injure hepatocytes, resulting in its associated malignant pathologies. Importantly, a hallmark of NAFLD is hepatic metabolic disruption and accumulation of lipids (88). Other mechanisms involved in the progression of NAFLD to NASH are oxidative and ER stress activation, which act to influence hepatocyte sensitivity and increase cellular injury (89;90).

Several VOCs are known to disrupt lipid metabolism and insulin signaling, in addition to inducing oxidative and ER stress activation, which contributes to their toxicity. For example, acrolein has been shown to form protein adducts and subsequent ER and oxidative stress activation (91;92). Additionally, TCE metabolites have been shown to cause lipid peroxidation adducts *in vivo* which produce oxidative damage (93). Therefore, the goal of this study was to determine if VC exposure induced hepatic metabolic changes to increase hepatocyte susceptibility to damage.

Here, VC exposure exacerbated HFD-induced macrovesicular steatosis and glycogen depletion at 12 weeks of exposure (Figure 4.1). It is known that accumulating lipids within hepatocytes can lead to lipotoxic effects (94-96).

Therefore, although no differences were observed between levels of hepatic free acids and cholesterol, it is probable that their accumulation is more susceptible to lipotoxic effects by becoming oxidized in the presence of VC exposure. Additionally, hepatocytes exposed to both HFD and VC may be more sensitive to these toxic lipid species and their ability to activate pro-apoptotic and stress pathways (97;98) which subsequently result in enhanced cellular injury.

Although significant alterations in histology were observed at 12 weeks of exposure, no associated change in mRNA expression in metabolic genes was observed for this time point. Therefore, an earlier exposure time point of 6 weeks was selected to provide mechanistic insight into the histological changes observed. This aim also sought to build upon previous work, which suggests that VC alone is enough to alter hepatic metabolic processes. Indeed, this work demonstrates that VC alone alters hepatic glucose metabolism (6 weeks) prior to the observation of enhanced hepatic oxidative and ER stress at 12 weeks of exposure. Several key regulators of the gluconeogenic pathway had significantly increased hepatic mRNA expression with VC alone compared to LFD control animals. One such regulator is *Pgc1α*, a transcription factor that positively regulates gluconeogenesis (99). Here, independent of diet, a VC effect is observed with increased *Pgc1α* mRNA expression. This suggests that an adaptive response has been activated in order for the hepatocytes to maintain both glucose and cellular energy levels (100). VC exposure causes a depletion in glucose levels which prompts the hepatocytes to upregulate gluconeogenic pathways, simultaneously depleting glycogen reserves. Additional support for this

hypothesis can be found from the pyruvate tolerance test (PTT) data at 6 weeks of exposure (Figure 4.4). The PTT can be used as an index of gluconeogenic capacity by the administration of pyruvate, a substrate for glucose production and subsequently measuring blood glucose levels. Although no effect was observed for animals fed a HFD, a significant peak in glucose levels occurred in animals exposed to LFD and VC after pyruvate challenge compared to controls, physiologically indicating enhanced gluconeogenic flux. These data may aid in the explanation of the increased insulin level observed in LFD + VC exposed animals at 12 weeks of exposure. The increased capacity for glucose production acts to combat the energy needs of the cell, however, it also increases circulating glucose levels (101;102). This may consequently aid in developing insulin resistance as seen through the increased insulin and resistin levels.

Oxidative and ER stress are often coupled events and part of a 'vicious cycle' hypothesized to propagate fatty liver diseases (103-105). Here, VC significantly enhanced the accumulation of lipid peroxidation products and oxidatively damage proteins caused by HFD feeding as seen by 4-HNE and MDA immunohistochemistry (Figure 4.5). Interestingly, LFD + VC exposed mice did show significantly more 4-HNE adducts when compared to LFD control animals. This indicates that VC is in fact sufficient to produce biomolecular damage without the addition of a secondary stimulus, however, with the addition of HFD, an enhanced effect is observed with increased accumulation over either factor alone. 4-HNE is an extremely toxic reactive aldehyde species with high intracellular levels causing irreversible biomolecule damage and cell death

(106;107). A diet effect was observed on levels of TBARS analyzed from hepatic tissue, although VC did not enhance this effect. However, TBARS is a notoriously non-specific assay due to MDA's instability and high reactivity with proteins (108;109), however is still a major index used to quantify the oxidative adduct, MDA. Therefore, it is possible that the results observed reflect this trait. Further, the VC-enhanced oxidative adducts were accompanied by concomitant enhancement of UPR activation and ER stress. Indeed, increased expression of ER stress markers were observed with the combination of HFD and VC and, notably, ER dilation was observed morphologically via electron microscopy for both VC exposed experimental groups (Figure 4.6). These data suggest that VC-induced metabolic dyshomeostasis plays a significant role in hepatocyte sensitization to further damage, such as oxidative stress. The metabolically damaged hepatocytes are unable to recover from oxidative damage and this incites the UPR and cell death pathways (Aim 1).

Taken together, the data from this aim describe a possible mechanism by which VC exposure enhances liver injury. Sub-OSHA concentrations of VC exposure alone cause alterations in hepatic glucose metabolism, which then render the cells to be susceptible to injury from both chronic exposure to VC and additive damage caused by a HFD. This was observed by increases in lipid peroxidation products and ER stress responses. This study revealed a novel impact of VC exposure on pathways known to be involved in NAFLD pathogenesis.

CHAPTER V

DETERMINING THE MECHANISM OF VINYL CHLORIDE INDUCED MITOCHONDRIAL DYSFUNCTION

A. Introduction

Mitochondria are essential for the function and health of all cell types. Mitochondria respond dynamically to changes in nutrient availability and a variety of stress signals. Their morphology and function varies depending on cell type and even can vary within the same cell (110). Mitochondrial dysfunction is involved in and also caused by many diseases. A hallmark of NAFLD pathogenesis is mitochondrial dysfunction (111). It is known that consuming a diet high in fat causes an abnormal increase in free fatty acids and triglycerides, affecting β-oxidation by mitochondria and impacting whole cell nutrient status. This abnormal influx of free fatty acids and triglycerides causes a metabolic shift within hepatocytes in which mitochondria increase their fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS) capacities as a compensatory mechanism (112). Mitochondrial abnormalities have been demonstrated in human populations presenting with NASH as evidenced by altered mitochondrial

structure and decreased ATP production (113;114). Further, Einer et al., have recently shown that both mitochondrial morphology and function are altered by a high fat diet in a mouse model (115).

Mitochondria are a significant source of endogenous ROS as electron leak occurs during normal oxidative respiration (116;117). However, as a consequence of mediating the altered nutrient flux, there is often an increase in mitochondrial electron leakage. In NAFLD, mitochondrial derived overproduction of reactive oxygen species contributes significantly to the oxidative damage observed and progression of injury (118).

One of the major hypotheses of the Beier laboratory is that VC and its reactive metabolites directly cause mitochondrial damage, impairing cellular energy homeostasis and sensitizing hepatocytes to other stressors. Indeed, the Beier lab has demonstrated that the VC metabolite, CAA, causes mitochondrial dysfunction prior to cell death processes being activated. Additionally, data from *in vivo* studies with CE demonstrate that hepatocytes are dying primarily via necrosis and not by the ATP-demanding apoptotic pathway. Independent of diet-induced alterations, several environmental pollutants have also been identified as mitochondrial poisons such as dioxin (119) and perfluorooctanoate (120-122).

Here, the major focus of this study was to determine if direct exposure to VC inhalation at sub-OSHA concentrations could incite mitochondrial dysfunction and to determine the mechanism by which this occurs.

B. Experimental Procedures

1. Animals and treatments

6 week old, male C57Bl/6J mice were exposed to sub-OSHA concentrations of VC via inhalation or room air in inhalation chambers for 6 hours per day, 5 days per week, for up to 12 weeks as described in Chapter II, Section A.1. Mice were fed either a low fat diet (LFD) or high fat diet (HFD) as detailed in Chapter II, Section A.4. Animals were sacrificed at either 6 or 12 weeks and tissues were collected as described in Chapter II, Section A.2.

2. Mitochondrial isolation

Hepatic mitochondria were isolated by differential centrifugation as described in Chapter II, Section A.5. Mitochondrial protein concentration was determined via Lowry Assay using bovine serum albumin as the standard control.

3. Seahorse analysis

Seahorse bioenergetic flux analysis was performed on isolated hepatic mitochondria as described in Chapter II, Section A.5.

4. Biochemical analyses

Plasma levels of lactate and β-hydroxybutyrate were determined using commercially available kits as described in Chapter II, Section C.1.

5. RNA isolation and real time qRT-PCR

Hepatic mRNA was isolated and quantified as described in Chapter II, Section F. Real time-qRTPCR was performed for *Tfam, Fis1, Drp1,* and *Cox4* as described in Chapter II, Section F.

6. Immunoblots

Whole liver and mitochondrial lysates were isolated and quantified as described in Chapter II, Section G. Western blots for OXPHOS complexes, phospho-Stat3, total Stat3, IP3-R1, and GAPDH were performed as detailed in Chapter II, Section G.

7. Quantitative cell analysis

Hepatocytes were isolated and plated as described in Chapter II, Section D. Quantitative cell analysis was performed as detailed in Chapter II, Section E.

8. Statistical analysis

Results are reported as means ± standard error (SEM, n=4-12) and were analyzed using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA). Unless otherwise specified, one-way or two-way ANOVA with Bonferroni's post-hoc test (for parametric data) or Mann-Whitney Rank Sum test (for nonparametric data) were used for determination of statistical significance among treatment groups, as appropriate. A *p* value less than 0.05 was selected before the study and the level of significance. [a], $p<0.05$ compared to the absence of HFD; [b], $p<0.05$ compared to the absence of VC.

C. Results

1. VC exposure's temporal effect on mitochondrial gene expression

In order to determine if VC exposure affected mitochondrial gene expression, several markers of mitochondrial biogenesis were examined via hepatic mRNA analysis as seen in Figure 5.1. *Mitochondria transcription factor A* (*Tfam*), *mitochondrial fission protein 1* (*Fis1*), *dynamin-related protein-1* (*Drp1*), and *cytochrome c oxidase 4* (*Cox4*) are all critically involved in mitochondrial dynamics. No alterations were observed for any of the experimental groups in *Cox4* or *Dmn1* expression at any time point. Interestingly, decreases were observed for *Tfam* and *Fis1* expression in animals that were exposed to HFD and VC at 6 weeks of exposure (Figure 5.1A). However, these changes were no longer observed at the 12 week time point (Figure 5.1B), which may indicate that key changes in mRNA expression are mediated early in chronic exposure, versus later time points.

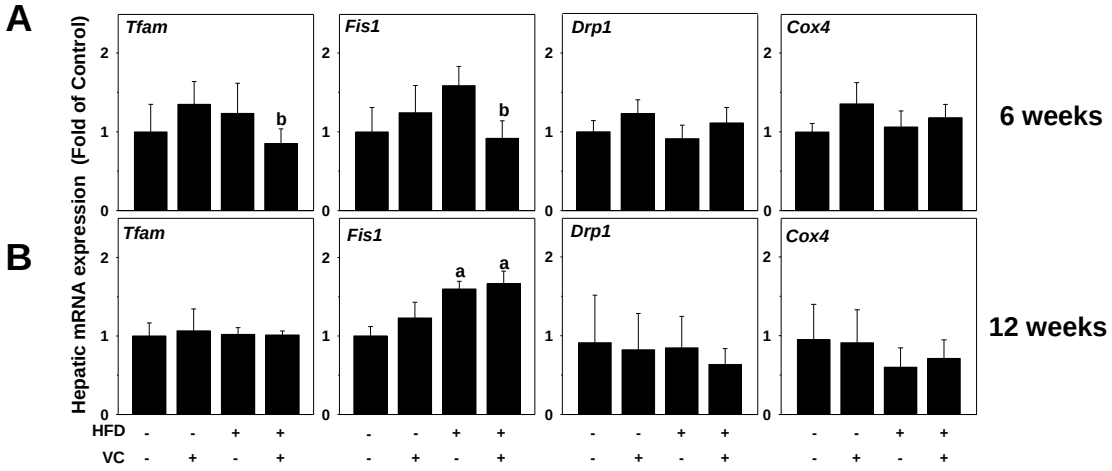


Figure 5.1. Effect of VC on mitochondrial gene expression. A: Hepatic mRNA expression of *Tfam, Fis1, Drp1,* and *Cox4* are shown as fold of control compared to LFD control animals at the 6 week time point. B: Hepatic mRNA expression of *Tfam, Fis1, Drp1,* and *Cox4* are shown as fold of control compared to LFD control animals at the 12 week time point. [a], $p<0.05$ compared to LFD control; [b], $p<0.05$ compared to absence of VC.

2. VC decreases mitochondrial protein abundance

In order to determine if mitochondrial protein levels were altered in this model, mitochondrial protein was analyzed by Lowry protein assay from mitochondria isolated from mice exposed to VC with or without HFD for 6 and 12 weeks of exposure (Figure 5.2). At the 6 week time point, there is a significant diet effect, with HFD fed animals having more mitochondrial protein compared to their LFD control counterparts, an effect that has also been observed by Turner et al., (123). However, at the 12 week time point, although there was still a significant diet effect, VC significantly decreased mitochondrial protein concentrations. Possibly indicating that the mitochondrial damage has become too severe, and/or a distinct change has occurred towards an adaptive mechanism.

Figure 5.2. Effect of VC on mitochondrial protein abundance. Mitochondrial protein concentration was determined via Lowry assay for mitochondria isolated from both the 6 and 12 weeks of exposure and is represented as mg/mL.

3. VC decreases protein levels of ETC associated complexes independent of HFD feeding

Since no increase in apoptosis in the hepatocyte population was observed in spite of increased caspase-3 cleavage (Chapter III), we hypothesize that VC may be impacting cells at the level of energy homeostasis. Indeed, previous data support this hypothesis. Anders et al., has demonstrated the CAA can cause mitochondrial damage and consequential ATP depletion (26). Therefore, in order to determine if VC exposure decreases protein levels of components of oxidative phosphorylation (OXPHOS), mitochondrial protein isolated from mice exposed to VC ± HFD for 6 weeks were analyzed via Western blot. Samples were blotted and probed for all five complexes of the electron transport chain (ETC) and quantified with total protein (Figure 5.3). Importantly, VC significantly decreased protein levels of Complex I, Complex III, Complex IV, and Complex V in the LFD group. This indicates that low-level VC exposure is capable of affecting oxidative phosphorylation by decreasing protein levels of select complexes. Interestingly, HFD alone decreases protein levels of Complex III and Complex V and the addition of VC paradoxically increased protein of these complexes in this group.

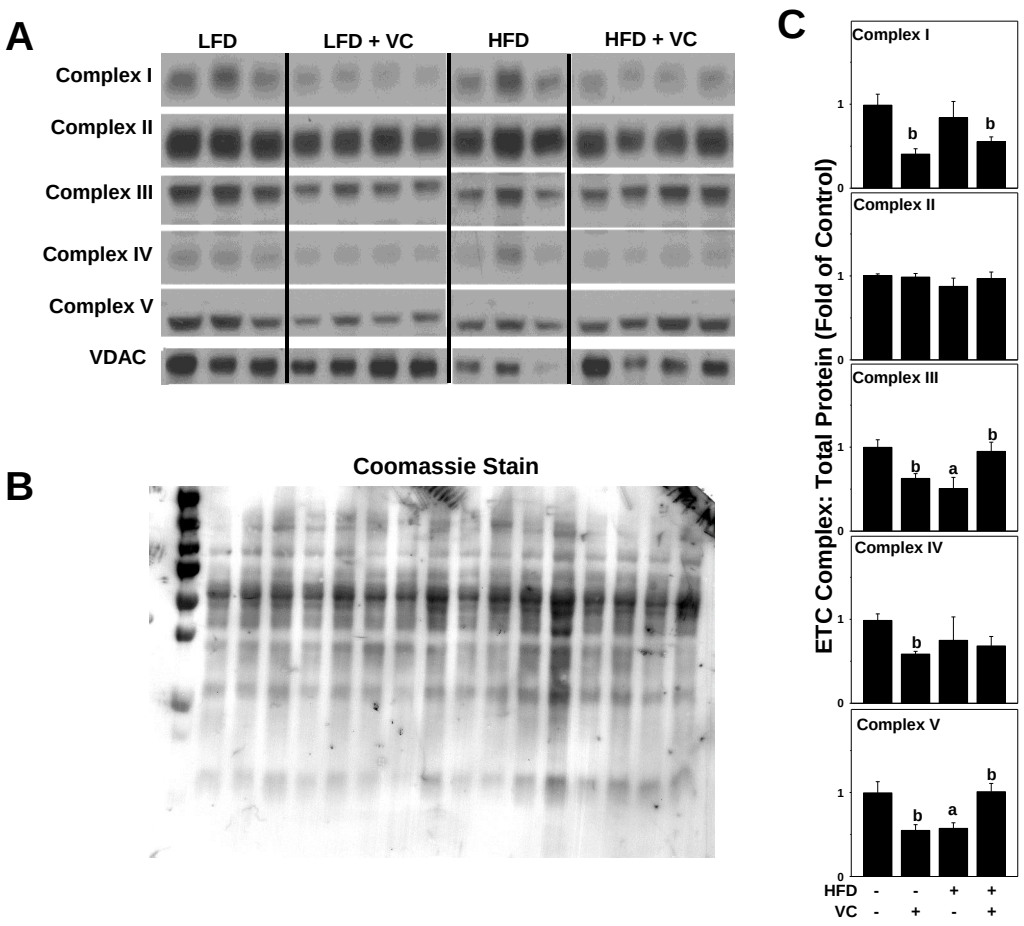


Figure 5.3. VC decreased OXPHOS protein levels independent of HFD.

A: Representative Western blots are shown for components of the ETC from mitochondrial isolates at 6 weeks of exposure. B: Coomassie stain for total protein is shown for the 6 week time point. C: Densitometric analysis for each complex is shown as fold of control compared to LFD control samples. [a], $p<0.05$ compared to LFD control; [b], $p<0.05$ compared to absence of VC.

4. VC's effect on mitochondrial respiration capacity

As previously stated, mitochondrial function is indicative of not only whole cell energy homeostasis, but also of altered mitochondrial respiration. Moreover, oxidative capacity is a hallmark of several liver diseases. Previous work from this group has demonstrated that VC metabolites decrease both oxygen consumption and maximum mitochondrial capacity in primary hepatocytes (26). Therefore, the effects of VC inhalation on mitochondrial function were examined in this model. Seahorse bioenergetic analysis was performed on isolated hepatic mitochondria from mice from all treatment groups at 6 weeks of exposure. Figure 5.4 depicts the analysis of Complex I, Complex II, and Complex V activity, in addition to maximum mitochondrial respiratory capacity. Importantly, independent of diet, VC exposure significantly decreased all of these indices. This indicates that VC directly impairs hepatic mitochondrial ETC function and respiratory capacity. Similar to previous findings (123), mitochondria isolated from HFD fed animals had higher respiration over their LFD counterparts. Importantly, VC still impaired respiration in this group.

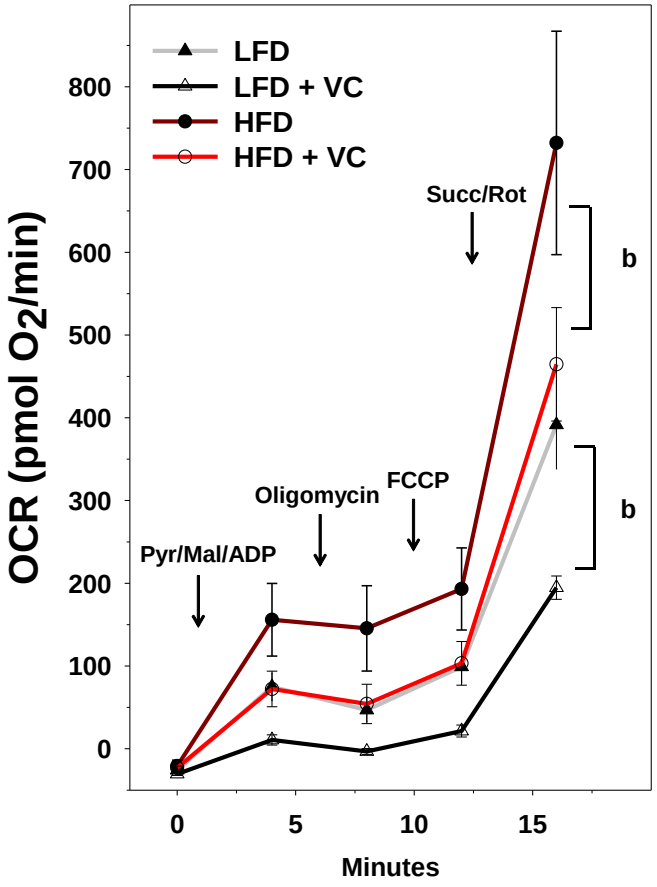


Figure 5.4. VC decreased mitochondrial respiration. Seahorse analysis of isolated hepatic mitochondria at 6 weeks of exposure and measurements of oxygen consumption are shown. Pyruvate/malate/ADP was administered as a Complex I substrate, oligomycin was administered as a Complex IV inhibitor, FCCP was a mitochondrial uncoupler, and succinate/rotenone was administered as Complex II substrates.

5. Potential role of phospho-Stat3 and calcium homeostasis

Mitochondria are dynamic organelles which actively communicate with other membrane bound organelles and the nucleus to coordinate cellular homeostasis (124). To this end, several signaling pathways are known to be modified by alterations in mitochondrial respiration. One such pathway is the signal transducer and activator of transcription 3 (STAT3) pathway. STAT3 phosphorylation at Serine-727 has been shown to non-transcriptionally regulate the electron transport chain to increase its activity (125). Here, we evaluated STAT3 phosphorylation both for the canonical Tyrosine-705 phosphorylation and Serine-727 phosphorylation via Western blot (Figure 5.5A). LFD + VC animals show no changes when compared to control animals at the Tyr-705 residue. A diet-effect was observed for P-STAT3-705 phosphorylation as seen as a decrease in activity, with no VC effect in this group. Upon analyzing Ser-727 protein levels, LFD + VC did not have a significant effect, although a p-value of 0.07 suggests that this pathway may be activated. A significant increase is observed with HFD feeding, however there is no VC effect for this group.

In addition to alterations in signaling cascades, mitochondrial dysfunction is also associated with aberrant intracellular calcium levels (126;127). Therefore, a key mediator of calcium release from the endoplasmic reticulum, IP3-R1, was examined via Western blot for whole liver lysates at 6 weeks of exposure (Figure 5.5B). LFD + VC exposed animals have significantly increased levels of IP3-R1 protein. HFD alone also caused an increase in IP3-R1 protein, while VC does not alter this effect in the HFD group.

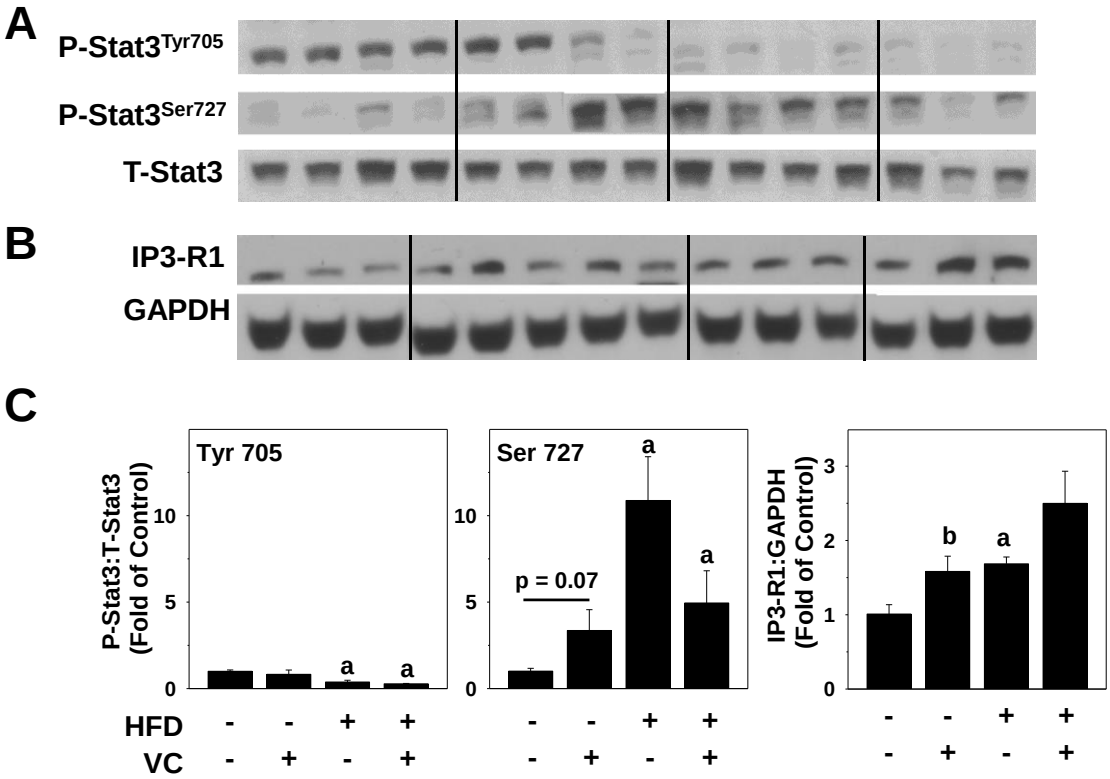


Figure 5.5. Effect of VC on Stat3 phosphorylation and calcium homeostasis. A: Representative Western blot for phospho-STAT3 (Tyr-705 and Ser-727) and total STAT3 are shown for the 6 week time point. B: Representative Western blot for IP3-R1 are shown for the 6 week time point. C: Densitometric analysis was performed and results are presented as fold of control compared to LFD control animals. [a], $p<0.05$ compared to LFD control; [b], $p<0.05$ compared to absence of VC.

6. VC's effect on lactate and ketone body production

Due to the observed changes in mitochondrial respiratory capacity, indices of both increased glycolysis (lactate, Figure 5.6A) and ketone body production (β-hydroxybutyrate, Figure 5.6B) were assessed to give additional insight into the mechanism of mitochondrial injury. Here, the 12 week time point was used for measurements, as this is when the pathological indices were greatest. Plasma lactate levels were analyzed as an index of glycolysis. VC significantly enhanced lactate levels in the LFD group, while the effect of HFD was not significant. β-hydroxybutyrate levels were assessed. No differences were observed for LFD fed animals with or without VC exposure. HFD did not significantly increase β-hydroxybutyrate levels, although HFD + VC did have a significant effect.

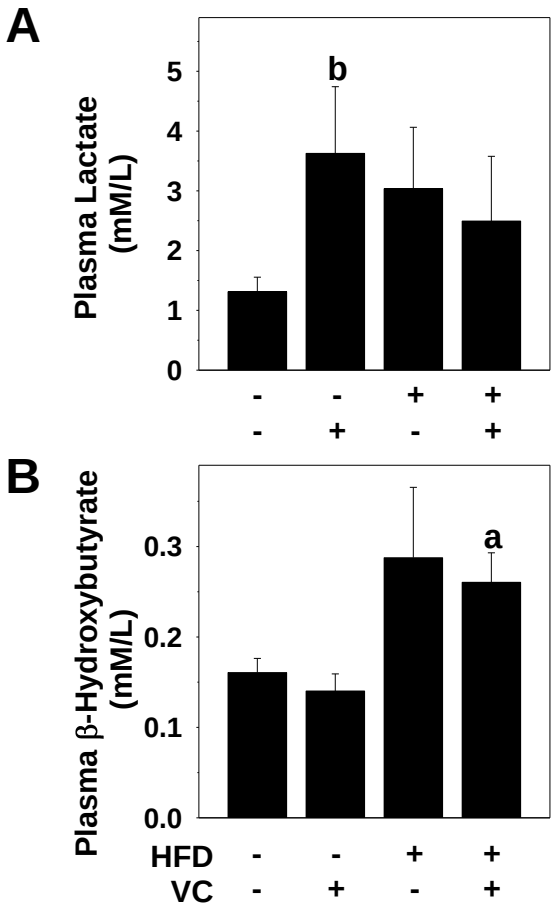


Figure 5.6. Effect of VC on lactate and ketone body production. A: Lactate levels were determined from plasma samples at 12 weeks of exposure and are represented as mM/L. B: Plasma β-hydroxybutyrate concentrations were determined in plasma as an index for ketone bodies for the 12-week time-point and results are represented in mM/L. [a], $p<0.05$ compared to LFD control; [b], $p<0.05$ compared to absence of VC.

7. VC sensitizes hepatocytes

In order to further investigate the effects of VC exposure on mitochondria in this model, primary hepatocytes were isolated from animals from each treatment group after 6 weeks of exposure and analyzed via Cellomics *ex vivo* with and without the additional challenge of CAA. Figure 5.7 depicts representative photomicrographs from animals exposed to LFD ± VC with and without CAA challenge. TOTO-3 was used as a marker of cell death and TMRM was used as a marker of mitochondrial membrane potential, in which a decrease is indicative of mitochondrial damage. Figure 5.8 displays the cell-by-cell analysis and quantification that was performed. Notably, LFD + VC exposed hepatocytes display increased mortality at baseline, with 41.7% of cell being TOTO-3 positive compared to 21.2% in LFD control hepatocytes. Indeed, upon the addition of CAA, VC further increases TOTO-3 positivity. Importantly, in hepatocytes administered 400μM CAA, VC increases the number of TMRM negative cells from 4.9% in LFD controls to 6.9%, indicating increased mitochondrial injury.

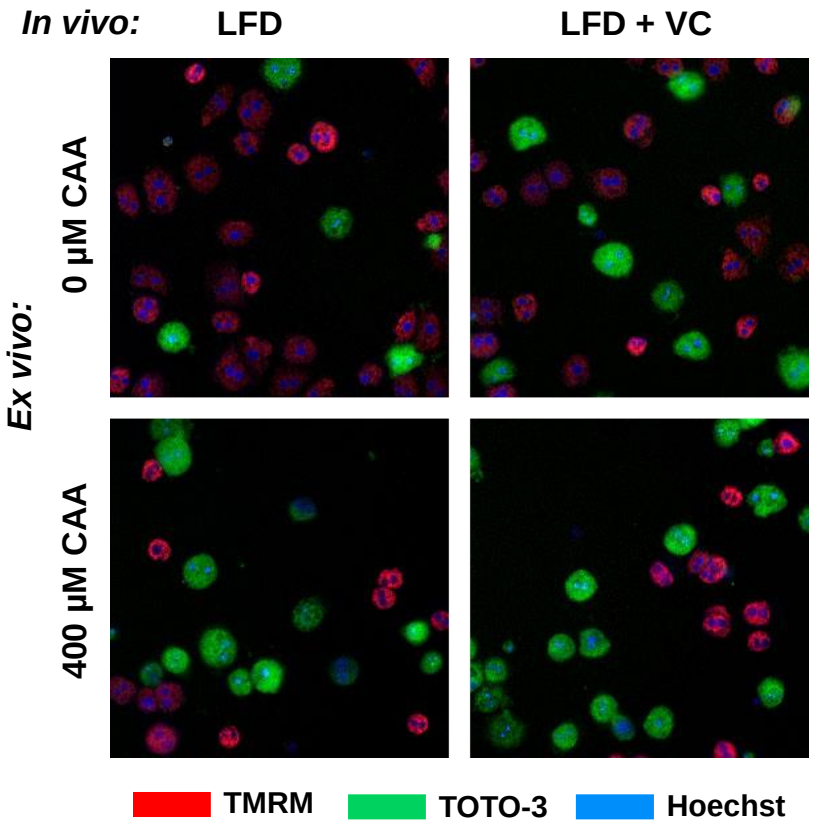


Figure 5.7. VC sensitized hepatocytes to cell death. Representative photomicrographs are shown for hepatocytes isolated from animals exposed to LFD ± VC at 6 weeks of exposure. TMRM (mitochondrial membrane potential), TOTO-3 (cell death), and Hoechst (nuclear) fluorescent staining are shown with and without CAA challenge.

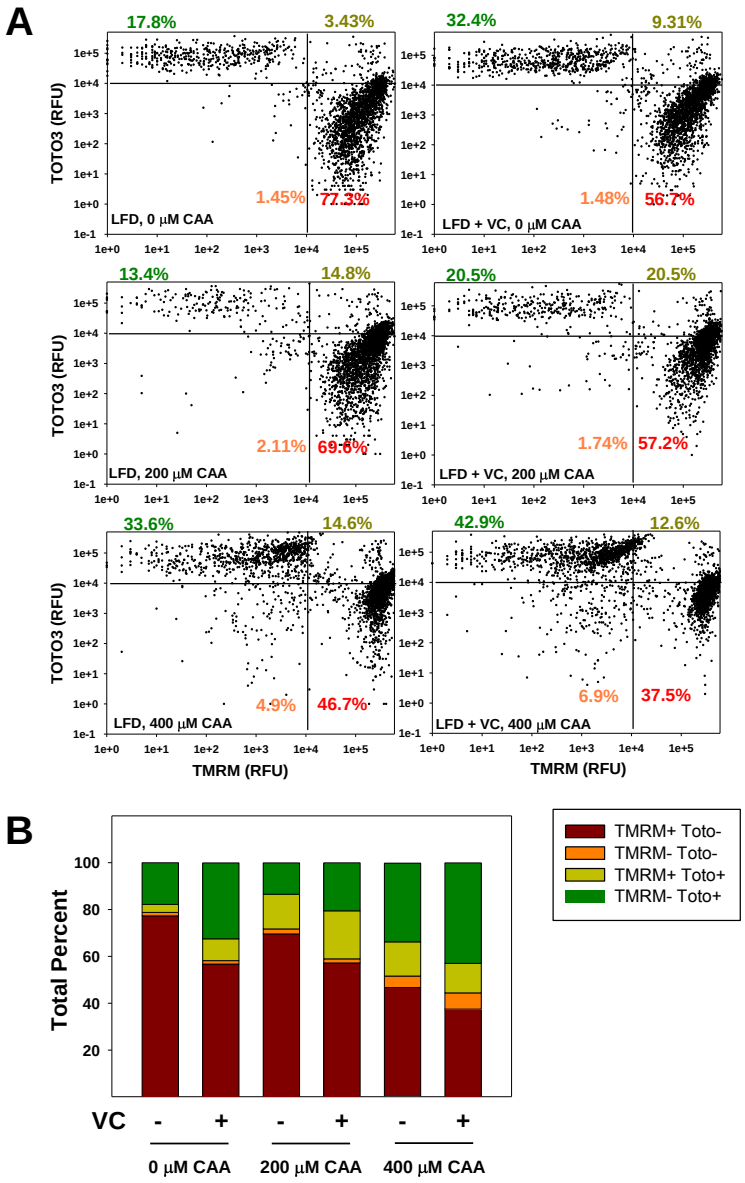


Figure 5.8. VC's effect on cell death. A: 2D scatter plots are shown for LFD±VC±CAA in which TMRM is depicted as a function of TOTO-3. Thresholds were calculated for positive and negative fluorescence of each marker. B: Relative percentage of cells in each quadrant are shown.

Similarly, hepatocytes from animals exposed to HFD ± VC were also analyzed. Figure 5.9A shows representative photographs and Figure 5.9B depicts the corresponding cell-by-cell analysis performed. HFD does increase cell death at baseline compared to LFD controls with 45.1% showing TOTO-3 positivity. However, no significant VC effect was observed in these groups, possibly due to HFD-induced sensitivity which may mask a VC effect.

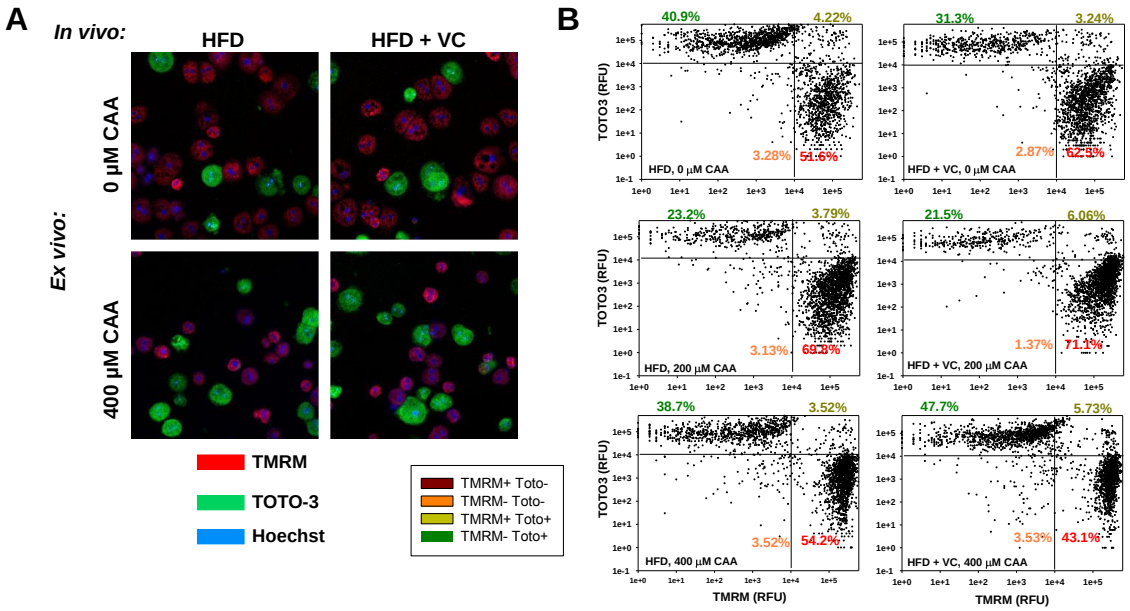


Figure 5.9. Effect of VC on hepatocyte viability with HFD. A: Representative photomicrographs are shown for hepatocytes isolated from animals exposed to HFD ± VC at 6 weeks of exposure. TMRM (mitochondrial membrane potential), TOTO-3 (cell death), and Hoechst (nuclear) fluorescent staining are shown with and without CAA challenge. B. 2D scatter plots are shown for HFD±VC±CAA exposed hepatocytes in which TMRM is depicted as a function of TOTO-3. Thresholds were calculated for positive and negative fluorescence of each marker and relative percentages are shown in each quadrant.

D. Discussion

Mitochondria are dynamic organelles that are constantly responding to their environment to promote cell survival and nutrient homeostasis. Indeed, mitochondrial function and morphology are known to be altered both in experimental NAFLD and in humans presenting with NAFLD and NASH (128;129). Control of mitochondrial biogenesis, fission and fusion, β-oxidation, and respiratory function are complex pathways that play key roles maintaining mitochondrial integrity. However, this is still an area that is actively being investigated, as these pathways have not been fully elucidated. In addition to diet-induced mitochondrial dysfunction, environmental toxicants have been found to negatively affect mitochondrial function by several mechanisms. For example, the VOC acrolein has been shown to decrease mitochondrial membrane potential and decrease ETC activity (91;130). Moreover, a recent study performed by Dezest et al., has shown that low-level exposure to a VOC mixture caused mitochondrial damage in keratinocytes (131). Further, the Beier lab has recently demonstrated the mitochondrial toxicity of the VC metabolite, CAA in hepatocytes (26). CAA has also been shown to be toxic to mitochondria in the context of kidney injury (132;133). However, the effects of direct exposure to VC inhalation at low concentrations on mitochondrial integrity and function have not been studied and were therefore the goal of this study.

The experiments conducted in this study analyzed several aspects of mitochondrial health and function and yielded that low-level VC exposure does indeed impact hepatocytes at the level of the mitochondria. First, the effects of

mitochondrial biogenesis was examined by analyzing mRNA expression of *Tfam*. Tfam is critical for mitochondrial biogenesis and maintaining the stability and transcription of mitochondrial DNA (134). Here, a decrease in *Tfam* was observed only in animals exposed to HFD and VC at 6 weeks, however, these changes were no longer seen at 12 weeks of exposure. Although observed earlier during the exposure, the decrease in this important regulator could indicate that the combination of VC and HFD has detrimental effects on mitochondrial biogenesis, which may lead to later injury observed in this model. Secondly, the balance of mitochondrial fission to fusion, that is the joining or separation of the organelle, is imperative for regulating mitochondrial bioenergetics and removing damage during periods of internal stress (135;136). To this point, increased fission, or the separation of mitochondria, has been associated with increased oxidative damage (137). Here, although a decrease in *Fis1* mRNA expression is observed at 6 week of exposure in HFD + VC animals, there is a diet-dependent effect for an increase in *Fis1* at 12 weeks of exposure. Although alterations were observed, future studies are needed to determine the exact role of these dynamics in this model.

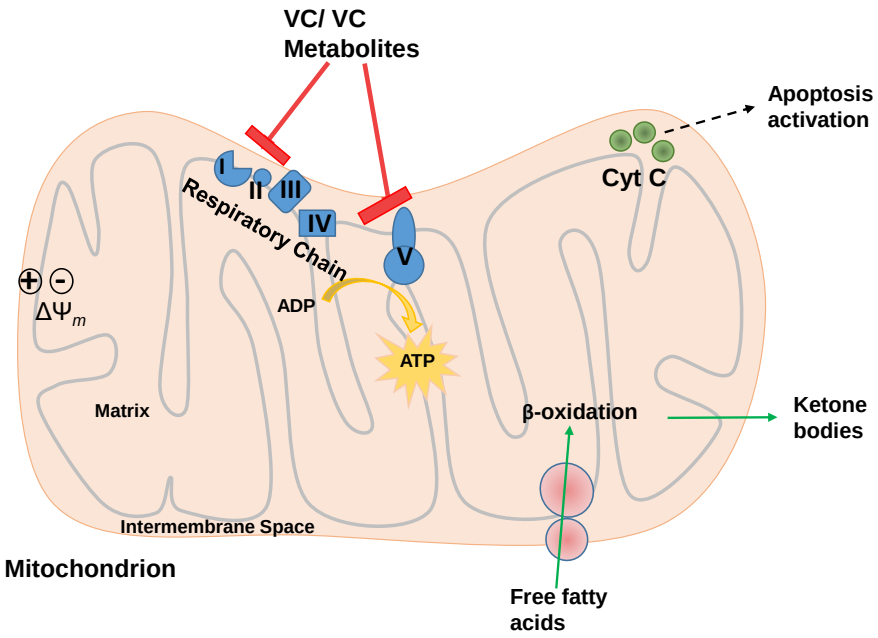
Mitochondria are also the site of fatty acid β-oxidative, which is the breakdown of fats to provide reducing equivalents necessary for oxidative phosphorylation (138). Here, ketone body production was measured as an index of β-oxidation capacity. During fasting conditions, β-oxidative capacity increases and the surplus of acetyl-CoA produced cannot be utilized by the TCA, so it is converted to ketone bodies, such as β-hydroxybutyrate (139). Indeed, here, the

animals were subjected to fasting and increased β-hydroxybutyrate levels were observed in HFD fed animals. However, because no deficits are observed in animals exposed to HFD + VC compared to HFD controls, it suggests that this mitochondrial process remains intact and functional (Scheme 5.1).

Perhaps the most critical function of mitochondria is their production of ATP via the electron transport chain (ETC) and oxidative phosphorylation. Here, several aspects of mitochondrial respiration and ETC function were assessed in the context of low-level VC exposure. Indeed, upon analysis of protein levels of ETC complexes, VC significantly decreased several complexes compared to LFD control animals. Additionally, VC significantly depressed mitochondrial respiration at the functional level as observed from the Seahorse analysis. HFD, however, decreased protein levels of certain complexes, an effect which has also been observed by Aharoni et al. (140). Paradoxically, the addition of VC in this group significantly enhanced protein levels of complexes III and V, which could be indicative of a compensatory mechanism. Importantly, despite the increased protein levels observed, HFD and VC exposed animals still had significant deficits upon functional analysis. This suggests an even stronger net deficit in this group. Further, increases in plasma lactate levels support the hypothesis that VC-exposed hepatocytes have increased glycolytic flux to compensate for the deficits in oxidative phosphorylation production of ATP. Collectively, these data suggest that VC exposed hepatocytes have decreased mitochondrial respiratory capacity and this has direct consequences on protein levels observed. Additionally, VC exposed hepatocytes were shown to be more sensitive to cell

death and mitochondrial damage as observed *ex vivo* in this study. This suggests that VC is sufficient to alter hepatocyte response to additional stressors, as they are more susceptible to damage.

A known positive regulator of ETC function is phosphorylated STAT3 at Serine-727 (141). Serine-727 activity has been documented as conferring protection against ETC damage and inducing an increase in oxidative phosphorylation. Here, a trend is observed for increased Serine-727 phosphorylation in LFD + VC exposed animals at 6 weeks, the same time point evaluated for the functional analysis. Interestingly, although a significant diet-induced increased is observed, VC does not increase this effect. This may indicate that the combination of HFD and VC activate and progress via different stress/metabolic pathways or could be demonstrative of exhaustion of this pathway. However, further experiments need to be conducted to determine the exact mechanism involved, but the activation of this signaling pathway may be a viable mechanism to pursue in VC-induced mitochondrial damage.



Scheme 5.1. Proposed effect of VC on mitochondrial function. Upon exposure to VC, reactive intermediates form though bio-activation processes and diet-induced obesity decreases their elimination. VC/VC metabolites directly impair ETC complex activity, which impairs oxidative phosphorylation. This increases the demand for ATP via glycolysis. The increased demand for glucose dysregulates metabolic homeostasis and depletes glycogen stores. Enhanced macrovesicular steatosis occurs, leaving β-oxidation of FFA intact, even under conditions of ATP depletion. The decreased mitochondrial potential facilitates cytochrome *c* release to activate apoptotic cascades, however the loss of ATP results in incomplete apoptosis and necrotic cell death. The combined metabolic stress of VC exposure causes increased oxidative stress, ER stress, and inefficient mitochondrial respiration and energy production, resulting in increased hepatocyte sensitivity to damage.

CHAPTER VI

DISCUSSION AND CONCLUSIONS

A. Restatement of goals and questions

The major goal of the work described in this dissertation was to expand on the unifying hypothesis that chronic exposure to sub-OSHA concentrations of VC is not only sufficient to exacerbate high fat diet-induced liver injury, but is capable of sensitizing hepatocytes via metabolic and mitochondrial dyshomeostasis. The Beier group has previously demonstrated the merit of evaluating the impact of sub-hepatotoxic exposures to VC metabolites in combination with other mitigating factors (i.e. inflammation and diet) (26;27). The experiments conducted in Chapter III describe a novel mouse model in which the effects of chronic VC inhalation can be evaluated in conjunction with exposure to a HFD; thereby, aiding in the characterization of nutrient:toxicant mechanisms of liver damage. Chapter IV characterized the alterations in hepatic metabolism after exposure to VC alone and described enhancement of both oxidative and ER stress pathways with the combination of a HFD and VC exposure. Finally, Chapter V investigated the effect of VC exposure on mitochondrial integrity and function, thereby

providing key insight into the mechanism of VC-induced hepatocyte injury. Taken together, these studies provide novel information into the complex mechanism of VC-induced hepatotoxicity and its ability to concomitantly enhance underlying liver injury.

B. Major findings of this dissertation

1. Chronic, low-level VC exposure enhances liver injury caused by a HFD feeding

Previous studies from the Beier laboratory have established that exposure to VC metabolites can enhance liver injury caused by both an inflammatory stimulus (LPS) and by metabolic disruption (HFD) in mouse models of exposure. Additionally, occupational exposures to extremely high levels of VC are known to produce both cancerous and non-cancerous liver diseases. However, the effect of low-level exposures to VC in the context of pre-existing liver injury has not been studied. As such, the goals of Chapter III were to 1.) Develop and characterize a mouse model for simultaneous exposure to sub-OSHA VC levels via inhalation and HFD feeding to better mimic human exposures, 2.) Evaluate indices of liver injury and inflammation to determine if VC exposure enhances diet-induced liver injury, and 3.) Determine the predominant mechanism of hepatocyte cell death in this model.

The first goal of Chapter III was to develop and characterize this model of VC inhalation and concomitant HFD feeding. Mice were exposed to sub-OSHA concentrations of VC (<1 ppm) in an inhalation chamber, whilst being fed either a low fat control diet or a high fat diet. In order to determine overall animal health

during the study, measurements of body weight, food consumption, and liver to body weight ratios were measured for each time point of exposure. Interestingly, although there was a diet-dependent effect on weight gain and liver to body weight ratios, VC did not enhance these indices as originally hypothesized. Additionally, VC did not alter body mass composition as determined via both fat and lean mass measurements. Importantly, no significant effect was observed in animals exposed to LFD + VC, indicating that this concentration of VC alone does not alter whole organism health.

A second major goal of Chapter III was to evaluate indices of liver injury to determine if VC exposure enhanced damage caused by a HFD. HFD feeding is known to cause NAFLD-associated pathologies in mice such as increased steatosis and elevated transaminase levels. Indeed, the studies conducted in Chapter III show that HFD + VC exposed animals had significantly higher levels of hepatic transaminases and increased fibrin accumulation. Fibrin can be indicative of increased tissue injury by activation of the coagulation cascade. Indeed, upon a more in depth analysis, it was determined that the increased fibrin accumulation observed was due to enhanced thrombin activity. To address the changes in overall liver injury observed, markers of hepatic inflammation were also examined. In addition, visualization of both hepatic neutrophil infiltration and macrophage recruitment was performed via histology. VC significantly increased hepatic neutrophil accumulation in the HFD group, while no changes were observed for any treatment group in regards to macrophage recruitment. This may indicate a neutrophil-dependent inflammatory response in

this model. Indeed, neutrophils are important innate immune mediators of local tissue injury and could be responding to the increase in tissue fibrin, as fibrin is a known attractant for neutrophils. However, intriguingly, VC did not alter protein levels of key cytokines involved in inflammation.

Finally, Chapter III sought to determine the major cell death pathway that occurs in this model. Historically, VC/VC metabolite exposure has been associated with necrotic cell death (26;34). The results presented from this study coalesce with the previous studies. Here, although there is a significant increase in caspase-3 cleavage with the combination of HFD and VC, there is not an increase in apoptotic hepatocytes as evidenced by TUNEL staining. Interestingly, there is a significant increase in NPCs undergoing apoptosis (e.g. macrophages) suggesting increased turnover or damage to this cell type specifically. As stated previously, it is important to note that LFD + VC exposed animals did not have any signs of liver injury or increased cell death in this model at 12 weeks of exposure. Again, this indicates that the concentration of VC does not overtly damage hepatocytes, but in conjunction with another stressor (diet), VC significantly enhanced hepatic injury.

2. VC exposure dysregulates hepatic metabolism and increases endogenous stress

As described in Chapter I, Section 4, liver disease progression to more severe pathologies is a complex and incompletely understood. In the seminal study by Cave et al., occupationally exposed VC workers had altered plasma insulin levels, indicating perturbed nutrient signaling (34) and a recent study

revealed highly exposed VC factory workers had significant changes to their plasma metabolome (79). Therefore, the driving hypothesis behind Chapter IV was that metabolic changes induced by VC exposure that may not be overtly toxic, but may contribute to development of more severe liver disease, is critical to understand the pathogenicity of VC exposure, especially at low-level concentrations. Additionally, examining the effect of VC on known pathways involved in NAFLD could provide mechanistic insight to VC hepatotoxicity. Therefore, Chapter IV of this dissertation aimed to 1.) Determine if VC inhalation exposure alone causes alterations in hepatic metabolism, and 2.) Evaluate the impact of VC exposure on both oxidative and ER stress processes as potential mechanisms by which VC enhances diet-induced liver damage.

In order to achieve the first goal of Chapter IV, histological indices of lipid (ORO) and carbohydrate (PAS) metabolism were examined for the same time point in which the enhanced hepatic injury was observed at 12 weeks of exposure. Hepatic lipids were also analyzed to give a more detailed breakdown of the increased lipid accumulation observed via the ORO stain. Indeed, a significant accumulation of macrovesicular lipids were observed as evidenced by liver triglyceride content in animals which were exposed to both HFD and VC. Interestingly, although VC did not cause changes in hepatic lipids in the absence of a HFD, it did significantly deplete hepatic glycogen reserves, which indicates alterations in carbohydrate, specifically glucose, metabolism. Looking to an earlier time point (6 weeks) to provide mechanistic insight to this phenomenon, mRNA expression of key glucose regulating enzymes was analyzed. Indeed,

LFD + VC exposed animals showed significant increases in the enzymes evaluated (e.g., *Pck1, G6Pase, Pgc1α*), suggesting that VC exposure disrupts cellular and whole organism (PTT results) glucose homeostasis.

Secondly, Chapter IV sought to investigate VC's effect on oxidative stress and ER stress pathways as possible mechanisms by which enhanced liver injury was observed with the co-exposure of HFD and VC (see Chapter III). To accomplish this, the studies performed analyzed histological and biochemical indices of hepatic oxidative stress. Results from these analyses determined that, in fact, low-level VC exposure significantly enhanced HFD-induced lipid peroxidation adducts, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA). Importantly, significant increases in 4-HNE positive staining were also observed for LFD + VC exposed animals, indicating that chronic, sub-OSHA exposures to VC alone is sufficient to induce biomolecular damage. As mentioned in Chapter I, Section 3, it is also likely the reactive metabolites of VC are the perpetrators of such damage as they are known to covalently bind to and damage both DNA and protein (19;21;22). Further, as part of the second goal of Chapter IV, several markers of ER stress activation were analyzed and electron microscopy was performed to macroscopically determine damage. Importantly, independent of diet at 12 weeks of exposure, there is compelling evidence of UPR activation via morphology changes to ER (e.g., ER dilation). Interestingly, significant, but minor changes in mRNA expression of key ER stress executors are observed, with no changes at the protein level. This suggests that other potential mechanisms of regulation may be involved. Taken together, the results

from Chapter IV suggest that VC metabolically sensitizes hepatocytes to secondary stressors, and although increased oxidative adducts and modest alterations to the ER are observed, it is likely that these are secondary pathologies which aid in/result from the metabolic and energy disruption observed in this model.

3. VC exposure targets the ETC and decreases mitochondrial function

Mitochondria are dynamic organelles capable of adapting to changes in cellular energy supply and responding to both endogenous and exogenous stressors in order to maintain homeostasis. Importantly, as they are crucial for maintaining cellular energy levels via ATP production, damage to this organelle often has profound consequences. Perturbations in nutrient homeostasis result in mitochondrial dysfunction. Both NAFLD and toxicant exposure have independently been shown to induce morphologic and functional changes in hepatic mitochondria. Therefore, the goals of Chapter V were to 1.) Investigate the role of low-level VC exposure on mitochondrial function and the impact of HFD and, 2.) Determine a mechanism by which VC exposure impacts mitochondrial integrity and energy production.

In order to accomplish the first goal, this study examined mRNA expression of key genes involved in mitochondrial biogenesis and organelle dynamics in addition to analyzing mitochondrial protein concentrations at both 6 and 12 weeks of exposure to VC with and without a HFD. Interestingly, decreases in mRNA expression of *Tfam* are seen at 6 weeks of VC exposure in the HFD and

VC exposed animals, suggesting impaired mitochondrial biogenesis. Additionally, alterations were observed in *Fis1* mRNA expression which varied depending on the time point examined. This is coupled with observations of a significant VC effect on mitochondrial respiration as seen via a functional assay on isolated hepatic mitochondria at 6 weeks of exposure independent of diet. Indeed, these data support the hypothesis of Chapter V, which is that low-level VC exposure is sufficient to cause effects at the mitochondrial level.

The second goal of Chapter V was to determine a possible mechanism by which the functional deficits were observed. To accomplish this, several proteins were examined via Western blot. Indeed, decreases were observed in several ETC complexes with VC exposure alone, indicating a direct effect on mitochondrial protein levels. Phosphorylated STAT3 at Serine-727 was examined as a possible signaling pathway at play in this model. Significant changes were observed at this phosphorylation site, suggesting that this pathway may be important in VC-induced mitochondrial damage. Further, VC was shown to cause hepatocytes to be more susceptible to cell death and mitochondrial damage in LFD fed animals.

4. Significance of new findings

The work presented in this dissertation has aided in filling a crucial knowledge gap regarding low-level VC exposure and its impact on existing liver injury caused by diet. The work presented herein sought to characterize a novel model of VC exposure that is currently below the OSHA regulation limit (<1 ppm) and its impact in a co-exposure paradigm with experimental NAFLD. As discussed in

Chapter I, potential for VC exposure remains prevalent as its current ranking as 4th on the ATSDR Hazardous Substance List attests to. Exposures at or below the OSHA limit are relevant not only for occupationally exposed workers, but also for populations living in areas surrounding both PVC producing facilities and EPA designated Superfund sites. An ever-growing concern globally and in the United States is the prevalence of obesity and NAFLD. Indeed, 25% of the global is estimated to have underlying NAFLD.

The experiments presented in Chapter III suggest a novel interaction between HFD and sub-OSHA exposures to VC via inhalation. The data presented in Chapter III demonstrate that the combination of VC inhalation and HFD feeding does not increase adiposity via obesogenic mechanisms at 12 weeks as some environmental pollutants are known to do (70). Although no changes in body weight gain or liver weight were observed with VC and HFD exposure, there were significant increases in indices of liver injury. Enhanced liver injury was observed as evidenced via increased liver enzymes, fibrin accumulation, and neutrophil infiltration all suggest that VC exposure enhances diet-induced liver damage. It is important to note that animals exposed to the same concentration of VC on a LFD did not have any signs of hepatic injury, indicating that the 'multiple-hit' paradigm of liver injury holds true in these studies. A second interesting result from the studies conducted in Chapter III reveal a differential effect of HFD and VC exposure on hepatic cell death. Similar to other studies with VC/ VC metabolites, it appears as though the primary route for hepatocyte

cell is not via apoptosis, despite increases in caspase cleavage and cytochrome *c* release from mitochondria.

The profound enhancement of liver injury observed in the combination of HFD and VC exposure prompted the study for mechanistic insight. Therefore, the experiments in Chapter IV provided key information regarding the hepatotoxicity of low-level VC exposure, both with and without the addition of HFD feeding. Indeed, the results gathered in Chapter IV suggest that VC exposure alone is sufficient to cause both metabolic and oxidative damage to hepatocytes. Importantly, these 'sub-hepatotoxic' alterations may be crucial in sensitizing hepatocytes to further injury. Sub-OSHA exposure to VC significantly altered hepatic glucose metabolism by causing a shift to increase systemic gluconeogenesis to compensate for decreased cellular ATP production (see below). Additionally, increased indices for oxidative stress and ER stress were observed in animals co-exposed to HFD and VC. Therefore, Chapter IV describes a novel potential mechanism by which low-level VC exposure enhances diet-induced liver injury.

Finally, Chapter V of this dissertation sought to develop the hypothesis that low-level VC exposure may directly damage mitochondrial function and the impact that HFD co-exposure may have on this effect. The experiments described in Chapter V demonstrate that low-level VC exposure is sufficient to induce mitochondrial damage as seen via decreased respiratory capacity at 6 weeks of exposure and decreased protein levels of key oxidative phosphorylation enzymes. Additionally, VC alone was observed to decrease cell viability

compared to LFD control animals at 6 weeks, suggesting that VC exposed hepatocytes are more susceptible to secondary damage. These data suggest a novel role for VC as an important risk-factor for developing liver disease. Notably, accumulating damage from adducted proteins, metabolic stress, and mitochondrial deficits all contribute to increased hepatocyte susceptibility to enhanced injury when combined with another stressor. This was demonstrated in this dissertation by co-exposure with a HFD to mimic NAFLD. Taken together, the results presented here provide new insight into low-level VC-induced hepatotoxicity and its potential for increasing liver injury when combined with experimental NAFLD.

C. Strengths and Weaknesses

1. Strengths

There are several strengths of the work presented in this dissertation. First, this work developed and used an animal model that more closely mimics human exposure to environmental VC exposures and concomitant exposure to a diet high in saturated fat. As mentioned previously, the field of environmental toxicology is shifting towards an ‘exposure biology’ approach which takes into account multiple factors when evaluating risk for developing disease. To this point, development of liver damage and disease progression is a complex process with multiple confounding factors. Therefore, the hypothesis evaluated here could likely not be sufficiently addressed by the use of simpler model systems (e.g., cultured cells). Particularly, this work used an animal model, in which chronic administration of sub-OSHA concentrations of VC exposure were

used. This is a major strength of this work. The results from these studies provide additional support for the continued research into environmentally relevant toxicant exposures and the impact they have on underlying liver injury.

Another strength of this work is the incorporation of state-of-the-art techniques in order to gain novel insight behind the mechanisms of VC hepatotoxicity. Indeed, these studies revealed the importance of VC exposure on hepatic metabolism, oxidative stress activation, and mitochondrial dysfunction. Importantly, this work has demonstrated that not only does VC independently cause hepatocytes to be more susceptible to further damage, but that concomitant exposure to experimental NAFLD significantly enhances liver injury and oxidative damage. These findings are critical as they demonstrate that current OSHA exposure thresholds may not be stringent enough given the prevalence of NAFLD in this country and around the world.

Finally, this dissertation has identified that sub-OSHA concentrations of VC are sufficient to cause functional deficits in hepatic mitochondria without the addition of a second factor. Building on previous work done with VC metabolites *in vitro*, this dissertation sought to determine the effects of VC exposure in a more relevant exposure paradigm. The experiments in Chapter V demonstrated the sensitivity of hepatic mitochondria to VC toxicity. While it is known that mitochondrial damage occurs in human populations with NAFLD and NASH, the findings herein reiterate that populations exposed to low-level VC concentrations may be uniquely susceptible to enhanced liver injury caused by diet.

2. Weaknesses

Although the animal model used in this dissertation is a key strength to this work, it is also a source of potential complexity. With increasing complexity of the model system, there is also an increase in uncertainty regarding mechanisms as play. Indeed, there is currently no rodent model that completely recapitulates human fatty liver disease progression or response to toxicants. It is therefore possible that the injury and metabolic alteration observed in this model are not applicable to human populations.

The work presented here has identified several potential mechanisms by which VC causes and enhances liver injury. However, due to the complexity of the factors at play, it is difficult to discern the exact pathways. To this point, a simpler model system would be ideal to investigate mechanistic details that are confounded in this murine model. Further, only male mice were used in this study. It is important to note the effects observed may vary with sex. Another source of potential weakness of work can be found within the exposure time line and time points chosen for sacrifice. Indeed, results from this work highlight the temporal changes associated with chronic VC exposure in mRNA expression that are no longer observed at the later time point of 12 weeks. Therefore, it is necessary to consider time points earlier than 6 weeks to evaluate injury and stress pathway activation. Additionally, time points past 12 weeks of exposure should be evaluated to examine continued effects of VC exposure and its potential to cause injury. The experiments described in Chapter V 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.

D. Future Directions

While the experiments described in this dissertation aided in filling knowledge gaps regarding chronic, low-level VC exposure and its potential to enhance underlying liver disease, it has also opened the door for new research questions which will need to be addressed in future studies. Some of these questions are addressed below.

1. Does VC exposure cause mitochondrial protein adducts?

The experiments described in Chapter V were mainly concerned with determining the functional effect of VC exposure on mitochondrial respiration and integrity. Although some alterations were observed at the mRNA and protein level, the most profound effect of VC could be seen at the level of mitochondrial function. As previously mentioned, the VC metabolite CAA is known to propagate intracellular damage via adducting proteins. Therefore, an interesting future study would be to perform a global mitochondrial proteomics analysis to determine if VC exposure induces covalent adduct formation, thereby decreasing mitochondrial function, while explaining why protein levels are not dramatically changing in this model.

2. Does VC exposure alter mitochondrial-ER membrane interactions?

Mitochondria and the endoplasmic reticulum (ER) communicate with each other through mitochondrial associated ER membranes (MAMs), sharing

metabolites and key signaling molecules (142). Recently, Arruda et al., have demonstrated that an increase in MAMs leads to mitochondrial dysfunction in HFD fed mice (143). Alterations in MAMs have also been implicated in regulating hepatocyte glucose metabolism (144) and in insulin resistance (145;146). Indeed, results from Chapter V of this dissertation regarding an increase in IP3-R1 protein levels with VC exposure give rise to the hypothesis that VC may be altering mitochondrial-ER cross talk and calcium homeostasis, contributing to hepatocyte injury and metabolic dyshomeostasis. Therefore, it would be of interest to investigate further whether enhanced mitochondria-ER contact and dysregulated calcium signaling is at play in this model.

3. **Will prevention of mitochondrial dysfunction protect against VC-induced hepatotoxicity?**

The results from Chapter V clearly demonstrate that VC-induced mitochondrial dysfunction may be a driving factor in injury progression especially when combined with a secondary factor. Therefore, it would be of great interest to determine if ameliorating mitochondrial damage could mitigate injury progression in this model. Several mitochondrial protective small molecules are being studied due to their antioxidant capabilities and ability to prevent liver injury. Indeed, several studies investigating the antioxidant Tempol have demonstrated its protective qualities in models of carbon tetrachloride induced liver injury (147;148). Further, a small antioxidant peptide, SS-31, has been evaluated for its protective qualities. SS-31 is cell permeable and localizes specifically to the inner mitochondrial membrane where it scavenges reactive

oxygen species, preventing damage. Indeed, studies have shown that SS-31 administration is protective in hepatic ischemia/reperfusion injury (149;150), kidney injury (151), and in experimental models of Parkinson's disease (152). Therefore, using one of these molecules or a compound that confers similar effects would be useful in determining a more specific mechanism of VC-induced hepatotoxicity.

4. Does VC alter epitranscriptomic regulation of mRNA expression?

The results from this dissertation reveal that although VC induces hepatic changes and enhances diet-induced liver injury, the corresponding mRNA and protein analyses performed do not yield the expecting alterations observed. It is therefore possible that VC exposure causes changes to the 'epitransciptome'. Indeed, it has recently been characterized that, similar to DNA, mRNA can be post-translationally modified. These modifications such as mRNA secondary structure stability and chemical modifications (i.e., methylation and acetylation) are able to influence cellular processes independent of mRNA abundance, per se. These modifications may also alter RNA-protein interactions and can influence response to environmental stressors(153;154). Moreover, preliminary data show significant VC-induced alterations in key enzymes involved in epitranscriptomic regulation. Based on the observations of this dissertation, it would be interesting to pursue the epitranscriptomic involvement in this model and identify novel mechanisms of VC hepatotoxicity.

E. Summary and Conclusions

Taken together, this dissertation describes a novel interaction between chronic, environmentally relevant exposures to the prevalent hepatotoxicant, VC, and its potential to increase liver injury when combined with experimental NAFLD. The experiments described in Chapter III underscore the need for research regarding low-level toxicant co-exposure with other risk factors. Here, experimental NAFLD was used due to its high prevalence in the United States and worldwide. Indeed, the combination of diet and VC exposure significantly enhanced indices of liver injury. Chapter IV describes potential mechanisms by which this injury is observed. Certainly, metabolic stress, ER stress, and oxidative damage are major contributors to the hepatic damage observed in this model. However, Chapter V revealed that VC-induced mitochondrial damage is likely the driving force behind the pathology observed. A decrease in mitochondrial integrity and respiration severely compromises VC-exposed hepatocytes and their ability to combat secondary stressors, culminating in overwhelming injury and cell death. Importantly, this work reinforces the relevance of environmental toxicant exposure and raises concerns that the current regulations in place may not be stringent enough given the health status of the current population.

REFERENCES

1. Stanger BZ. Cellular homeostasis and repair in the mammalian liver. Annu.Rev.Physiol 2015;77:179-200

2. Dixon LJ, Barnes M, Tang H, Pritchard MT, Nagy LE. Kupffer cells in the liver. Compr.Physiol 2013 Apr;3(2):785-97. PMCID:PMC4748178

3. Raucy JL, Kraner JC, Lasker JM. Bioactivation of halogenated hydrocarbons by cytochrome P4502E1. Crit Rev.Toxicol 1993;23(1):1-20

4. Technical Overview of Volatile Organic Compounds United States Environmental Protection Agency. 2017.

5. Cleary E, Asher M, Olawoyin R, Zhang K. Assessment of indoor air quality exposures and impacts on respiratory outcomes in River Rouge and Dearborn, Michigan. Chemosphere 2017 Nov;187:320-9

6. Sakai K, Norback D, Mi Y, Shibata E, Kamijima M, Yamada T, Takeuchi Y. A comparison of indoor air pollutants in Japan and Sweden: formaldehyde, nitrogen dioxide, and chlorinated volatile organic compounds. Environ.Res. 2004 Jan;94(1):75-85

7. Volatile organic compounds' impact on indoor air quality United States Environmental Protection Agency. 2017.

8. ATSDR's Substance Priority List Agency for Toxic Substances and Disease Registry. 2017.

9. Maslia ML, Aral MM, Ruckart PZ, Bove FJ. Reconstructing Historical VOC Concentrations in Drinking Water for Epidemiological Studies at a U.S. Military Base: Summary of Results. Water (Basel) 2016;8(10):449. PMCID:PMC5580837

10. Bove FJ, Ruckart PZ, Maslia M, Larson TC. Evaluation of mortality among marines and navy personnel exposed to contaminated drinking water at USMC base Camp Lejeune: a retrospective cohort study. Environ.Health 2014;13(1):10. PMCID:PMC3943370

11. Sass JB, Castleman B, Wallinga D. Vinyl chloride: a case study of data suppression and misrepresentation. Environ.Health Perspect. 2005 Jul;113(7):809-12

12. G.Daniel Todd. Toxicological Profile for Vinyl Chloride (VC) (update). 2011.

13. Falk H, Creech JL, Jr., Heath CW, Jr., Johnson MN, Key MM. Hepatic disease among workers at a vinyl chloride polymerization plant. JAMA. 1974 Oct 7;230(1):59-63

14. Creech JL, Jr., Johnson MN. Angiosarcoma of liver in the manufacture of polyvinyl chloride. J Occup Med. 1974 Mar;16(3):150-1

15. Chaudhary P, Bhadana U, Singh RA, Ahuja A. Primary hepatic angiosarcoma. Eur.J Surg Oncol. 2015 Sep;41(9):1137-43

16. Elliott P, Kleinschmidt I. Angiosarcoma of the liver in Great Britain in proximity to vinyl chloride sites. Occup.Environ.Med 1997 Jan;54(1):14-8. PMCID:PMC1128629

17. Occupational Safety and Health Standards 2016.

18. Antweiler H. Studies on the metabolism of vinyl chloride. Environ.Health Perspect. 1976 Oct;17:217-9. PMCID:PMC1475241

19. Guengerich FP, Mason PS, Stott WT, Fox TR, Watanabe PG. Roles of 2-haloethylene oxides and 2-haloacetaldehydes derived from vinyl bromide and vinyl chloride in irreversible binding to protein and DNA. Cancer Res 1981;1981/11/01:4391-8

20. Guengerich FP, Crawford WM, Jr., Watanabe PG. Activation of vinyl chloride to covalently bound metabolites: roles of 2-chloroethylene oxide and 2-chloroacetaldehyde. Biochemistry 1979 Nov 13;18(23):5177-82

21. Bolt HM. Metabolic activation of vinyl chloride, formation of nucleic acid adducts and relevance to carcinogenesis. IARC Sci Publ. 1986;(70):261-8

22. Bolt HM. Vinyl chloride-a classical industrial toxicant of new interest. Crit Rev.Toxicol. 2005 Apr;35(4):307-23

23. Morinello EJ, Koc H, Ranasinghe A, Swenberg JA. Differential induction of N(2),3-ethenoguanine in rat brain and liver after exposure to vinyl chloride. Cancer Res. 2002 Sep 15;62(18):5183-8

24. Maltoni C, Lefemine G, Ciliberti A, Cotti G, Carretti D. Carcinogenicity bioassays of vinyl chloride monomer: a model of risk assessment on an

experimental basis. Environ.Health Perspect. 1981 Oct;41:3-29. PMCID:PMC1568874

25. Swenberg JA, Ham A, Koc H, Morinello E, Ranasinghe A, Tretyakova N, Upton PB, Wu K. DNA adducts: effects of low exposure to ethylene oxide, vinyl chloride and butadiene. Mutat.Res. 2000 Jan 3;464(1):77-86

26. Anders LC, Lang AL, Anwar-Mohamed A, Douglas AN, Bushau AM, Falkner KC, Hill BG, Warner NL, Arteel GE, Cave M, et al. Vinyl Chloride Metabolites Potentiate Inflammatory Liver Injury Caused by LPS in Mice. Toxicol.Sci. 2016 Jun;151(2):312-23. PMCID:PMC4880135

27. Anders LC, Yeo H, Kaelin BR, Lang AL, Bushau AM, Douglas AN, Cave M, Arteel GE, McClain CJ, Beier JI. Role of dietary fatty acids in liver injury caused by vinyl chloride metabolites in mice. Toxicol.Appl.Pharmacol. 2016 Nov 15;311:34-41. PMCID:PMC5079761

28. Brun P, Castagliuolo I, Di Leo V, Buda A, Pinzani M, Palu G, Martines D. Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis. Am J Physiol Gastrointest Liver Physiol 2007 Feb;292(2):G518-G525

29. Sayiner M, Koenig A, Henry L, Younossi ZM. Epidemiology of Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis in the United States and the Rest of the World. Clin.Liver Dis. 2016 May;20(2):205-14

30. Day CP, James OF. Steatohepatitis: a tale of two "hits"? Gastroenterology. 1998 Apr;114(4):842-5

31. Yang SQ, Lin HZ, Lane MD, Clemens M, Diehl AM. Obesity increases sensitivity to endotoxin liver injury: implications for the pathogenesis of steatohepatitis. Proc Natl Acad Sci U S A. 1997;94:2557-62

32. Joshi-Barve S, Kirpich I, Cave MC, Marsano LS, McClain CJ. Alcoholic, Nonalcoholic, and Toxicant-Associated Steatohepatitis: Mechanistic Similarities and Differences. Cell Mol Gastroenterol Hepatol. 2015 Jul;1(4):356-67. PMCID:PMC5301292

33. Piacentini M, Baiocchini A, Del NF, Melino G, Barlev NA, Rossin F, D'Eletto M, falasca L. Non-alcoholic fatty liver disease severity is modulated by transglutaminase type 2. Cell Death.Dis. 2018 Feb 15;9(3):257. PMCID:PMC5833377

34. Cave M, Falkner KC, Ray M, Joshi-Barve S, Brock G, Khan R, Bon Homme M, McClain CJ. Toxicant-associated steatohepatitis in vinyl chloride workers. Hepatology. 2010 Feb;51(2):474-81. PMCID:PMC4019991

35. Cotrim HP, De Freitas LA, Freitas C, Braga L, Sousa R, Carvalho F, Parana R, Santos-Jesus R, Andrade Z. Clinical and histopathological features of NASH in workers exposed to chemicals with or without associated metabolic conditions. Liver Int 2004 Apr;24(2):131-5

36. Perez CA, Bosia JD, Cantore MS, Chiera A, Cocozzella DR, Adrover RE, Borzi S, Curciarello JO. [Liver damage in workers exposed to hydrocarbons]. Gastroenterol Hepatol. 2006 Jun;29(6):334-7

37. Obesity and Overweight World Health Organization. 2018.

38. Overweight and Obesity Statistics National Institute of Diabetes and Digestive and Kidney Diseases. 2018.

39. Adult Obesity Facts Center for Disease Control and Prevention. 2018.

40. Grundy SM, Brewer HB, Jr., Cleeman JI, Smith SC, Jr., Lenfant C. Definition of metabolic syndrome: Report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. Circulation. 2004 Jan 27;109(3):433-8

41. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology 2016 Jul;64(1):73-84

42. Younossi Z, Anstee QM, Marietti M, Hardy T, Henry L, Eslam M, George J, Bugianesi E. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. Nat.Rev.Gastroenterol Hepatol. 2018 Jan;15(1):11-20

43. McPherson S, Hardy T, Henderson E, Burt AD, Day CP, Anstee QM. Evidence of NAFLD progression from steatosis to fibrosing-steatohepatitis using paired biopsies: implications for prognosis and clinical management. J Hepatol. 2015 May;62(5):1148-55

44. Calzadilla BL, Adams LA. The Natural Course of Non-Alcoholic Fatty Liver Disease. Int J Mol Sci 2016 May 20;17(5). PMCID:PMC4881593

45. Anstee QM, Seth D, Day CP. Genetic Factors That Affect Risk of Alcoholic and Nonalcoholic Fatty Liver Disease. Gastroenterology 2016 Jun;150(8):1728-44

46. Romeo S, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, Boerwinkle E, Cohen JC, Hobbs HH. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat.Genet 2008 Dec;40(12):1461-5. PMCID:PMC2597056

47. Dongiovanni P, Anstee QM, Valenti L. Genetic predisposition in NAFLD and NASH: impact on severity of liver disease and response to treatment. Curr.Pharm.Des 2013;19(29):5219-38. PMCID:PMC3850262

48. Boyle M, Masson S, Anstee QM. The bidirectional impacts of alcohol consumption and the metabolic syndrome: Cofactors for progressive fatty liver disease. J Hepatol. 2018 Feb;68(2):251-67

49. Drew RT, Boorman GA, Haseman JK, McConnell EE, Busey WM, Moore JA. The effect of age and exposure duration on cancer induction by a known carcinogen in rats, mice, and hamsters. Toxicol.Appl.Pharmacol. 1983 Mar 30;68(1):120-30

50. Wieckowski MR, Giorgi C, Lebiedzinska M, Duszynski J, Pinton P. Isolation of mitochondria-associated membranes and mitochondria from animal tissues and cells. Nat Protoc 2009 Oct 8;4(11):1582-90. PMCID:n/a

51. Beier JI, Luyendyk JP, Guo L, von Montfort C, Staunton DE, Arteel GE. Fibrin accumulation plays a critical role in the sensitization to lipopolysaccharide-induced liver injury caused by ethanol in mice. Hepatology. 2009 Jan 23;49(5):1545-53. PMCID:PMC2852109

52. Tan M, Schmidt RH, Beier JI, Watson WH, Zhong H, States JC, Arteel GE. Chronic subhepatotoxic exposure to arsenic enhances hepatic injury caused by high fat diet in mice. Toxicol Appl Pharmacol 2011 Dec 15;257(3):356-64

53. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can.J.Biochem.Physiol. 1959;37:911-7

54. von Montfort C, Beier JI, Guo L, Kaiser JP, Arteel GE. Contribution of the sympathetic hormone epinephrine to the sensitizing effect of ethanol on LPS-induced liver damage in mice. Am.J.Physiol Gastrointest.Liver Physiol. 2008 Mar 6;294(5):G1227-G1234. PMCID:PMC2660374

55. Berry MN, Friend DS. High yield preparation of isolated rat liver parenchymal cells: A biochemical and fine structural study. J.Cell Biol. 1969;43:506-20

56. Smedsrod B, Pertoft H. Preparation of pure hepatocytes and reticuloendothelial cells in high yield from a single rat liver by means of Percoll centrifugation and selective adherence. J Leukoc Biol 1985;38:213-30

57. Ding X, Beier JI, Baldauf KJ, Jokinen JD, Zhong H, Arteel GE. Acute ethanol preexposure promotes liver regeneration after partial hepatectomy

in mice by activating ALDH2. Am J Physiol Gastrointest Liver Physiol. 2014 Jan;306(1):G37-G47. PMCID:PMC3920082

58. Yang H, Ni HM, Guo F, Ding Y, Shi YH, Lahiri P, Frohlich LF, Rulicke T, Smole C, Schmidt VC, et al. Sequestosome 1/p62 Protein Is Associated with Autophagic Removal of Excess Hepatic Endoplasmic Reticulum in Mice. J.Biol.Chem. 2016 Sep 2;291(36):18663-74. PMCID:PMC5009243

59. U.S.Department of Health and Human Services PHS. Agency for Toxic Substances and Disease Registry (ATSDR): Toxicological profile for Vinyl Chloride. 2006.

60. Kielhorn J, Melber C, Wahnschaffe U, Aitio A, Mangelsdorf I. Vinyl chloride: still a cause for concern. Environ.Health Perspect. 2000 Jul;108(7):579-88

61. McKone TE, Knezovich JP. The transfer of trichloroethylene (TCE) from a shower to indoor air: experimental measurements and their implications. J.Air Waste Manage.Assoc. 1991 Mar;41(3):282-6

62. U.S.Environmental Protection Agency (U.S.EPA). Toxicological review of vinyl chloride in support of summary information on the Integrated Risk Information System (IRIS). 2000.

63. Flegal KM, Kruszon-Moran D, Carroll MD, Fryar CD, Ogden CL. Trends in Obesity Among Adults in the United States, 2005 to 2014. JAMA 2016 Jun 7;315(21):2284-91

64. Alkhouri N, Carter-Kent C, Feldstein AE. Apoptosis in nonalcoholic fatty liver disease: diagnostic and therapeutic implications. Expert.Rev.Gastroenterol Hepatol. 2011 Apr;5(2):201-12. PMCID:PMC3119461

65. Wieckowska A, Zein NN, Yerian LM, Lopez AR, McCullough AJ, Feldstein AE. In vivo assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. Hepatology 2006 Jul;44(1):27-33

66. Garrido C, Galluzzi L, Brunet M, Puig PE, Didelot C, Kroemer G. Mechanisms of cytochrome c release from mitochondria. Cell Death.Differ. 2006 Sep;13(9):1423-33

67. Shan Q, Huang F, Wang J, Du Y. Effects of co-exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin and polychlorinated biphenyls on nonalcoholic fatty liver disease in mice. Environ.Toxicol 2015 Dec;30(12):1364-74

68. Wei J, Sun X, Chen Y, Li Y, Song L, Zhou Z, Xu B, Lin Y, Xu S. Perinatal exposure to bisphenol A exacerbates nonalcoholic steatohepatitis-like

phenotype in male rat offspring fed on a high-fat diet. J Endocrinol. 2014 Sep;222(3):313-25

69. Tan X, Xie G, Sun X, Li Q, Zhong W, Qiao P, Sun X, Jia W, Zhou Z. High fat diet feeding exaggerates perfluorooctanoic acid-induced liver injury in mice via modulating multiple metabolic pathways. PLoS One 2013;8(4):e61409. PMCID:PMC3634078

70. Wahlang B, Falkner KC, Gregory B, Ansert D, Young D, Conklin DJ, Bhatnagar A, McClain CJ, Cave M. Polychlorinated biphenyl 153 is a diet-dependent obesogen that worsens nonalcoholic fatty liver disease in male C57BL6/J mice. J Nutr Biochem. 2013 Sep;24(9):1587-95. PMCID:PMC3743953

71. Mastrangelo G, Fedeli U, Fadda E, Valentini F, Agnesi R, Magarotto G, Marchi T, Buda A, Pinzani M, Martines D. Increased risk of hepatocellular carcinoma and liver cirrhosis in vinyl chloride workers: synergistic effect of occupational exposure with alcohol intake. Environ.Health Perspect. 2004 Aug;112(11):1188-92. PMCID:PMC1247480

72. Kubes P, Mehal WZ. Sterile inflammation in the liver. Gastroenterology 2012 Nov;143(5):1158-72

73. Xu R, Huang H, Zhang Z, Wang FS. The role of neutrophils in the development of liver diseases. Cell Mol Immunol. 2014 May;11(3):224-31. PMCID:PMC4085492

74. Loike JD, el Khoury J, Cao L, Richards CP, Rascoff H, Mandeville JT, Maxfield FR, Silverstein SC. Fibrin regulates neutrophil migration in response to interleukin 8, leukotriene B4, tumor necrosis factor, and formyl-methionyl-leucyl-phenylalanine. J.Exp.Med. 1995 May 1;181(5):1763-72

75. Beier JI, Arteel GE. Alcoholic liver disease and the potential role of plasminogen activator inhibitor-1 and fibrin metabolism. Exp.Biol.Med.(Maywood.). 2012 Jan 1;237(1):1-9. PMCID:n/a

76. Cave M, Falkner KC, Henry L, Costello B, Gregory B, McClain CJ. Serum cytokeratin 18 and cytokine elevations suggest a high prevalence of occupational liver disease in highly exposed elastomer/polymer workers. J.Occup.Environ.Med. 2011 Oct;53(10):1128-33. PMCID:PMC3190062

77. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res. 2009 Jun;29(6):313-26. PMCID:PMC2755091

78. Hsiao TJ, Wang JD, Yang PM, Yang PC, Cheng TJ. Liver fibrosis in asymptomatic polyvinyl chloride workers. J Occup.Environ.Med 2004 Sep;46(9):962-6

79. Guardiola JJ, Beier JI, Falkner KC, Wheeler B, McClain CJ, Cave M. Occupational exposures at a polyvinyl chloride production facility are associated with significant changes to the plasma metabolome. Toxicol.Appl.Pharmacol. 2016 Dec 15;313:47-56

80. Sozen E, Ozer NK. Impact of high cholesterol and endoplasmic reticulum stress on metabolic diseases: An updated mini-review. Redox.Biol. 2017 Aug;12:456-61. PMCID:PMC5357672

81. Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Gorgun C, Glimcher LH, Hotamisligil GS. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science 2004 Oct 15;306(5695):457-61

82. Mehal WZ. The Gordian Knot of dysbiosis, obesity and NAFLD. Nat.Rev.Gastroenterol Hepatol. 2013 Nov;10(11):637-44

83. Yoon JC, Puigserver P, Chen G, Donovan J, Wu Z, Rhee J, Adelmant G, Stafford J, Kahn CR, Granner DK, et al. Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. Nature 2001 Sep 13;413(6852):131-8

84. Sies H. Oxidative stress: introductory remarks. In: Sies H, editor. Oxidative Stress. London: Academic Press; 1985. p. 1-8.

85. Bellentani S, Scaglioni F, Marino M, Bedogni G. Epidemiology of non-alcoholic fatty liver disease. Dig.Dis. 2010;28(1):155-61

86. Zheng X, Xu F, Liang H, Cao H, Cai M, Xu W, Weng J. SIRT1/HSF1/HSP pathway is essential for exenatide-alleviated, lipid-induced hepatic endoplasmic reticulum stress. Hepatology 2017 Sep;66(3):809-24

87. Bove FJ, Ruckart PZ, Maslia M, Larson TC. Mortality study of civilian employees exposed to contaminated drinking water at USMC Base Camp Lejeune: a retrospective cohort study. Environ.Health 2014;13:68. PMCID:PMC4237831

88. Cohen JC, Horton JD, Hobbs HH. Human fatty liver disease: old questions and new insights. Science 2011 Jun 24;332(6037):1519-23. PMCID:PMC3229276

89. Takaki A, Kawai D, Yamamoto K. Multiple hits, including oxidative stress, as pathogenesis and treatment target in non-alcoholic steatohepatitis

(NASH). Int J Mol Sci 2013 Oct 15;14(10):20704-28. PMCID:PMC3821639

90. Rolo AP, Teodoro JS, Palmeira CM. Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. Free Radic.Biol.Med. 2012 Jan 1;52(1):59-69

91. Mohammad MK, Avila D, Zhang J, Barve S, Arteel G, McClain C, Joshi-Barve S. Acrolein cytotoxicity in hepatocytes involves endoplasmic reticulum stress, mitochondrial dysfunction and oxidative stress. Toxicol.Appl.Pharmacol. 2012 Nov 15;265(1):73-82. PMCID:PMC3501104

92. Moghe A, Ghare S, Lamoreau B, Mohammad M, Barve S, McClain C, Joshi-Barve S. Molecular mechanisms of acrolein toxicity: relevance to human disease. Toxicol Sci 2015 Feb;143(2):242-55. PMCID:PMC4306719

93. Hassoun E, Mettling C. Dichloroacetate and Trichloroacetate Toxicity in AML12 Cells: Role of Oxidative Stress. J Biochem.Mol Toxicol 2015 Nov;29(11):508-12

94. Csak T, Ganz M, Pespisa J, Kodys K, Dolganiuc A, Szabo G. Fatty acid and endotoxin activate inflammasomes in mouse hepatocytes that release danger signals to stimulate immune cells. Hepatology 2011 Jul;54(1):133-44. PMCID:PMC4158408

95. Xu B, Jiang M, Chu Y, Wang W, Chen D, Li X, Zhang Z, Zhang D, Fan D, Nie Y, et al. Gasdermin D plays a key role as a pyroptosis executor of non-alcoholic steatohepatitis in humans and mice. J.Hepatol. 2017 Dec 19;

96. Hirsova P, Ibrahim SH, Gores GJ, Malhi H. Lipotoxic lethal and sublethal stress signaling in hepatocytes: relevance to NASH pathogenesis. J Lipid Res. 2016 Oct;57(10):1758-70. PMCID:PMC5036373

97. Cazanave SC, Mott JL, Elmi NA, Bronk SF, Werneburg NW, Akazawa Y, Kahraman A, Garrison SP, Zambetti GP, Charlton MR, et al. JNK1-dependent PUMA expression contributes to hepatocyte lipoapoptosis. J Biol.Chem. 2009 Sep 25;284(39):26591-602. PMCID:PMC2785347

98. Malhi H, Barreyro FJ, Isomoto H, Bronk SF, Gores GJ. Free fatty acids sensitise hepatocytes to TRAIL mediated cytotoxicity. Gut 2007 Aug;56(8):1124-31. PMCID:PMC1955518

99. Finck BN, Kelly DP. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. J Clin.Invest 2006 Mar;116(3):615-22. PMCID:PMC1386111

100. Liang H, Bai Y, Li Y, Richardson A, Ward WF. PGC-1alpha-induced mitochondrial alterations in 3T3 fibroblast cells. Ann.N.Y.Acad.Sci 2007 Apr;1100:264-79

101. Herzig S, Long F, Jhala US, Hedrick S, Quinn R, Bauer A, Rudolph D, Schutz G, Yoon C, Puigserver P, et al. CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. Nature 2001 Sep 13;413(6852):179-83

102. Gusdon AM, Song KX, Qu S. Nonalcoholic Fatty liver disease: pathogenesis and therapeutics from a mitochondria-centric perspective. Oxid.Med Cell Longev. 2014;2014:637027. PMCID:PMC4211163

103. Videla LA, Rodrigo R, Orellana M, Fernandez V, Tapia G, Quinones L, Varela N, Contreras J, Lazarte R, Csendes A, et al. Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients. Clin.Sci (Lond) 2004 Mar;106(3):261-8

104. Egnatchik RA, Leamy AK, Jacobson DA, Shiota M, Young JD. ER calcium release promotes mitochondrial dysfunction and hepatic cell lipotoxicity in response to palmitate overload. Mol Metab 2014 Aug;3(5):544-53. PMCID:PMC4099508

105. Puri P, Mirshahi F, Cheung O, Natarajan R, Maher JW, Kellum JM, Sanyal AJ. Activation and dysregulation of the unfolded protein response in nonalcoholic fatty liver disease. Gastroenterology 2008 Feb;134(2):568-76

106. Ayala A, Munoz MF, Arguelles S. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid.Med Cell Longev. 2014;2014:360438. PMCID:PMC4066722

107. Esterbauer H, Eckl P, Ortner A. Possible mutagens derived from lipids and lipid precursors. Mutat.Res. 1990 May;238(3):223-33

108. Shin BC, Huggins JW, Carraway KL. Effects of pH, concentration and aging on the malonaldehyde reaction with proteins. Lip. 1972 Apr;7(4):229-33

109. Siu GM, Draper HH. Metabolism of malonaldehyde in vivo and in vitro. Lip. 1982 May;17(5):349-55

110. Meyer JN, Leung MC, Rooney JP, Sendoel A, Hengartner MO, Kisby GE, Bess AS. Mitochondria as a target of environmental toxicants. Toxicol Sci 2013 Jul;134(1):1-17. PMCID:PMC3693132

111. Ajith TA. Role of mitochondria and mitochondria-targeted agents in non-alcoholic fatty liver disease. Clin.Exp.Pharmacol Physiol 2017 Nov 7;

112. Simoes ICM, Fontes A, Pinton P, Zischka H, Wieckowski MR. Mitochondria in non-alcoholic fatty liver disease. Int J Biochem.Cell Biol. 2018 Feb;95:93-9

113. Caldwell SH, Swerdlow RH, Khan EM, Iezzoni JC, Hespenheide EE, Parks JK, Parker WD, Jr. Mitochondrial abnormalities in non-alcoholic steatohepatitis. J Hepatol. 1999 Sep;31(3):430-4

114. Cortez-Pinto H, Chatham J, Chacko VP, Arnold C, Rashid A, Diehl AM. Alterations in liver ATP homeostasis in human nonalcoholic steatohepatitis: a pilot study. JAMA 1999 Nov 3;282(17):1659-64

115. Einer C, Hohenester S, Wimmer R, Wottke L, Artmann R, Schulz S, Gosmann C, Simmons A, Leitzinger C, Eberhagen C, et al. Mitochondrial adaptation in steatotic mice. Mitochondrion. 2018 May;40:1-12

116. Savini I, Catani MV, Evangelista D, Gasperi V, Avigliano L. Obesity-associated oxidative stress: strategies finalized to improve redox state. Int J Mol Sci 2013 May 21;14(5):10497-538. PMCID:PMC3676851

117. Kozlov AV, Lancaster JR, Jr., Meszaros AT, Weidinger A. Mitochondria-meditated pathways of organ failure upon inflammation. Redox.Biol. 2017 Oct;13:170-81. PMCID:PMC5458092

118. Begriche K, Massart J, Robin MA, Bonnet F, Fromenty B. Mitochondrial adaptations and dysfunctions in nonalcoholic fatty liver disease. Hepatology 2013 Oct;58(4):1497-507

119. Biswas G, Srinivasan S, Anandatheerthavarada HK, Avadhani NG. Dioxin-mediated tumor progression through activation of mitochondria-to-nucleus stress signaling. Proc.Natl.Acad.Sci U.S.A 2008 Jan 8;105(1):186-91. PMCID:PMC2224183

120. O'Brien TM, Wallace KB. Mitochondrial permeability transition as the critical target of N-acetyl perfluorooctane sulfonamide toxicity in vitro. Toxicol Sci 2004 Nov;82(1):333-40

121. Starkov AA, Wallace KB. Structural determinants of fluorochemical-induced mitochondrial dysfunction. Toxicol Sci 2002 Apr;66(2):244-52

122. Walters MW, Bjork JA, Wallace KB. Perfluorooctanoic acid stimulated mitochondrial biogenesis and gene transcription in rats. Toxicol. 2009 Oct 1;264(1-2):10-5

123. Turner N, Bruce CR, Beale SM, Hoehn KL, So T, Rolph MS, Cooney GJ. Excess lipid availability increases mitochondrial fatty acid oxidative capacity in muscle: evidence against a role for reduced fatty acid oxidation

in lipid-induced insulin resistance in rodents. Diabetes 2007 Aug;56(8):2085-92

124. Chae S, Ahn BY, Byun K, Cho YM, Yu MH, Lee B, Hwang D, Park KS. A systems approach for decoding mitochondrial retrograde signaling pathways. Sci Signal. 2013 Feb 26;6(264):rs4

125. Wegrzyn J, Potla R, Chwae YJ, Sepuri NB, Zhang Q, Koeck T, Derecka M, Szczepanek K, Szelag M, Gornicka A, et al. Function of mitochondrial Stat3 in cellular respiration. Science 2009 Feb 6;323(5915):793-7. PMCID:PMC2758306

126. Patergnani S, Suski JM, Agnoletto C, Bononi A, Bonora M, De ME, Giorgi C, Marchi S, Missiroli S, Poletti F, et al. Calcium signaling around Mitochondria Associated Membranes (MAMs). Cell Commun.Signal. 2011 Sep 22;9:19. PMCID:PMC3198985

127. Rizzuto R, Marchi S, Bonora M, Aguiari P, Bononi A, De SD, Giorgi C, Leo S, Rimessi A, Siviero R, et al. Ca(2+) transfer from the ER to mitochondria: when, how and why. Biochim.Biophys.Acta 2009 Nov;1787(11):1342-51. PMCID:PMC2730423

128. Sunny NE, Bril F, Cusi K. Mitochondrial Adaptation in Nonalcoholic Fatty Liver Disease: Novel Mechanisms and Treatment Strategies. Trends Endocrinol.Metab 2017 Apr;28(4):250-60

129. Pessayre D, Fromenty B. NASH: a mitochondrial disease. J.Hepatol. 2005;42:928-40

130. Jia L, Liu Z, Sun L, Miller SS, Ames BN, Cotman CW, Liu J. Acrolein, a toxicant in cigarette smoke, causes oxidative damage and mitochondrial dysfunction in RPE cells: protection by (R)-alpha-lipoic acid. Invest Ophthalmol.Vis.Sci 2007 Jan;48(1):339-48. PMCID:PMC2597695

131. Dezest M, Le BM, Chavatte L, Desauziers V, Chaput B, Grolleau JL, Descargues P, Nizard C, Schnebert S, Lacombe S, et al. Oxidative damage and impairment of protein quality control systems in keratinocytes exposed to a volatile organic compounds cocktail. Sci Rep. 2017 Sep 6;7(1):10707. PMCID:PMC5587662

132. Knouzy B, Dubourg L, Baverel G, Michoudet C. Targets of chloroacetaldehyde-induced nephrotoxicity. Toxicol.In Vitro 2010 Feb;24(1):99-107

133. Springate JE. Ifosfamide metabolite chloroacetaldehyde causes renal dysfunction in vivo. J.Appl.Toxicol. 1997 Jan;17(1):75-9

134. Rantanen A, Jansson M, Oldfors A, Larsson NG. Downregulation of Tfam and mtDNA copy number during mammalian spermatogenesis. Mamm.Genome 2001 Oct;12(10):787-92

135. Youle RJ, van der Bliek AM. Mitochondrial fission, fusion, and stress. Science 2012 Aug 31;337(6098):1062-5. PMCID:PMC4762028

136. Chan DC. Fusion and fission: interlinked processes critical for mitochondrial health. Annu.Rev.Genet 2012;46:265-87

137. Galloway CA, Lee H, Brookes PS, Yoon Y. Decreasing mitochondrial fission alleviates hepatic steatosis in a murine model of nonalcoholic fatty liver disease. Am J Physiol Gastrointest Liver Physiol 2014 Sep 15;307(6):G632-G641. PMCID:PMC4166723

138. Eaton S, Bartlett K, Pourfarzam M. Mammalian mitochondrial beta-oxidation. Biochem.J 1996 Dec 1;320 (Pt 2):345-57. PMCID:PMC1217938

139. Grabacka M, Pierzchalska M, Dean M, Reiss K. Regulation of Ketone Body Metabolism and the Role of PPARalpha. Int J Mol Sci 2016 Dec 13;17(12). PMCID:PMC5187893

140. Aharoni-Simon M, Hann-Obercyger M, Pen S, Madar Z, Tirosh O. Fatty liver is associated with impaired activity of PPARgamma-coactivator 1alpha (PGC1alpha) and mitochondrial biogenesis in mice. Lab Invest 2011 Jul;91(7):1018-28

141. Camporeale A, Demaria M, Monteleone E, Giorgi C, Wieckowski MR, Pinton P, Poli V. STAT3 Activities and Energy Metabolism: Dangerous Liaisons. Cancers.(Basel) 2014 Jul 31;6(3):1579-96. PMCID:PMC4190557

142. Annunziata I, Sano R, d'Azzo A. Mitochondria-associated ER membranes (MAMs) and lysosomal storage diseases. Cell Death.Dis. 2018 Feb 28;9(3):328. PMCID:PMC5832421

143. Arruda AP, Pers BM, Parlakgul G, Guney E, Inouye K, Hotamisligil GS. Chronic enrichment of hepatic endoplasmic reticulum-mitochondria contact leads to mitochondrial dysfunction in obesity. Nat.Med 2014 Dec;20(12):1427-35. PMCID:PMC4412031

144. Theurey P, Tubbs E, Vial G, Jacquemetton J, Bendridi N, Chauvin MA, Alam MR, Le RM, Vidal H, Rieusset J. Mitochondria-associated endoplasmic reticulum membranes allow adaptation of mitochondrial metabolism to glucose availability in the liver. J Mol Cell Biol. 2016 Apr;8(2):129-43

145. Lim JH, Lee HJ, Ho JM, Song J. Coupling mitochondrial dysfunction to endoplasmic reticulum stress response: a molecular mechanism leading to hepatic insulin resistance. Cell Signal. 2009 Jan;21(1):169-77

146. Rieusset J. Mitochondria and endoplasmic reticulum: mitochondria-endoplasmic reticulum interplay in type 2 diabetes pathophysiology. Int J Biochem.Cell Biol. 2011 Sep;43(9):1257-62

147. Abdel-Hamid NM, Wahid A, Mohamed EM, Abdel-Aziz MA, Mohafez OM, Bakar S. New pathways driving the experimental hepatoprotective action of tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) against acute hepatotoxicity. Biomed.Pharmacother. 2016 Apr;79:215-21

148. Abouzied MM, Eltahir HM, Taye A, Abdelrahman MS. Experimental evidence for the therapeutic potential of tempol in the treatment of acute liver injury. Mol Cell Biochem. 2016 Jan;411(1-2):107-15

149. Szeto HH. Mitochondria-targeted cytoprotective peptides for ischemia-reperfusion injury. Antioxid.Redox.Signal. 2008 Mar;10(3):601-19

150. Zhao K, Zhao GM, Wu D, Soong Y, Birk AV, Schiller PW, Szeto HH. Cell-permeable peptide antioxidants targeted to inner mitochondrial membrane inhibit mitochondrial swelling, oxidative cell death, and reperfusion injury. J Biol.Chem. 2004 Aug 13;279(33):34682-90

151. Sweetwyne MT, Pippin JW, Eng DG, Hudkins KL, Chiao YA, Campbell MD, Marcinek DJ, Alpers CE, Szeto HH, Rabinovitch PS, et al. The mitochondrial-targeted peptide, SS-31, improves glomerular architecture in mice of advanced age. Kidney Int 2017 May;91(5):1126-45. PMCID:PMC5392164

152. Yang L, Zhao K, Calingasan NY, Luo G, Szeto HH, Beal MF. Mitochondria targeted peptides protect against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity. Antioxid.Redox.Signal. 2009 Sep;11(9):2095-104. PMCID:PMC2819801

153. Helm M, Motorin Y. Detecting RNA modifications in the epitranscriptome: predict and validate. Nat.Rev.Genet. 2017 May;18(5):275-91

154. Zhou J, Wan J, Gao X, Zhang X, Jaffrey SR, Qian SB. Dynamic m(6)A mRNA methylation directs translational control of heat shock response. Nature 2015 Oct 22;526(7574):591-4. PMCID:PMC4851248

ABBREVIATIONS

ALD	Alcoholic liver disease
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
Atf4	Activating transcription factor 4
ATSDR	Agency for toxic substance disease registry
BMI	Body mass index
CAA	Chloroacetaldehyde
CAE	Chloroacetate esterase
CE	Chloroethanol
CEO	Chloroethylene oxide
CHOP	Ccat-enhancer-binding protein homologous protein
Cox4	Cytochrome C oxidase 4
Cpt1a1	Carnitine palmitoyltransferase 1A
DEXA	Dual x-ray absorptiometry
Dgat2	Diacylglycerol O-acyltransferase 2

Drp1	Dynamin related protein-1
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ER	Endoplasmic reticulum
ETC	Electron transport chain
FFA	Free fatty acids
Fis1	Mitochondrial fission protein 1
G6Pase	Glucose-6-phosphotase
Gsk3β	Glycogen synthase kinase 3 beta
H&E	Hematoxylin and eosin
HFD	High fat diet
4-HNE	4-hydroxynonenol
Hsp90	Heat shock protein 90
IL-6	Interleukin-6
ITT	Insulin tolerance test
LFD	Low fat diet
MAM	Mitochondrial associated ER membrane

MCP-1	Monocyte chemotactic protein-1
MDA	Malondialdehyde
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatotis
NPC	Non-parenchymal cell
OGTT	Oral glucose tolerance test
ORO	Oil Red-O
OSHA	Occupational safety and health administration
OXPHOS	Oxidative phosphorylation
Pai-1	Plasminogen activator inhibitor 1
PAS	Periodic acid Schiff
Pgc1α	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
Pparα	Peroxisome proliferator alpha
Ppm	Parts per million
PTT	Pyruvate tolerance test
PVC	Polyvinyl chloride
RER	Respiratory exchange ratio
ROS	Reactive oxygen species

STAT-3	Signal transducer and activator of transcription factor 3
TAFLD	Toxicant-associated fatty liver disease
TASH	Toxicant-associated steatohepatitis
TAT	Thrombin anti-thrombin
TBARS	Thiobarbituic acid reactive substances
Tfam	Mitochondrial transcription factor A
TG	Triglyceride
TNFα	Tumor necrosis factor alpha
VC	Vinyl chloride
VOC	Volatile organic compound

CURRICULUM VITAE

Anna L. Lang
505 South Hancock Street, Room 552G
Louisville, KY 40202
Phone: 859-486-5887
Email: allang02@louisville.edu

Education

05/2013	B.S. in Biology, Genetics, Molecular and Cellular Track, Cum Laude
	Northern Kentucky University, Highland Heights, KY
05/2016	M.S. in Pharmacology and Toxicology
	University of Louisville, Louisville, KY
05/2018	Ph.D. in Pharmacology and Toxicology
	University of Louisville, Louisville, KY

Academic Appointments

08/2014 - 05/2016	Master's Research Assistant, Pharmacology and Toxicology, University of Louisville, Louisville, KY
05/2016 – 05/2018	Doctoral Research Assistant, Pharmacology and Toxicology, University of Louisville, Louisville, KY
07/2016 – 05/2018	NRSA (T32) Pre-doctoral Fellow, Pharmacology and Toxicology, University of Louisville, Louisville, KY

Professional Memberships and Activities

2011 – 2013	Society of Toxicology (SOT), Undergraduate Student Affiliate
2015 – present	Ohio Valley Society of Toxicology (OVSOT), Student Member
2015 – present	Society of Toxicology (SOT), Graduate Student Member
2015 – present	Society of Toxicology, Women in Toxicology
2015 – present	Society of Toxicology, Mechanisms Specialty Section

Honors and Awards

2009 – 2013 David and Ruth B. Iler Full Tuition Scholarship

2012	First Place in Best Poster Presentation: Undergraduate Student. Ohio Valley Society of Toxicology, Columbus, OH
2013	Pfizer Undergraduate Student Travel Award funded travel to SOT annual meeting, San Antonio, TX
2013	Graduation B.S. (Cum Laude), Northern Kentucky University, KY
2016	Graduation M.S., University of Louisville, KY
2016	Awarded NRSA Pre-doctoral Fellowship (T32-ES011564)
2016	3rd Place Graduate Student Poster Presentation, Research! Louisville, Louisville, KY
2016	Graduate Student Travel Award, Graduate Student Council, University of Louisville
2017	Graduate Student Travel Award, Graduate Student Council, University of Louisville
2018	OVSOT Graduate Student Representative for the annual SOT meeting, San Antonio, TX
2018	Dean's Citation Award, University of Louisville, KY
2018	Graduation Ph.D., University of Louisville, KY

Other Positions and Employments

07/2013 – 11/2013	Project Assistant, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health (NIOSH), Cincinnati, OH
11/2013 – 07/2014	Research Assistant II, Cincinnati Children's Research Hospital, Department of Psychiatry, Cincinnati, OH

Mentoring of Undergraduates, Highschool students, etc.

08/2015-05/2016	Heegook Yeo, University Honors Program, UofL, KY. (Assisted Dr. Beier with mentorship)
05/2016-present	Brenna Kaelin, undergraduate research associate, UofL, KY. (Assisted Dr. Beier with mentorship)
05/2017-08/2017	Karl Hempel, T35 NIDDK SRSP research fellow, UofL, KY. (Assisted Dr. Beier with mentorship)
05/2017-08/2017	Austin Krueger, R25 NCI undergraduate research fellow, UofL, KY. (Assisted Dr. Beier with mentorship)
08/2017-12/2017	Max Rakutt, undergraduate research associate, UofL, KY. (Assisted Dr. Beier with mentorship)

Presentations

Oral Presentations

1. Research seminar, 03/2015. Vinyl Chloride enhances alcohol-induced liver injury. University of Louisville, Department of Pharmacology and Toxicology, Louisville, KY

2. Master's Defense/Ph.D Proposal. 05/2016. Vinyl chloride-diet interactions: Potential roles of autophagy and energy management. Department of Pharmacology and Toxicology, Louisville, KY.

3. Platform presentation, 06/2016. Critical Role of Mammalian Target of Rapamycin (mTOR) in Liver Damage Caused by VC Metabolites in Mice. OVSOT summer meeting, Cincinnati, OH

4. Research symposium, 05/2017. Environment/diet interaction in fatty liver diseases. Local meeting with Nobel laureate Phillip Sharp, Louisville, KY

5. Research seminar, 06/2017. Vinyl chloride inhalation enhances experimental fatty liver disease in mice. EPA CSS Task1.1d; Steatosis AOP webinar/seminar series.

6. Platform presentation, 07/2017. Vinyl chloride inhalation exacerbates experimental fatty liver disease in mice. OVSOT summer meeting, Louisville, KY
7. Research seminar, 01/2018. Vinyl chloride inhalation enhances experimental fatty liver disease in mice. Interview with Dr. Emmanuel Thomas, University of Miami, Miami, FL.
8. Dissertation Defense. 04/2018. Vinyl chloride enhances diet-induced liver injury via metabolic homeostasis: Critical role of mitochondria. Department of Pharmacology and Toxicology, Louisville, KY.

Poster Presentations

1. Poster, 10/2015. Inhibiting mammalian target of rapamycin (mTOR) via rapamycin blunts liver damage caused by VC metabolites in mice. Research! Louisville annual meeting, Louisville, KY.

2. Poster, 03/2016. Critical Role of Mammalian Target of Rapamycin (mTOR) in Liver Damage Caused by VC Metabolites in Mice. *The Toxicologist. Supplement to Toxicological Sciences* 150(1):231.

3. Poster, 06/2016. Critical role of mammalian target of rapamycin (mTOR) in liver damage caused by VC metabolites in mice. OVSOT Student Summer Meeting, Cincinnati, OH. (Selected for Podium Presentation).

4. Poster, 10/2016. Critical Role of Mammalian Target of Rapamycin (mTOR) In Liver Damage Caused by VC Metabolites in Mice. Research!Louisville, Louisville, KY. (3rd place Graduate Student Poster Award).

5. Poster, 10/2016. Rapamycin protects liver from the enhancement of LPS induced liver injury caused by experimental vinyl chloride exposure: potential role of mTOR in toxicant/toxin interactions in mice. OVSOT annual meeting, Indianapolis, IN.

6. Poster, 11/2016. Rapamycin protects liver from the enhancement of LPS induced liver injury caused by experimental vinyl chloride exposure: potential role of mTOR in toxicant/toxin interactions in mice. *Hepatology* 64:347A.
7. Poster, 03/2017. Vinyl Chloride Inhalation Exacerbates Experimental Fatty Liver Disease in Mice. *The Toxicologist. Supplement to Toxicological Sciences* 156(1):359.
8. Poster, 07/2017. Vinyl Chloride Inhalation Exacerbates Experimental Fatty Liver Disease in Mice. OVSOT Student Summer Meeting, Louisville, KY. (Selected for Podium Presentation).
9. Poster, 10/2017. Vinyl Chloride Inhalation Exacerbates Experimental Fatty Liver Disease in Mice. Research!Louisville, Louisville, KY.
10. Poster, 10/2017. Vinyl chloride inhalation causes mitochondrial dysfunction and exacerbates experimental fatty liver disease in mice. *Hepatology*, 66:415A.
11. Poster, 03/2018. Vinyl chloride inhalation causes hepatic metabolism dysfunction in mice. SOT annual meeting, San Antonio, TX.

Abstracts

Local/Regional

12. Bushau AM, Anders LC, Douglas AN, Poole LG, Massey VL, **Lang AL**, Falkner KC, Cave M, McClain CJ and Beier JI (2014) Mechanistic Insight Into Vinyl Chloride-Induced Liver Injury. Research!Louisville. Louisville, KY.
13. Anders LC, Bushau AM, Douglas AN, **Lang AL**, Falkner KC, Arteel GE, Cave MC, McClain MJ and Beier JI (2014) Exposure to Vinyl Chloride Metabolites Exacerbates Liver Injury Caused by High Fat Diet in Mice. Research!Louisville. Louisville, KY. Research!Louisville, Louisville, KY. (Basic Science Research Faculty Award).
14. Anders LC, Bushau AM, Douglas AN, **Lang AL**, Falkner KC, Arteel GE, Cave MC and McClain MJ and Beier JI (2014) Exposure to Vinyl Chloride Metabolites Exacerbates Liver Injury Caused by High Fat Diet in Mice. American College of Physicians (ACP) Kentucky Chapter Meeting, Louisville, KY.
15. Bushau AM, Anders LC, Douglas AN, Poole LG, Massey VL, **Lang AL**, Falkner KC, Cave M, McClain CJ and Beier JI (2014) Mechanistic Insight Into Vinyl Chloride-Induced Liver Injury. OVSOT annual meeting, Dayton, OH.
16. Bushau AM, Anders LC, Douglas AN, Poole LG, Massey VL, **Lang AL**, Falkner KC, Cave M, McClain CJ and Beier JI (2015) Mechanistic Insight Into Vinyl Chloride-Induced Liver Injury. Posters at the Capitol, Frankfort, KY.
17. **Lang AL**, Kaelin BR, Yeo H, Hudson SV, McKenzie CM, Sharp CN, Poole LG, Arteel GE, and Beier JI (2015) Inhibiting mammalian target of rapamycin (mTOR) via rapamycin blunts liver damage caused by VC metabolites in mice. Research! Louisville annual meeting, Louisville, KY.
18. Kaelin BK, Bushau AM, Douglas AN, **Lang AL**, Falkner KC, Arteel GE, Cave MC, McClain MJ and Beier JI (2015) Mechanistic Insight Into Vinyl Chloride-Induced Liver Injury: Role of Dietary Fatty Acids. Research! Louisville annual meeting, Louisville, KY.

19. McKenzie CM, Anders LC, Poole LG, Hudson SV, Bushau AM, **Lang AL**, Arteel GE, McClain MJ and Beier JI (2015) Enhancement of NAFLD Risk by Vinyl Chloride: Role of Adipose Tissue in a Mouse Model. Research! Louisville annual meeting, Louisville, KY.
20. Kaelin BK, Bushau AM, Douglas AN, **Lang AL**, Falkner KC, Arteel GE, Cave MC, McClain MJ and Beier JI (2016) Mechanistic Insight Into Vinyl Chloride-Induced Liver Injury: Role of Dietary Fatty Acids. Southern Regional Honors Council: 2016 Conference. Orlando, FL.
21. **Lang AL**, Kaelin BR, Yeo H, Hudson SV, McKenzie CM, Sharp CN, Poole LG, Arteel GE, and Beier JI (2016) Critical Role of Mammalian Target of Rapamycin (mTor) in Liver Damage Caused by VC Metabolites in Mice. *OVSOT Student Summer Meeting*, Cincinnati, OH. (Selected for Podium Presentation).
22. **Lang AL**, Kaelin BR, Yeo H, Sharp CN, Arteel GE, and Beier JI (2016) Critical Role Of Mammalian Target Of Rapamycin (mTOR) In Liver Damage Caused By VC Metabolites In Mice. Research!Louisville, Louisville, KY. (3rd place Graduate Student Poster Award).
23. **Lang AL**, Kaelin BR, Yeo H, Poole LG, Arteel GE and Beier JI (2016) Rapamycin protects liver from the enhancement of LPS induced liver injury caused by experimental vinyl chloride exposure: potential role of mTOR in toxicant/toxin interactions in mice. OVSOT annual meeting, Indianapolis, IN.
24. Kaelin BK, Bushau AM, Douglas AN, **Lang AL**, Falkner KC, Arteel GE, Cave MC, McClain MJ and Beier JI (2016) Mechanistic Insight Into Vinyl Chloride-Induced Liver Injury: Role of Dietary Fatty Acids. Posters at the Capitol, Frankfort, KY.
25. **Lang AL**, Chen L, Poff GD, and Beier JI (2017) Vinyl Chloride Inhalation Exacerbates Experimental Fatty Liver Disease in Mice. *OVSOT Student Summer Meeting*, Louisville, KY. (Selected for Podium Presentation).
26. Chen L, **Lang AL**, and Beier JI (2017) Vinyl Chloride exacerbates liver injury induced by high-fat diet via causing ALDH2 dysfunction in mice. *OVSOT Student Summer Meeting*, Louisville, KY.
27. **Lang AL**, Chen L, Poff GD, and Beier JI (2017) Vinyl Chloride Inhalation Exacerbates Experimental Fatty Liver Disease in Mice. Research!Louisville, Louisville, KY.
28. Chen L, **Lang AL**, and Beier JI (2017) Vinyl Chloride exacerbates liver injury induced by high-fat diet via causing ALDH2 dysfunction in mice. Research!Louisville, Louisville, KY.
29. Hempel KW, **Lang AL**, Head KZ, and Beier JI (2017) Effect of Vinyl Chloride Metabolites on 3T3-L1 Adipocytes. Research!Louisville, Louisville, KY.
30. Krueger AM, **Lang AL**, Kaelin BK, and Beier JI (2017) Mechanisms by which Rapamycin Protects from Liver Damage Caused by VC Metabolites in Mice. Research!Louisville, Louisville, KY.

National

1. Bushau AM, Anders LC, Douglas AN, **Lang AL**, Joshi-Barve S, Poole LG, Massey VM, Falkner KC, Cave M, McClain CJ and Beier JI (2015) Mechanistic Insight Into

Vinyl Chloride-Induced Liver Injury. *The Toxicologist. Supplement to Toxicological Sciences* 144:25.

2. Anders LC, Douglas AN, Bushau AM, **Lang AL**, Falkner KC, Arteel GE, Cave M, McClain CJ and Beier JI (2015) Exposure to Vinyl Chloride Metabolites Exacerbates Liver Injury Caused by High Fat Diet in Mice. *The Toxicologist. Supplement to Toxicological Sciences* 144:26.
3. Anders LC, Bushau AM, **Lang AL**, Falkner KC, Arteel GE, Cave M, McClain CJ and Beier JI (2015) Mechanistic Insight Into Vinyl Chloride Metabolite-Induced Liver Injury Caused by High Fat Diet in Mice. *Gastroenterology* 148(4): S-980. (Selected for oral presentation).
4. Anders LC, Bushau AM, **Lang AL**, Arteel GE, Cave MC, McClain CJ and Beier JI (2015) Inflammasome Activation Due to Vinyl Chloride Metabolite Exposure in NAFLD Caused by High Fat Diet in Mice. *Hepatology*, 62(1):1250A. (Resident Research Award).
5. **Lang AL**, Kaelin BR, Yeo H, Hudson SV, McKenzie CM, Sharp CN, Poole LG, Arteel GE, and Beier JI (2016) Critical Role of Mammalian Target of Rapamycin (mTor) in Liver Damage Caused by VC Metabolites in Mice. *The Toxicologist. Supplement to Toxicological Sciences* 150(1):231.
6. Kaelin BK, Bushau AM, Douglas AN, **Lang AL**, Falkner KC, Arteel GE, Cave MC, McClain MJ and Beier JI (2016) Mechanistic Insight Into Vinyl Chloride-Induced Liver Injury: Role of Dietary Fatty Acids. ACC Meeting of the Minds Undergraduate Research Conference. Book of Abstracts: 22.
7. Anders LC, Yeo H, Kaelin BR, Bushau AM, **Lang AL**, Arteel GE, McClain CJ and Beier JI (2016) Role of Dietary Fatty Acids in Liver Injury Caused by Vinyl Chloride Metabolites in Mice. *Hepatology* 64:769A. (Presidential Poster of Distinction).
8. **Lang AL**, Kaelin BR, Yeo H, Poole LG, Arteel GE and Beier JI (2016) Rapamycin protects liver from the enhancement of LPS induced liver injury caused by experimental vinyl chloride exposure: potential role of mTOR in toxicant/toxin interactions in mice. *Hepatology* 64:347A.
9. **Lang AL**, Chen L, and Beier JI (2017) Vinyl Chloride Inhalation Exacerbates Experimental Fatty Liver Disease in Mice. *The Toxicologist. Supplement to Toxicological Sciences* 156(1):359.
10. **Lang AL**, Chen L, Poff GD, and Beier JI (2017) Vinyl chloride inhalation causes mitochondrial dysfunction and exacerbates experimental fatty liver disease in mice. *Hepatology*, 66:415A.
11. Chen L, **Lang AL**, and Beier JI (2017) Vinyl Chloride exacerbates liver injury induced by high-fat diet via causing ALDH2 dysfunction in mice. *Hepatology*, 66:413A.
12. Kaelin BK, McKenzie CM, Hempel KW, **Lang AL**, and Beier JI (2017) Enhancement of NAFLD Risk by Vinyl Chloride: Role of Adipose Tissue in a Mouse Model. *Hepatology*, 66:153A.
13. **Lang AL**, Chen L., Poff GD, Beier JI, (2018). Vinyl chloride inhalation causes hepatic metabolism dysfunction in mice. *The Toxicologist. Supplement to Toxicological Sciences* 162(1):1131.

Publications

Complete List of Published Work:

1. Curran CP, Altenhofen E, Ashworth A, Brown A, Kamau-Cheggeh C, Curran M, Evans A, Floyd R, Fowler JC, Garber H, Hays B, Kraemer S, **Lang AL**, Mynhier A, Samuels A, Strohmaier C. Ahrd Cyp1a2(-/-) mice show increased susceptibility to PCB-induced developmental neurotoxicity. *Neurotoxicology* 33(6):1436-42. PMID: 22935098. PMCID: PMC3518762

2. Anders LA, **Lang AL**, Anwar-Mohamed A, Douglas AD, Bushau AM, Falkner KC, Hill BG, Warner NL, Arteel GE, Cave M, McClain CJ and Beier JI. Vinyl Chloride Metabolites Potentiate Inflammatory Liver Injury caused by LPS in Mice. *Toxicol Sci.* 2016; 151(2):312-23. PMID: 26962056. PMCID: PMC4880135. (Data highlighted on journal cover page)

3. Anders LA, Yeo H, Kaelin BR, Bushau AM, **Lang AL**, Douglas AD, Cave M, Arteel GE, McClain CJ and Beier JI. Role of Dietary Fatty Acids in Liver Injury Caused by Vinyl Chloride Metabolites in Mice. *Toxicol Appl Pharmacol.* 2016; 311:33-41. PMID: 27693805. PMCID: PMC5079761

4. Schaefer, T.L.; Davenport, M.H.; Grainger, L.M. Robinson, C.K.; Earnheart, A.T.; Stegman, M.S.; **Lang, A.L.**; Ashworth, A.A.; Molinaro, G.; Huber, K.M.; Erickson, C.A. Acamprosate in a mouse model of Fragile X Syndrome: modulation of UP states, ERK1/2 activation, locomotor behavior, and anxiety. *Journal of Neurodevelopmental Disorders* 2017; 9:6. PMID: 28616095. PMCID: PMC5467053

5. **Lang AL**, Chen L, Poff GD, Ding WX, Arteel GE, and Beier JI. Vinyl chloride dysregulates metabolic homeostasis and enhances diet-induced liver injury in mice. *Hepatol Commun.* 2018; 2(3): 270-284.PMID: 29507902. PMCID: PMC5831023

6. Colter BT, Garber HF, Fleming SM, Fowler JP, Harding GD, Hooven MK, Howes AA, Infante SK, **Lang AL**, MacDougall MC, Stegman M, Taylor KR, Curran CP. Ahr and Cyp1a2 genotypes both affect susceptibility to motor deficits following gestational and lactational exposure to polychlorinated biphenyls. *Neurotoxicology* 2018, 65:125-134. PMID: 29409959. PMCID: PMC5857246

7. **Lang AL** and Beier JI. Interaction of volatile organic compounds and underlying liver disease: a new paradigm for risk. *Biol Chem.* Invited review, revision under review.

8. Liang Y, **Lang AL**, Zhang J, Chen J, Wang K, Chen L, Beier JI, Qian Y, and Cai L. Exposure to vinyl chloride and its influence on western diet-induced cardiac remodeling. *Chem Res Toxicol.*, revision under review.

9. **Lang AL**, Kreuger A, Kaelin BR, Chen L, Rakutt MJ, Beier JI. Rapamycin attenuates liver injury caused by VC metabolite exposure. *Toxicol Sci.* Submitted.

10. Kaelin BR, Yeo H, Anders LA, **Lang AL**, Beier JI. Vinyl chloride metabolite exposure: role of the adipose: liver axis. In preparation.