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Vinyl chloride-diet interactions in liver disease : potential roles of autophagy and energy management.

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VINYL CHLORIDE-DIET INTERACTIONS IN LIVER DISEASE:
POTENTIAL ROLES OF AUTOPHAGY AND ENERGY MANAGEMENT

By

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B.S Northern Kentucky University, 2013

A Thesis Submitted to the Faculty of the
School of Medicine of the University of Louisville
In Partial Fulfillment of the
Requirements for the Degree of

Master of Science

In Pharmacology and Toxicology

Department of Pharmacology and Toxicology

University of Louisville

Louisville, KY

August 2016

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ACKNOWLEDGMENTS

First, I would like to thank my mentor, Dr. Juliane Arteel, for her encouragement and guidance over the past two years. I would like to thank her for encouraging me to think independently as a scientist and her continued support in my graduate career. I would like to thank Dr. Gavin Arteel for constantly pushing me to do my best. I would like to thank my undergraduate mentor, Dr. Christine Curran, for cultivating in me the scientific curiosity that led to where I am today.

I would also like to thank my parents, Frank and Linda Lang, for their constant love, support, and understanding.

ABSTRACT

VINYL CHLORIDE-DIET INTERACTIONS IN LIVER DISEASE: POTENTIAL ROLES OF AUTOPHAGY AND ENERGY MANAGEMENT

Anna L. Lang

May 12, 2016

Vinyl chloride (VC) is a prevalent environmental toxicant that has been shown to cause liver injury at high, occupational exposures. However, most studies have not addressed interactions of low doses with risk-modifying factors. This study aims to explore low-level VC metabolite exposure interactions with other potential risk-modifying factors and their effect on underlying liver disease. We examined sub-hepatotoxic effects of a VC metabolite (chloroethanol, CE) in two murine models of liver injury using ethanol and lipopolysaccharide (LPS). In both, CE significantly enhanced liver injury when compared to either ethanol or LPS alone. Previous studies have shown an increase in mTOR activity with CE alone. Here, we used a pharmacologic inhibitor of mTOR, rapamycin, to study its effect on injury progression. Indeed, the addition of rapamycin significantly attenuated liver injury, hepatic steatosis, and inflammatory markers in the CE + LPS model.

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INTRODUCTION

I. Vinyl Chloride

Vinyl chloride monomer (VC) is a pervasive environmental organochlorine toxicant. It is ranked #4 on the CDC's Agency for Toxic Substances and Disease Registry (ATSDR) Substance Priority List;(1) with an annual production recently estimated at 27 million metric tons (2). VC is used in industry to produce polyvinyl chloride (PVC), a plastic involved in commercial production. Facilities that manufacture PVC products emit VC gas into the air as a waste product. VC is also present as a solvent at many EPA Superfund sites across the United States. Aside from being a direct contaminant at these sites, VC is a degradation product of other chlorinated chemicals such as trichloroethylene (TCE; ATSDR rank #16) tetrachloroethylene (PCE; ATSDR rank #33) and dichloroethene (DCE; ATSDR rank #216) via soil microorganisms (3). Owing to its widespread prevalence, VC is present in landfill leachates and many military Superfund sites across the country. For example, up to 1,000,000 military, civilian personnel, and their families were exposed to VC at Camp Lejeune, NC alone (3). Additionally, more than 80,000 American chemical workers have been occupationally exposed to VC (4, 5).

VC is present in the air surrounding production facilities at significant concentrations. A major route of environmental exposure stems from

contaminated groundwater. Residential areas surrounding both manufacturing and Superfund sites are susceptible to VC migrating through soil and home foundations where it readily volatilizes to enter showers, basements, and living spaces where these vapors can recirculate (4, 5). Due to the high risk of low-level human exposure in residential areas around VC-using facilities, understanding the effects of this toxicant on human health is necessary, but current knowledge is sparse on low-level VC exposure.

The major target of VC exposure is the liver. VC is a known hepatotoxicant and at high, occupational levels of exposure, VC can cause steatosis (fat accumulation), inflammation (steatohepatitis), fibrosis, and necrosis (6). Recently, a pathology unique to VC exposure has been described, toxicant-associated steatohepatitis (TASH), in chemical plant workers occupationally exposed to VC (7, 8). These individuals showed most of the typical clinical signs of liver injury. Within that cohort, 84% of patients exhibited hepatic steatosis and 85% exhibited steatohepatitis. However, of the patients with steatohepatitis, only 55% went on to develop fibrosis. Interestingly, plasma transaminase levels, which are typically elevated with liver injury, were at normal levels in these individuals (8). Although the effects of high level VC exposure have been studied, the effects of low level exposure and its interactions with other risk-modifying factors, such as diet or alcohol consumption, on hepatic and overall health have not been determined. Therefore, it is crucial that these knowledge gaps are filled.

II. Liver Diseases

The liver is a major site of nutrient metabolism, detoxification, and is crucial to maintaining a healthy digestive system. Liver function is determined not only by genetics, but by diet, alcohol consumption, and toxicant exposure. As such, the liver is susceptible to injury from a variety of agents. The most common manifestation of liver damage is fatty liver disease (FLD), which is characterized by an accumulation of triglycerides in the liver (9). Depending on the source of the insult, it can be termed alcoholic liver disease (ALD) or non-alcoholic fatty liver disease (NAFLD). Both diseases share similar pathological manifestations, but have unique etiologies. In general, liver disease is a spectrum of pathologies ranging from benign steatosis to active inflammation (steatohepatitis) to fibrosis, cirrhosis (10), and potentially, hepatocellular carcinoma (11, 12). Processes hypothesized that contribute to the initiation and progression of FLD include lipotoxicity, (13) oxidative stress (13, 14) and inflammation (15, 16).

Although many people have FLD, only a fraction of that population will progress from steatosis to a more severe form of the disease, such as steatohepatitis (17, 18). Many factors play a role in determining severity of disease and its progression, including genetics and environmental agents. Due to an increase in triglycerides within hepatocytes, regular fatty acid metabolism is disrupted, leading to alterations in other metabolic pathways such as protein and carbohydrate metabolism (19). These changes on a molecular level cause the liver to be more susceptible to insult from a second agent, such as VC. The effects of low-level VC exposure on underlying liver injury have not been studied.

It is known that the liver becomes susceptible to injury from a second agent after changes occur in response to a first agent (20, 21). For example, Yang et al. (21) demonstrated that livers from genetically obese (fa/fa) rats have enhanced sensitivity to inflammation produced by the injection of bacterial lipopolysaccharide (LPS) compared to their lean littermates; this exacerbation of liver damage is characterized by a more robust inflammatory response and enhanced cell death. Recent studies from our group have demonstrated that sub-hepatotoxic doses of a major VC metabolite, chloroethanol (CE), enhanced liver injury in response to LPS administration in a murine model (22). In that study, animals who received CE alone showed no histological changes in their liver tissue morphology. However, on a protein level, many key metabolic enzymes were altered. There was also an increase in steatosis and a decrease in glycogen stores, indicating alterations in lipid and carbohydrate metabolism. The combination of both CE and LPS caused enhanced damage when compared to either exposure alone as characterized by enhanced inflammatory cytokine expression, histology and gross liver damage (22). This study demonstrates a proof-of-concept that low levels of a VC metabolite can enhance liver injury caused by LPS administration. Alterations in hepatocyte metabolic enzymes and pathways that are not overly toxic can sensitize the liver to be more susceptible to an insult from another agent. Although the hypothesis of multiple “hits” in liver injury progression is not a new one, the application of this paradigm to explore low-level VC exposure and underlying liver damage is novel.

A. Alcohol and the liver

Millions of people worldwide consume alcoholic beverages. According to the World Health Organization (WHO), 3.3 million (5.9%) of deaths worldwide are due to the harmful use of alcohol (23). ALD encompasses several pathologies from mild fat accumulation (steatosis), liver inflammation (alcoholic steatohepatitis; ASH), to severe fibrosis, and cirrhosis (end-stage liver disease). Cirrhosis of the liver is the 12th leading cause of death in the United States and in 2013, nearly 50% of all liver disease-related deaths were caused by ALD (24).

As the liver is the primary location of alcohol metabolism, it is the organ most affected by alcohol-induced injury. Alcohol is metabolized via alcohol dehydrogenase (ADH1), mitochondrial aldehyde dehydrogenase (ALDH2), and CYP2E1. A majority of its detrimental effects are a result of its toxic metabolite, acetaldehyde, accumulation, induction of an inflammatory response, and oxidative stress (25).

In ALD, steatosis can be caused directly by ethanol metabolites' disruption of the balance between NAD^+ and NADH , leading to inhibition of β -oxidation and an accumulation of free fatty acids (FFA) and triglycerides (25). Alcohol exposure can also alter transcription factors that mediate lipid-metabolizing enzymes. For example, acetaldehyde can directly upregulate sterol regulatory element-binding protein 1c (SREBP-1c) resulting in an increase in lipogenesis. Conversely, acetaldehyde interacts with the DNA binding domain of peroxisome proliferator activator protein alpha ($\text{PPAR}\alpha$), which inhibits the breakdown of FFA via β -oxidation (18). Combined, the result is an increase of lipids within the tissue. Another major mediating factor of alcohol-induced liver injury is the inflammatory

response, particularly the cytokine tumor necrosis factor alpha (TNF α). Upon stimulation by alcohol consumption, TNF α is released from the liver's resident macrophages, known as Kupffer cells. It is upregulated and is involved in tissue damage in response to alcohol exposure (26). The production of TNF α results in recruitment of other inflammatory cells, such as neutrophils, as their infiltration into tissue results in a more pronounced inflammatory response (18).

The combination of increased hepatic lipids and increased cytokine and inflammatory cell recruitment allows for a cyclic pattern of liver damage. If alcohol consumption continues, injury worsens and is a risk factor for developing more serious injury. Due to the fact that VC and alcohol share several mechanisms of liver injury, alcohol consumption may be a risk factor for developing more serious liver disease when co-exposed with VC.

B. Obesity and the liver

Over 30% of US adults are obese (BMI \geq 30) with another >30% being overweight (BMI = 25-30) (27). This metabolic syndrome is characterized by insulin resistance, hypertension, and dyslipidemia (28). Due to overconsumption of dietary fats, NAFLD is a major hepatic manifestation of obesity and it was recently estimated that 25% of the global population has NAFLD (29). Hepatic steatosis occurs when the balance between fatty acid synthesis and uptake is greater than its oxidation (30). This causes a disruption in energy homeostasis and an accumulation of lipid droplets to occur within the liver tissue. NAFLD is also associated with other metabolic dysfunction manifestations such as insulin

resistance and type 2 diabetes, and can be mediated by the inflammatory response (31).

Within the past decade, the perceived role of adipocytes has shifted from simple lipid storing vessels to more complex mediators of metabolic, hormonal, and inflammatory responses (32). Obesity causes an increase in the size of adipocytes within adipose tissue and immune cells, such as macrophages, are able to infiltrate into the adipose tissue and release cytokines such as TNF α and interleukin-6 (IL-6) (33, 34), which cause further stress and are able to travel through circulation and exert effects on the liver and other organs. In addition to the cytokines being released from the adipose tissue, an increase in hepatic adiposity also results in an increase in hepatic pro-inflammatory cytokine release. These cytokines, like TNF α , activate Kupffer cells, which then release more inflammatory signals and cause tissue damage (35). These metabolic changes within hepatocytes, such as lipid accumulation, make these cells more susceptible to insults from other factors and may result in exacerbated liver injury upon co-exposure with toxicants, such as VC.

III. VC Mechanism

A. VC metabolism

As mentioned previously, VC is metabolized by many of the same enzymes that are involved in alcohol metabolism. After exposure via inhalation, absorption is rapid and distribution is widespread. Due to the fact that the enzymes involved in VC metabolism are primarily located in the liver, it is the most sensitive to VC exposure. There are several pathways that VC can be metabolized into its reactive

intermediates which all follow Michaelis-Menten enzyme kinetics (36). The first pathway involves microsomal mixed function oxidases, primarily CYP2E1, which oxidizes VC to 2-chloroethylene oxide, which is then conjugated to glutathione and excreted. An alternate pathway of VC metabolism is oxidation to 2-chloroethanol. 2-chloroethanol can then enter into the same pathway as ethanol metabolism. It is transformed into 2-chloroacetaldehyde by alcohol dehydrogenase. Aldehyde dehydrogenase converts 2-chloroacetaldehyde into chloroacetic acid which is then conjugated to glutathione and excreted in urine, ultimately, as thioacetic acid (37).

VC is inhaled and absorbed through the lungs and its half-life is relatively short, being only a few hours. Therefore, it is not likely that VC gas directly damages tissue. Its two main reactive metabolites, chloroethanol (CE) and chloroacetaldehyde (CAA), are able to enter cells and cause damage. Once inside the cell, VC metabolites are able to generate reactive oxygen and nitrogen species (ROS/RNS) and form protein adducts. These then go on to further damage the cells by causing oxidative damage, increasing inflammatory cytokine recruitment (22), and increasing endoplasmic reticulum (ER) stress.

B. VC metabolites damage mitochondria

Mitochondria are essential to the health of all cells as they produce ATP and regulate major cell death pathways such as apoptosis and necrosis. Mitochondria respond dynamically to stress, nutritional status, and environmental signals. It has been hypothesized that the VC metabolite, CAA, inhibits oxidative phosphorylation in mitochondria (38, 39) and our group has recently shown that CAA is mitotoxic *in vitro* and *ex vivo*. Data from that study demonstrate that CAA

decreases mitochondrial membrane potential in a concentration-dependent manner, and with increasing concentrations, decreases plasma membrane potential as well. This finding was corroborated by cellular bioenergetics analyses that showed a decrease in ATP production and decreased oxygen consumption rate with increasing CAA concentrations (22).

Another aspect of mitochondrial damage besides impaired function is the accumulation and elimination of damaged mitochondria. This is accomplished by the complex process of autophagy.

C. Autophagy

Autophagy is the intracellular process of “self-eating” or degradation of macromolecules and organelles during times of starvation or stress to provide nutrients or to protect the cell. Autophagy is a highly conserved process and as such so are the proteins involved. Several autophagy related genes (Atg's) have been identified along with associated proteins which aid in the degradative process (40, 41). There are four main phases of autophagy (Figure 1). The first involves initiation and formation of the autophagosome. It begins as an isolated membrane portion (the phagophore) from the ER or golgi apparatus (42). Through nucleation and expansion of the membrane, the phagophore becomes an autophagosome; a double membraned structure that engulfs targeted macromolecules, proteins, or organelles for degradation. The autophagosome matures and fuses with a lysosome, releasing its contents for enzymatic degradation (43).

Autophagy is crucial for normal cell homeostasis and has been shown to be a critical player in several models of liver disease. Its activation has been shown

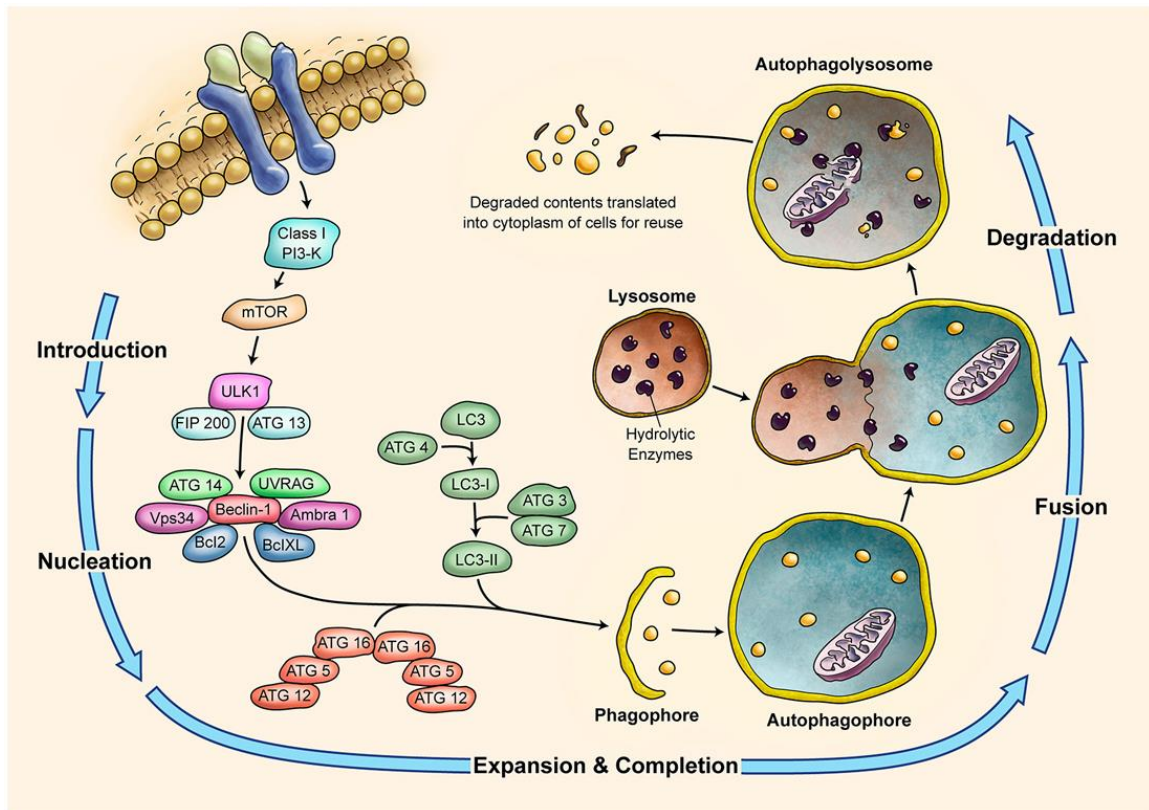


Figure 1. Scheme of autophagic pathway. The main stages of autophagy are depicted. Autophagy begins with introduction or initiation of the process. This is followed by nucleation, phagophore formation, and autophagosome expansion. Finally, the autophagosome fuses with a lysosome and degradation of its contents (44).

to be protective (45-47) while its inhibition has been associated with increased liver damage (48, 49). One probable mechanism of its protective effects is the removal of damaged organelles, such as mitochondria (mitophagy) (50, 51). If the affected cells are unable to clear and degrade damaged mitochondria, their accumulation would add to the cellular injury and further impair normal cell function.

mTOR, (mechanistic target of rapamycin) is a major regulator of autophagy. With mTOR being the major sensor for nutritional status of the cell, its activation blocks initiation of autophagy while mTOR inhibition allows autophagy to proceed (52). As mentioned previously, our group recently showed that mTOR is activated in mice administered VC metabolites alone. We hypothesize that pharmacologically inhibiting mTOR with rapamycin would allow us to determine if autophagy inhibition is involved in liver damage caused by VC metabolite exposure.

IV. Overall significance

VC is present in the ambient air and ground water surrounding chemical and PVC production facilities, making residents living in those areas at risk for low-level VC exposure. Many of the at-risk areas are urbanized and of a lower socioeconomic status than more suburban residential areas. Other risk factors associated with highly urban populations are an increased risk for alcohol consumption and a high fat diet resulting in being overweight or obese. Both of these factors cause changes on the molecular level in the liver, such as steatosis, and cause these individuals to be more susceptible to a second hepatic insult.

There is a knowledge gap regarding co-exposure of underlying liver disease such as ALD or NAFLD and the effects of low-level, subclinical VC exposure. Therefore, the goals of the current work are two-fold: a) to determine whether VC exposure may be acting to enhance liver injury in underlying ALD and b) to determine the role of mTOR in liver injury progression caused by VC exposure and whether or not activation of autophagy could have protective effects in an experimental model of liver injury involving VC metabolites and LPS.

MATERIAL AND METHODS

I. Animals and Treatments

Eight 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 local Institutional Animal Care and Use Committee.

A. Acute-on-chronic alcohol exposure

Mice were fed either an ethanol-containing Lieber-DeCarli diet or an iso-calorically matched control diet. Because of the relatively high caloric content of ethanol, pair-fed control animals received an iso-caloric control diet; the calories in the iso-caloric diet were matched by adding a calorie equivalent of maltose-dextrin. During the exposure period, animals were housed in pairs in shoebox cages in a room held at 75°F. Diet was provided in vacuum tubes and replaced between 3 and 5 pm daily. Both ethanol-fed animals and their pair-fed counterparts received control diet for the first five days of liquid diet feeding to allow acclimation to the liquid diet feeders. On the sixth day, ethanol-fed animals received diet containing 5% (vol/vol) ethanol. They continued to receive the ethanol-containing diet for 10 days. On the eleventh day, the two diet groups were further separated into additional groups that either received a bolus gavage of chloroethanol (CE; 50 mg/kg) or vehicle (saline), modified from Bertola et al., (53). The concentration of CE was

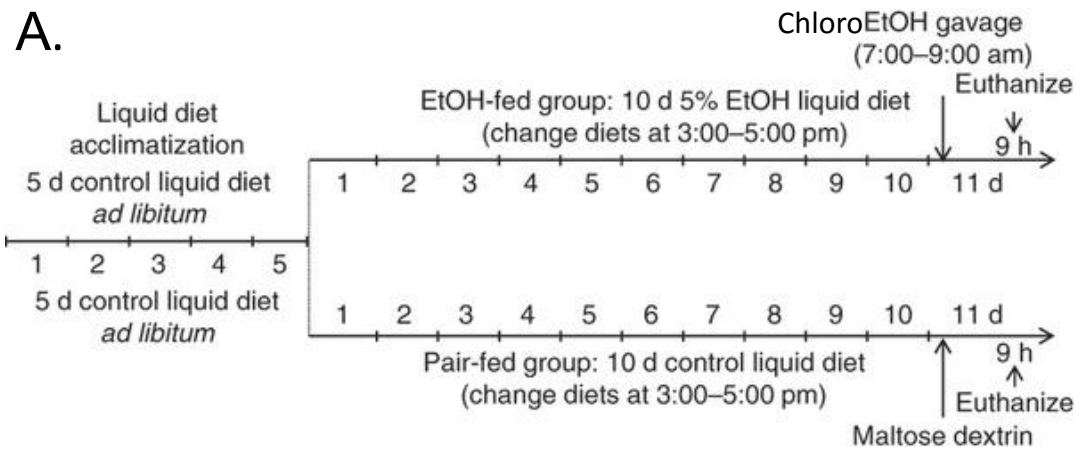
determined by others to not directly cause liver damage (54) and was validated (22); mice were ethanol fasted for 4 hours prior to oral gavage and were sacrificed 4, 9, or 24 hours later (Figure 2A).

B. Rapamycin administration

Mice were administered CE (50 mg/kg) via oral gavage. One hour after, rapamycin (1mg/kg, i.p., LC Laboratories, Woburn, MA,) was administered. 24 hours after CE administration, mice were administered LPS (*E. Coli*, 10 mg/kg, i.p., Sigma, St. Louis, MO) and sacrificed 24 hours later (Figure 2B).

C. Sample collection

At the time of sacrifice, animals were anesthetized with ketamine/xylazine (100/15 mg/kg, i.p.). Blood was collected from the vena cava just prior to sacrifice (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.



B.

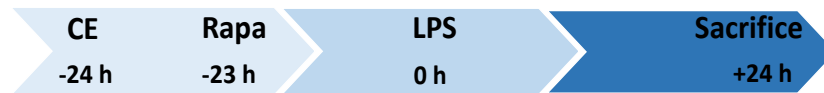


Figure 2. Timelines of experiments. Panel A shows the NIAAA model timeline modified with the CE bolus gavage on the eleventh day. Panel B depicts the timeline for CE, rapamycin, and LPS administration.

II. Biochemical Analysis

Plasma activity levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined spectrophotometrically using standard kits (Thermotrace, Melbourne, Australia).

III. Histology

A. General Morphology

Formalin fixed, paraffin embedded liver tissue was cut at 5 μm and mounted on charged glass slides. Sections were deparaffinized with Citrisolv (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 alcohol, washed in Citrisolv and then mounted with Permount (Fisher, Waltham, MA).

B. Neutrophil accumulation

Neutrophil accumulation in liver tissue was measured using chloracetate esterase (CAE) staining. Briefly, formalin fixed, paraffin embedded liver tissue was cut 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. Tissue specimens were incubated in a solution of naphthol AS-D chloroacetate (1 mg/ml) in N,N-dimethylformamide, with 4% sodium nitrite and 4% new fuchsin. The naphthol AS-D chloroacetate is enzymatically hydrolyzed by chloroacetate esterase in neutrophils, liberating the naphthol compound. Naphthol

combines with a freshly formed diazonium salt, leaving bright pink color deposits at the site of enzymatic activity.

C. Apoptosis

Apoptosis was detected via terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL; Millipore, Billerica, MA). In brief, formalin fixed, paraffin embedded liver tissue was cut 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. TUNEL-positive cells (hepatocytes and non-parenchymal cells, NPCs) were counted using Image J software and are expressed as positive cells per 1000 hepatocytes.

D. Metabolism

Lipid accumulation was detected via Oil Red-O (ORO) staining. Frozen sections of liver were cut at 10 μm and stained with Oil Red-O solution (Sigma, St. Louis, MO) for 10 minutes, washed, and counterstained with hematoxylin for 45 seconds. Samples were then 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 cut 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, washed in water, and incubated with Schiff reagent for 15 minutes. Samples were then counterstained

with hematoxylin for 45 seconds, washed in water, dehydrated through graded ethanol, washed in Citrisolv, and then mounted with Permount.

IV. Immunoblots

Liver samples were homogenized in RIPA buffer (55), containing protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO). Samples were loaded onto SDS-polyacrylamide gels (Invitrogen, Thermo Fisher Scientific, Grand Island, NY), followed by electrophoresis and Western blotting onto PVDF membranes (Hybond P, GE Healthcare Bio-Sciences, Pittsburgh, PA). Primary antibodies against phosphorylated and total mTOR, total GAPDH, and total LC-3 I/II (Cell Signaling Technology; Beverly, MA) were used. Densitometric analysis was performed using UN-SCAN-IT gel (Silk Scientific Inc., Orem, UT) software.

V. RNA Isolation and Real-Time RT-PCR

RNA was extracted immediately following sacrifice from fresh liver samples using RNA Stat60 (Tel-Test, Ambion, Austin, TX) and chloroform. RNA concentrations were determined spectrophotometrically and 1 μ g of total RNA was reverse transcribed using a kit (Quanta Biosciences, Gaithersburg MD). Real-time RT-PCR was performed using a StepOne real time PCR system (Thermo Fisher Scientific, Grand Island, NY). Primers and probes were ordered as commercially available kits (Thermo Fisher Scientific, Grand Island, NY). The comparative C_T method was used to determine fold differences between the target genes and an endogenous reference (18S).

VI. Statistical Analyses

Results are reported as means \pm SEM (n = 4-8) and were analyzed using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA). ANOVA with Bonferroni's post-hoc test (for parametric data) or Mann-Whitney Rank Sum test (for nonparametric data) were used for the determination of statistical significance among treatment groups, as appropriate. A *p* value less than 0.05 was selected before the study as the level of significance.

RESULTS

CE exacerbates liver injury caused by ethanol

In order to assess status of overall liver health in the given groups, plasma transaminase levels were measured as an index of liver injury. Figure 3A shows plasma transaminase levels at 4, 9, and 24 hours after CE administration. Pair-fed mice that did or did not receive CE showed no elevation in either ALT or AST at any time point. However, ethanol-fed mice who received CE show a significant increase in AST levels at the 24 hour time point. Figure 3B depicts representative photomicrographs of general liver morphology at the 9 hour time point as seen through H&E staining. Neither pair-fed control nor pair-fed CE animals showed signs of tissue damage. Ethanol-fed animals showed an increase in inflammatory cell infiltration. CE enhanced these effects, increasing inflammatory and necrotic foci as highlighted in Figure 3B with an arrow.

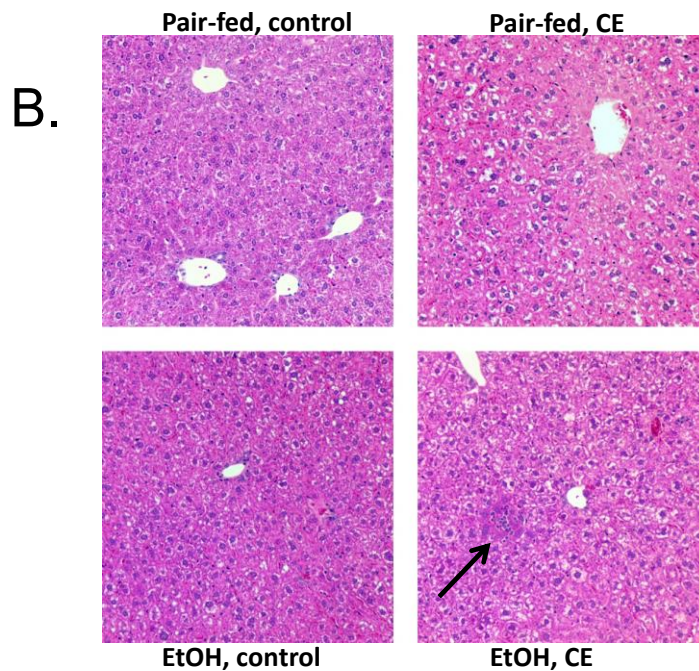
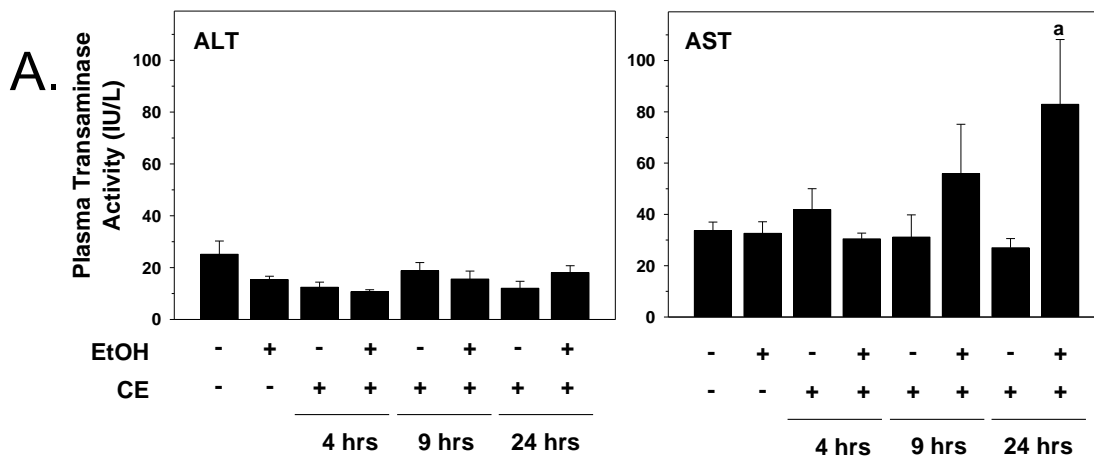


Figure 3. CE enhances ethanol-induced liver injury. Plasma transaminases (A) and representative photomicrographs for paraffin-embedded liver sections stained for H&E at 9 hours (B) are shown (200x). Ethanol-fed, CE administered animals show elevated AST levels at the 24 hour time point and damaged liver morphology at the 9 hour time point. ^a, $p < 0.05$ compared to pair-fed controls.

CE enhances ethanol-induced steatosis

Ethanol feeding is known to enhance steatosis in models of liver injury (53). In line with this, Figure 4 depicts lipid accumulation in the liver visualized via ORO staining. Pair-fed control animals showed no increase in ORO staining and CE alone did not significantly increase ORO positive staining. However, ethanol increased ORO positive staining. CE enhanced the intensity of ORO staining in ethanol-fed animals.

Rapamycin blunts liver damage caused by CE and LPS

In order to elucidate mechanisms by which CE acts as an enhancing factor in a multiple-hit model, a more robust model of liver injury was used with CE + LPS, as previously described by Anders et al., (22). To verify that rapamycin inhibited mTOR activation, mTOR phosphorylation was analyzed via Western Blot (Figure 5). No significant differences were observed between the groups. To examine the effects of rapamycin on liver damage, plasma transaminase levels were analyzed (Figure 6A). Control, CE alone, and CE with rapamycin administered animals show normal transaminase activity. LPS alone increased ALT and AST values and CE enhanced this effect. Significantly, the addition of rapamycin attenuated both ALT and AST values. Liver morphology was examined via H&E staining and CAE positive cells were visualized as an index of neutrophil infiltration (Figure 6B). Control animals show normal morphology and little CAE positive staining. LPS caused inflammatory foci and an increase in CAE positive cells, CE enhanced this effect. Notably, rapamycin administration resulted in more normal morphology and fewer CAE positive cells.

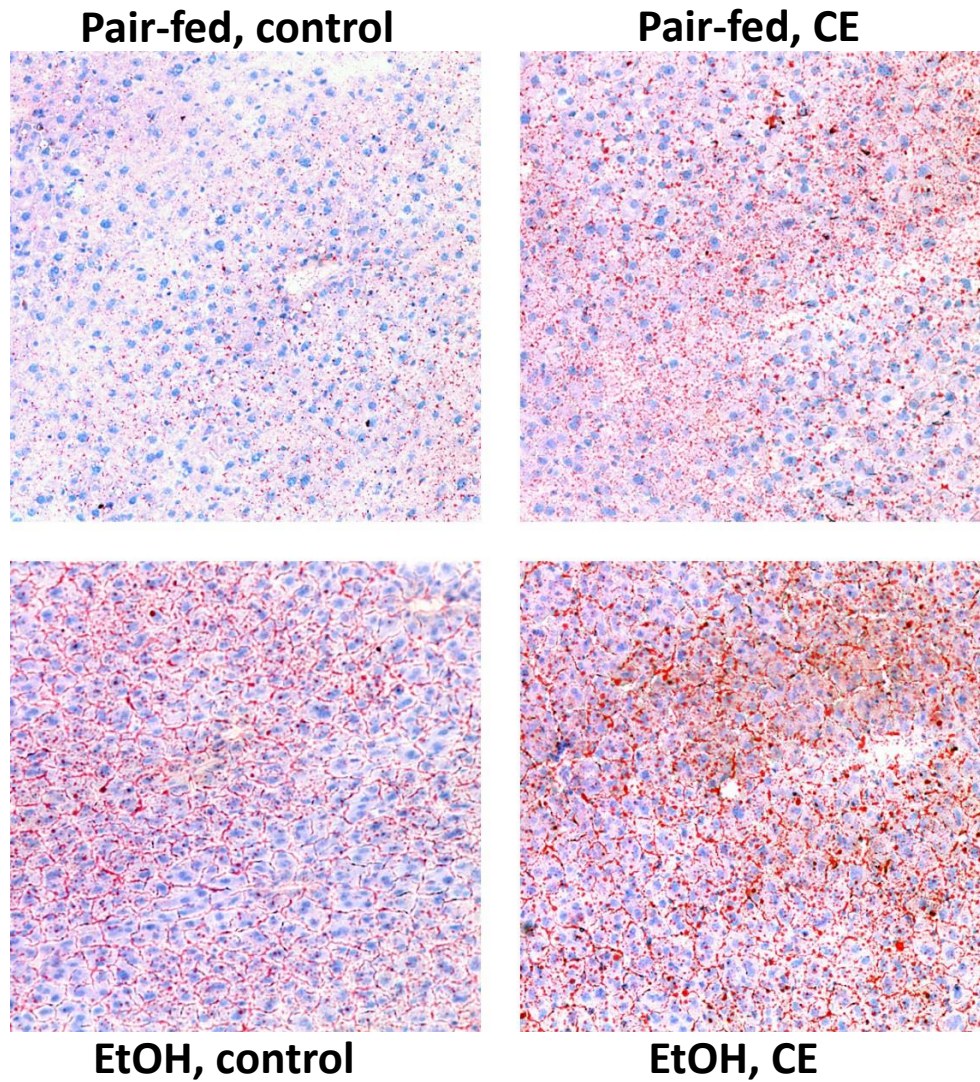


Figure 4. CE's effect on ethanol-induced steatosis. Representative photomicrographs are depicted for frozen liver sections stained with ORO for lipids as described in Materials and Methods (200x).

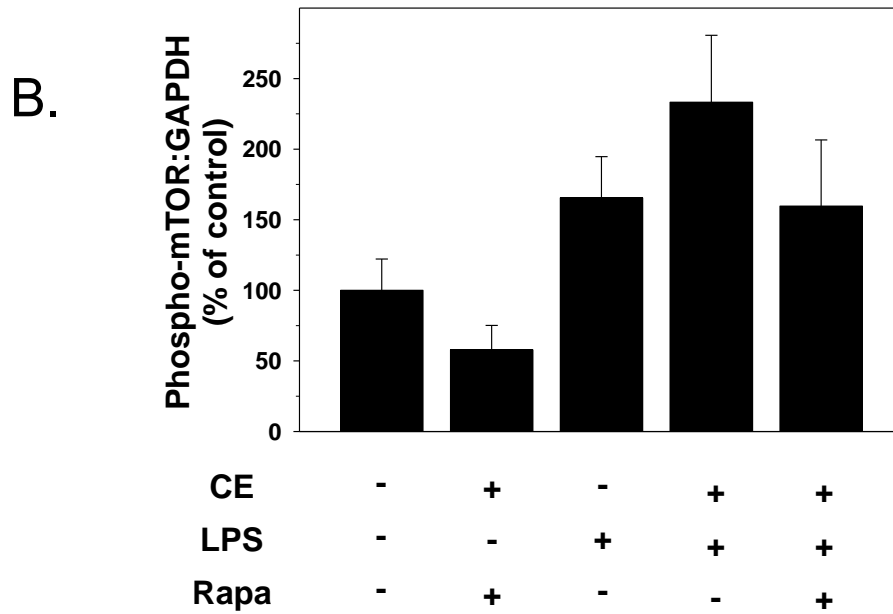
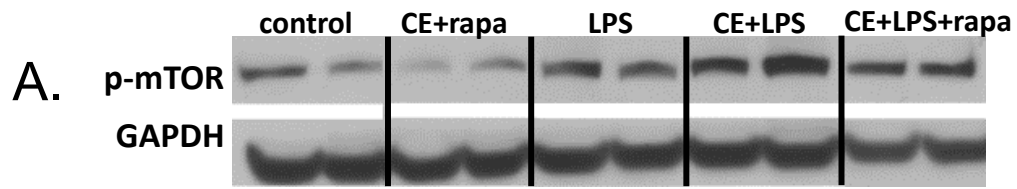


Figure 5. I Western blot for phosphorylated mTOR and GAPDH (A) are depicted and densitometry analysis was used to analyze differences amongst groups (B) as described in Materials and Methods.

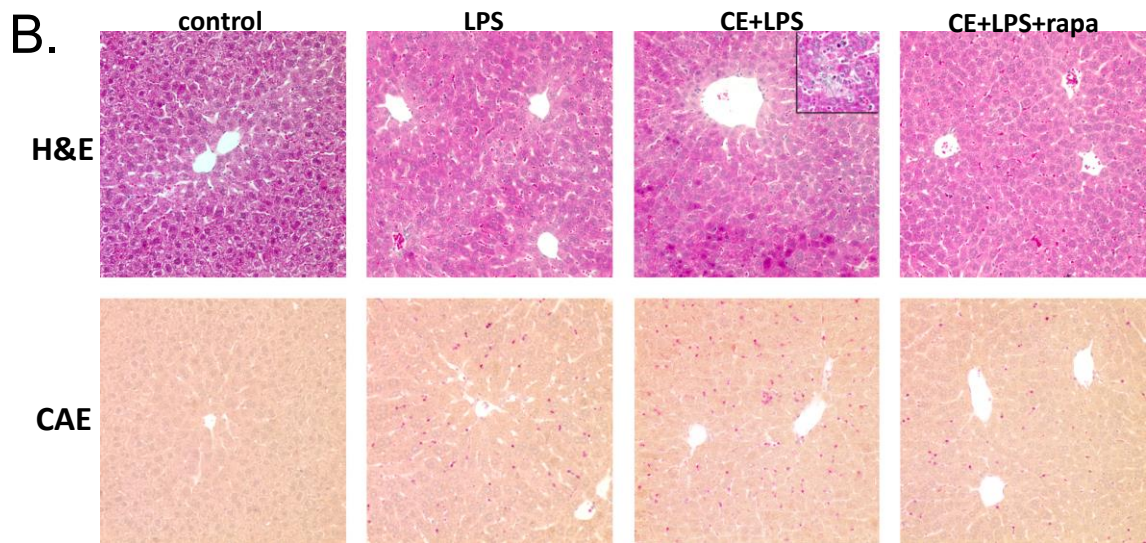
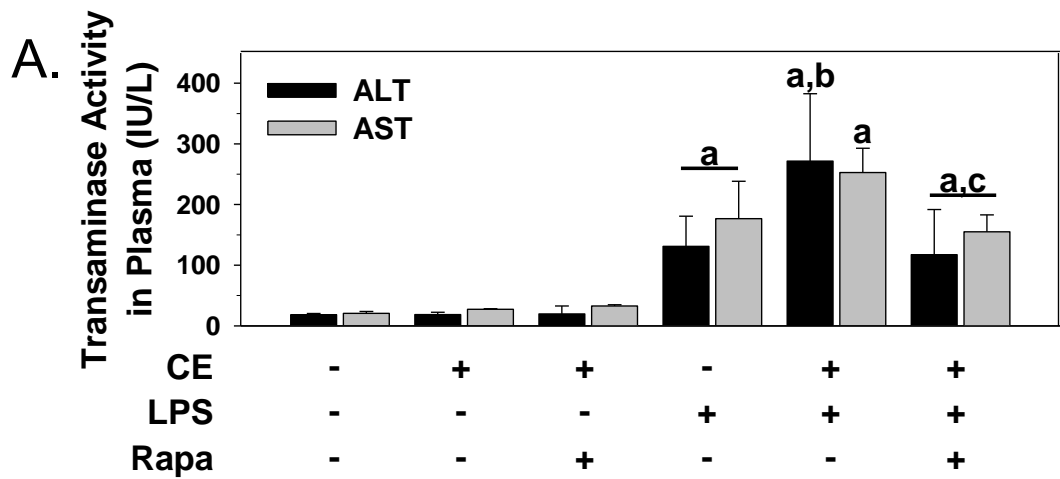


Figure 6. Rapamycin blunts liver injury caused by CE + LPS. Plasma transaminase levels (A) and representative photomicrographs for paraffin-embedded livers stained with H&E and CAE (B) are shown as described in Materials and Methods. (200x). Data are reported at mean \pm SEM. ^a, $p < 0.05$ compared to control, ^b, $p < 0.05$ compared to LPS alone, ^c, $p < 0.05$ compared to CE + LPS.

Effect of rapamycin on changes in apoptosis caused by CE and LPS

The type of cell death has been shown to play an important role in many models of liver injury. Indeed, previously it has been shown that VC causes necrotic rather than apoptotic cell death (8, 22). In order to determine the cell death pathway in this model of injury, TUNEL-positive cells were visualized as an index of apoptosis and TUNEL-positive hepatocytes and non-parenchymal cells were counted. Consistent with previous work with this model, an increase of TUNEL-positive cells with LPS administration and a decrease in that number with the addition of CE was observed (22). Importantly, the administration of rapamycin significantly attenuates the number of TUNEL-positive hepatocytes as seen in Figure 7B, while it had no effect on the number of TUNEL-positive non-parenchymal cells.

Rapamycin's effect on changes in carbohydrate and lipid metabolism caused by CE and LPS

It is known that CE alone causes alterations in hepatic metabolic pathways (22). In order to explore the effects of mTOR inhibition on lipid and glycogen levels in liver tissue, ORO staining for lipids (Figure 8A) and PAS for glycogen (Figure 8B) were visualized. LPS alone caused an increase in ORO positive staining and a depletion of glycogen reserves. The combination of both CE and LPS resulted in enhanced ORO positive staining and almost complete depletion of hepatic glycogen stores. Notably, the addition of rapamycin prevented glycogen depletion and attenuated the accumulation of lipids.

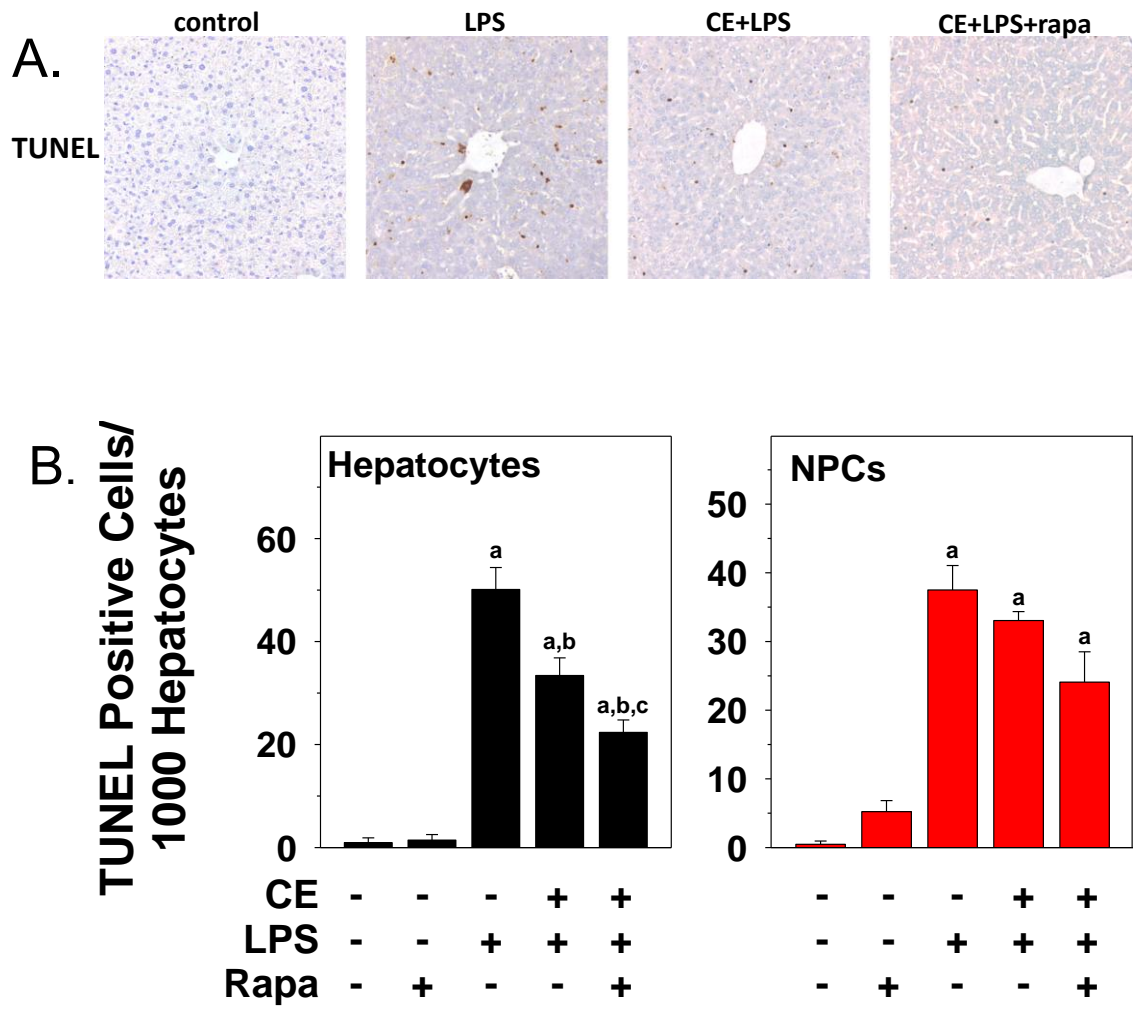


Figure 7. Rapamycin blunts number of TUNEL-positive cells. Representative photomicrographs of paraffin-embedded liver stained for TUNEL-positive cells are depicted (A) and positive cells were counted (B) as described in Materials and Methods (200x). Data are reported at mean \pm SEM. ^a, $p < 0.05$ compared to control, ^b, $p < 0.05$ compared to LPS alone, ^c, $p < 0.05$ compared to CE + LPS.

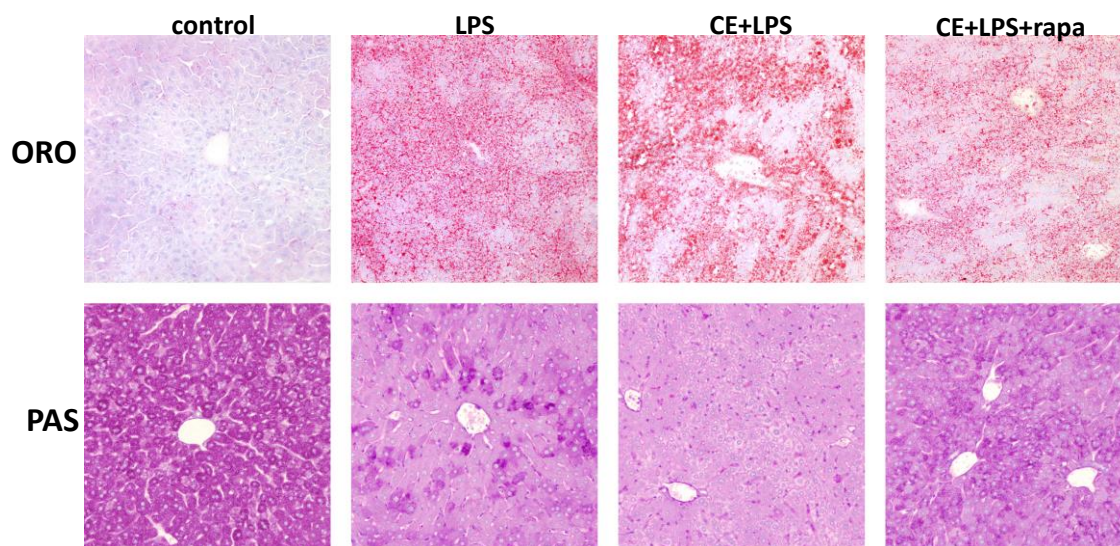


Figure 8. Rapamycin's effect on lipid and glycogen stores. Representative photomicrographs of frozen liver sections stained with ORO (top) and paraffin-embedded liver sections stained with PAS for glycogen (bottom) are depicted as described in Materials and Methods (200x).

Rapamycin blunts CE and LPS induced inflammatory cytokine expression

In order to determine the effect of mTOR inhibition on the inflammatory response, hepatic mRNA expression was analyzed to determine relative levels of pro- and anti-inflammatory markers. As mentioned in the introduction, TNF α is a major cytokine involved in liver inflammation. Plasminogen activator inhibitor-1 (PAI-1), a mediator of inflammation, is involved in liver injury progression (56). IL-6 and IL-1 β are pro-inflammatory cytokines involved in the acute-phase inflammatory response (57). Interleukin-10 (IL-10) is an anti-inflammatory cytokine and can be protective during liver inflammation (58). Inducible nitric oxide synthase (iNOS) is activated by pro-inflammatory cytokines and contributes to the inflammatory process (59). Across all markers, there was an increase in expression with LPS alone. As expected, the combination of CE and LPS enhanced expression of *Tnfa*, *Pai-1*, *Il-6*, *iNos*, *Il-1 β* , and *Il-10*. Rapamycin administration resulted in a significant blunting of *Pai-1*, *iNos*, *Il-1 β* and *Il-10* expression, indicating an attenuation of the inflammatory response. However, no significant differences were observed for *Tnfa* or *Il-6* expression with rapamycin.

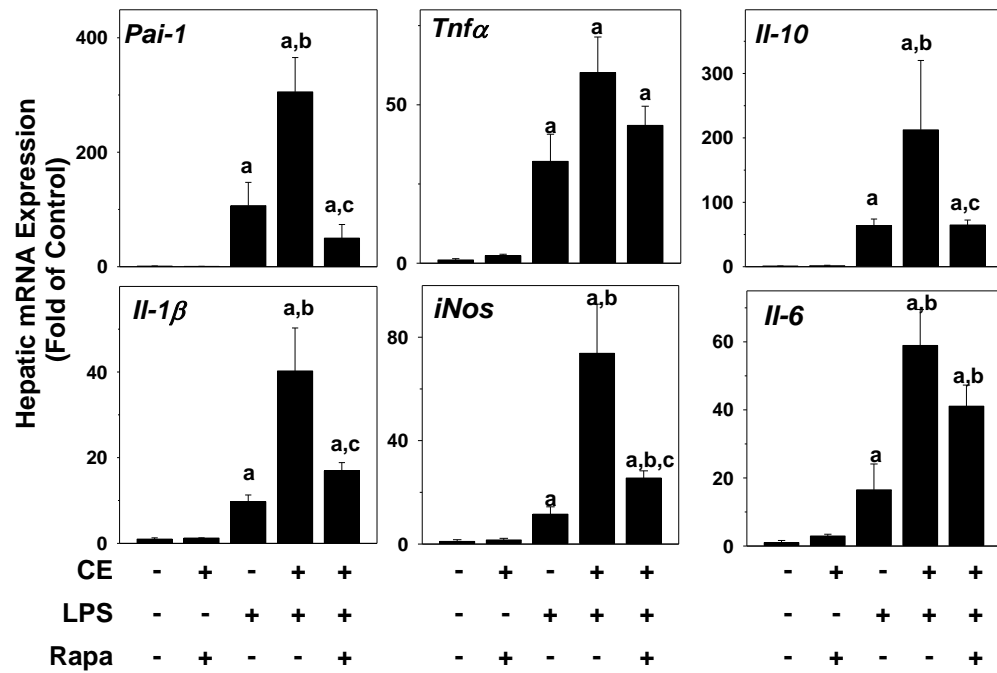


Figure 9. Rapamycin's effect on inflammatory cytokines. Hepatic mRNA expression data for inflammatory mediators were measured by qRT-PCR are shown. Data are reported at mean \pm SEM. ^a, $p < 0.05$ compared to control, ^b, $p < 0.05$ compared to LPS alone, ^c, $p < 0.05$ compared to CE + LPS.

Autophagy marker changes with CE, LPS and rapamycin

Rapamycin's allosteric inhibition of mTOR is known to result in an induction of autophagic processes (45-47). Several markers are indicative of autophagy progression and activation. Atg 5 is necessary for the formation and elongation of the autophagosome (60). Rapamycin and CE administered animals showed a significant increase in *Atg 5* expression. LPS caused no increase in *Atg5* expression, however there was a significant increase in expression with CE and LPS with and without rapamycin. No differences were observed between those groups. Microtubule associated protein-1 light chain 3 (LC-3) is cleaved and conjugated to phosphatidylethanolamine to form LC-3 II which remains associated with the autophagosomal membrane until ultimate fusion with the lysosome (41, 61, 62), and has been used as a marker of autophagy progression. LC-3 II protein was observed via Western Blot. There were no significant differences across the groups.

A major target of autophagic degradation is cellular inflammatory machinery known as the inflammasome. Therefore, mRNA expression of a protein involved in inflammasome formation, NLR family pyrin domain containing 3 (NLRP3), was analyzed. A significant increase was observed with LPS administration. There was also a significant increase with CE and LPS with or without rapamycin, but no differences were observed amongst the groups.

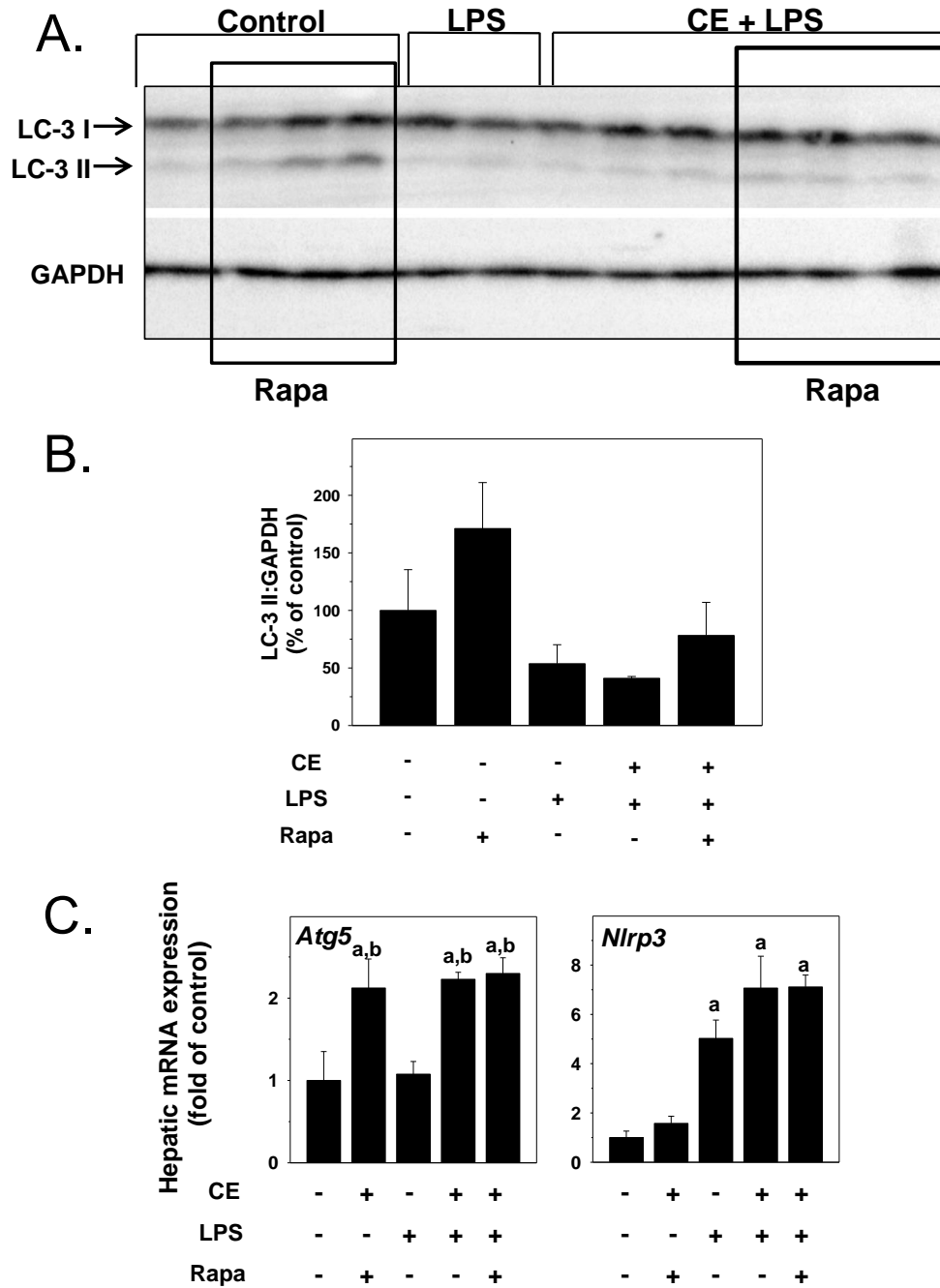


Figure 10. Rapamycin's effect on autophagy markers. LC-3 protein was analyzed by Western Blot (A) and densitometry was used to analyze differences across groups (B) as described in Material and Methods. mRNA expression of markers of autophagy (*Atg5*) and inflammasome activity (*Nlrp3*) were measured by RT-qPCR (C). Data are reported as the mean \pm SEM. ^a, $p < 0.05$ compared to control, ^b, $p < 0.05$ compared to LPS alone.

DISCUSSION

Since the realization that high, occupational exposure to VC was hepatotoxic, workplace regulations have been implemented to avoid this severe type of exposure (63). However, PVC producing facilities still release VC as a waste product into the environment. This potentially puts the individuals around these facilities at risk for low-level, chronic VC exposure. As humans are rarely exposed to one factor at a time, other exposures, such as alcohol consumption or diet, may modify risk for developing more severe liver injury upon co-exposure with low-level VC.

CE enhances ethanol-induced liver injury and steatosis

Alcohol consumption is a common risk-modifying factor for the development of more severe liver diseases, especially upon exposure with other modifying factors, such as VC. Since alcohol abuse is common in urban areas (64), the first goal of this project was to examine the potential risk for alcohol and low-level VC co-exposure using a novel model for liver injury modified from the NIAAA acute-on-chronic model (53). In the NIAAA model, mice are given alcohol-containing diet for 10 days followed by a binge dose of ethanol on the final day in order to examine a more relevant pattern of human alcohol exposure. Here, this model was adapted for the purpose of examining a potential synergistic effect of two known liver-damaging agents, alcohol and VC. With the modified model, mice were

administered CE instead of an ethanol binge on the final day. Indeed, increased liver injury was observed when animals were exposed to both ethanol and CE (Figure 3). Chronic ethanol exposure induces its metabolizing enzyme, CYP2E1. (65). Acetaminophen toxicity is known to be enhanced in individuals who consume alcohol because the two compounds share metabolizing enzymes. Ethanol is preferentially metabolized by ADH, causing acetaminophen to be alternately metabolized to its toxic metabolite via CYP2E1, which accumulates and causes severe liver injury (66). However, in this model, CE is the toxic metabolite of VC and as such, the already induced CYP2E1 more efficiently metabolizes CE. Therefore, less accumulation of CE is not as damaging, resulting in less tissue injury and inflammation.

Serum AST levels were significantly elevated at the 24 hour time point in animals who received the ethanol diet and CE, although no differences were seen in ALT levels at any time point. AST is known to be present in both hepatocyte cytoplasm and mitochondria and its isolated elevation is attributed to release from non-hepatic tissue, particularly damaged muscle (67). However, previous data (22) gives evidence that VC metabolites damage hepatic mitochondria. Therefore, the addition of CE to already injured hepatocytes is sufficient to cause damage to those mitochondria and release AST. The ratio of AST to ALT values can be a useful tool when determining cause of liver damage. When AST and ALT values respond the same, it is indicative of an acute viral or toxicant induced liver injury due to the release of only the cytoplasmic enzymes. However, when AST values are higher than ALT values, it is indicative of alcohol-induced liver injury and due

to the release of the mitochondrial AST from necrotic cell death (68, 69). Therefore, the isolated elevated AST levels could be attributed to the ethanol-feeding alone. However, the addition of CE significantly enhanced AST levels compared to ethanol-fed control animals, suggesting a synergistic effect on liver damage.

In ALD, steatosis is seen in 90% of subjects who consume significant amounts of alcohol (70). Here, steatosis was observed in ethanol-fed animals (Figure 4). Ethanol-fed animals mainly showed evidence of macrovesicular lipid droplets, being indicative of triglyceride accumulation. However, the presence of both micro- and macrovesicular lipid droplets was seen with the addition of CE, indicating an accumulation of FFA and triglycerides, respectively. The presence of microvesicular lipid droplets and their associated FFA have detrimental effects on cellular function and sensitizes the liver to be more susceptible to more severe injury (71). FFA accumulation has also been shown to be indicative of impaired mitochondrial β -oxidation (72), which is in line with previous data that VC metabolites are damaging hepatic mitochondria. Indeed, the addition of CE in ethanol-fed animals resulted in enhanced steatosis as seen by the presence of microvesicular lipid droplets, indicative of FFA accumulation and impaired mitochondrial function in this model.

mTOR activation is critical in CE-induced liver injury

Exploring the interaction and possible multiple-hit phenomenon with ethanol and VC co-exposure resulted in interesting findings. However, we were interested in examining the mechanisms by which VC acts as a risk-modifying factor for underlying liver disease. Therefore, another model of liver injury established at the

same time in our laboratory was also employed to characterize molecular changes and mechanistic endpoints in response to VC metabolite exposure. In that model, our laboratory demonstrated that administration of the VC metabolite, CE, alone was sufficient to alter metabolic enzyme expression although no hepatic tissue damage was observed (22). With the addition of LPS administration, liver damage was enhanced in animals exposed to CE. Findings from that study include enhanced liver damage, neutrophil infiltration, oxidative stress, and metabolic dysfunction. Alterations of key metabolic enzymes were observed some which include mTOR, AMP-activated protein kinase (AMPK), and protein kinase B or AKT. Specifically, a significant activation of mTOR was observed in mice who were administered CE alone. Therefore, the second goal of this project was to investigate the role of mTOR in liver injury caused by VC metabolite exposure.

mTOR inhibition blunts CE-induced metabolic changes

mTOR activity is known to be dysregulated in several disease states (73) and its inhibition has been shown to be protective in many models of liver injury (74, 75). A common method of mTOR inhibition involves administration of rapamycin. Rapamycin has anti-proliferative and immunosuppressive effects (76). It also enhances autophagy processes by allosteric inhibition of mTOR complex I (77). The metabolic profiles of animals in this experiment were analyzed histologically through ORO and PAS stains for lipids and glycogen, respectively. The addition of rapamycin blunted the accumulation of hepatic lipids and prevented glycogen depletion, indicative of a protective effect on hepatocyte metabolism.

One possible mechanism by which rapamycin attenuated lipid accumulation is by affording mitochondrial protection from CE. Mitochondrial protection results in unhindered oxidative phosphorylation and β -oxidation. With oxidative respiration intact, the switch to anaerobic respiration (glycolysis) and subsequent glycogen depletion was not required. Additionally, Singh et al. demonstrated that autophagy is crucial for regulating lipid metabolism via an autophagic process unique to lipid droplets, lipophagy (78). Lipophagy is a specific type of autophagy in which lipid droplets are sequestered and enzymatically degraded by lysosomes (79). Lin and colleagues showed that induction of autophagy was protective against both alcohol and NAFLD-induced hepatic steatosis and reduced liver injury (80). Therefore, the results of this model of liver injury suggest that autophagic activity can blunt hepatic lipid droplet formation via lipophagy. FFA are known to be lipotoxic and preventing their accumulation would afford another mechanism of protection by rapamycin administration.

mTOR inhibition attenuates CE-induced liver injury

Upon rapamycin administration, the liver damage and inflammation observed with LPS and CE was significantly attenuated (Figures 6 and 9). Chen et al., demonstrated in a murine model of methionine-choline deficient diet induced NASH, that rapamycin decreased histological damage, decreased *Il-1 β* expression, and decreased myeloperoxidase activity, indicative of less neutrophil infiltration (84). Here, rapamycin administration significantly attenuated hepatic mRNA expression of inflammatory markers such as *iNos*, *Il-10*, and *Il-1 β* . A significant attenuation of both ALT and AST liver enzymes was also observed.

Histologically, a normal gross morphology and fewer CAE positive cells were observed in rapamycin-administered animals, indicating that, indeed, mTOR is crucial for VC metabolite-induced injury. VC metabolite administration causes mitochondrial damage and, consequently, a decrease in cellular energy levels leading to cell death and injury (22). ROS and RNS, produced by damaged mitochondria, mediate hepatocyte inflammation and account for the increased injury observed. The data support that rapamycin attenuates injury via mitochondrial protection by increasing mitophagy. Mitophagy alleviates the burden of injured mitochondria within the cytoplasm by their degradation, which allows for maintenance of mitochondrial homeostasis and provides protection against enhanced damage (85). Indeed, rapamycin and CE administration caused a significant increase in *Atg5* mRNA expression (Figure 10C), supporting the hypothesis of the autophagic process being active in this model.

In addition to its role in mediating cellular protection, autophagy is a known negative regulator of inflammasome activity (86, 87). Inflammasomes are multi-protein complexes that assemble in the cytoplasm in response to pathogens or cellular damage (88). A major function of inflammasome formation is caspase-1 activation and IL-1 β maturation (87). The inflammasome complex NLRP3 is known to be activated by ROS (89) and endogenous damage signals or damage associated molecular patterns (DAMPs) such as high mobility group box 1 protein (HMGB1) and heat shock proteins (90, 91). In this study, rapamycin significantly blunted *Il-1 β* expression, which suggests that enhanced autophagy and therefore

elimination of excess, active inflammasomes may be important players in injury attenuation.

Summary and conclusions

In conclusion, the current study demonstrated that VC metabolite exposure may be one factor in a “multiple-hit” phenomenon of liver damage in two different models. mTOR plays a critical role in VC metabolite-induced liver damage. Indeed, its inhibition attenuated indices of liver damage and steatosis. Moreover, mTOR inhibition caused blunting of key inflammatory mediator expression, attenuation of lipid accumulation and prevention of glycogen depletion. Autophagy may be a player in VC metabolite induced liver injury. Indeed, the data support that autophagy afforded mitochondrial protection via mitophagy and provided a more homeostatic lipid metabolic profile through lipophagy and degradation of excess, lipotoxic FFA. Attenuated IL-1 β expression with the addition of rapamycin suggests that the inflammasome may be a key regulator in the progression of VC-metabolite induced liver injury. However, further studies are necessary to analyze these mechanisms in greater detail.

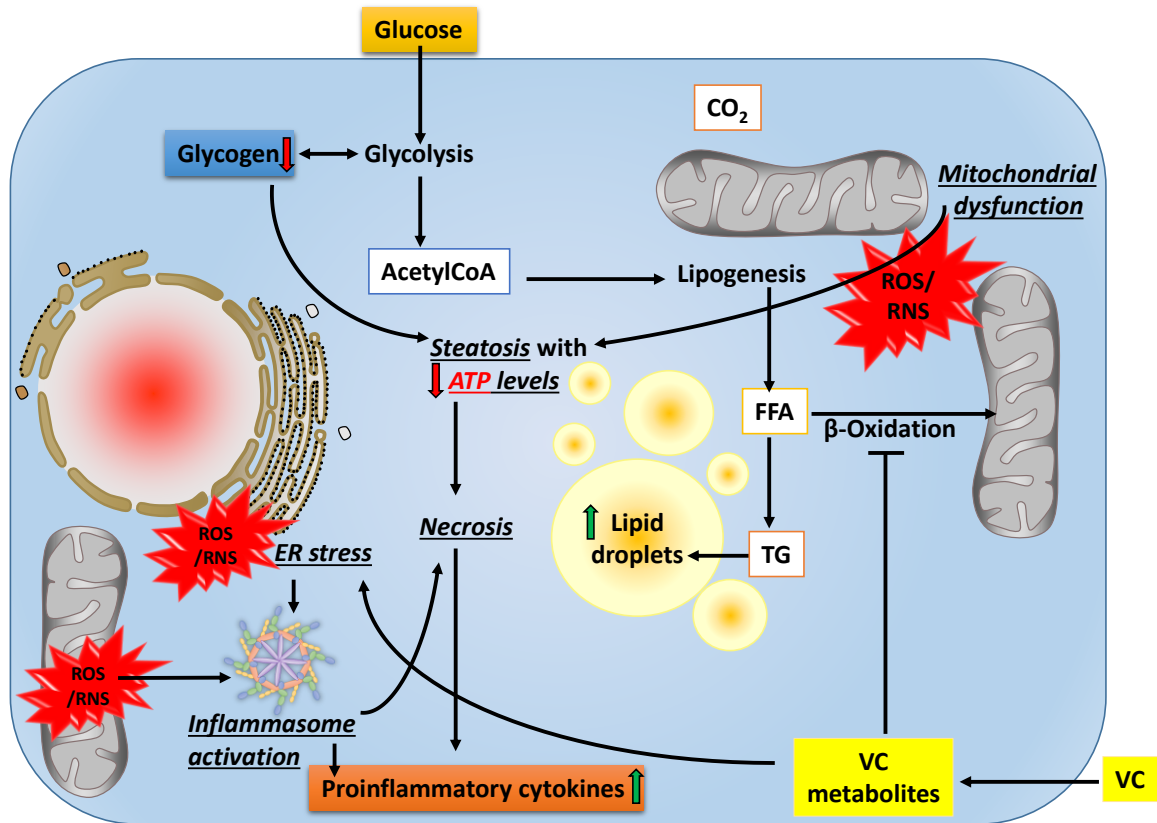


Figure 11. Working hypothesis. Upon exposure to VC, 1) reactive solvent intermediates form though bio activation processes and diet-induced obesity decreases their elimination. 2) Through carbonyl stress and the generation of reactive oxygen and nitrogen species (ROS/RNS), solvent metabolites cause ER stress leading to the production of proinflammatory cytokines and mitochondrial damage, which impairs oxidative phosphorylation; the cell increases flux through anaerobic glycolysis to compensate for this loss of ATP yield. The increased demand for glucose depletes glycogen stores 3) acetylCoA is being shunted to lipid synthesis (causing steatosis) rather than β -oxidation, even under conditions of ATP depletion. 4) The combined metabolic stress of solvent metabolite exposure and ATP depletion likely causes 'liponecrosis' associated with increased proinflammatory cytokines and inflammasome activation.

STRENGTHS OF THIS WORK

There are many strengths of this work. This study demonstrated that sub-hepatotoxic levels of the VC metabolite, CE, enhanced liver damage in two separate models of injury. We show that underlying liver injury caused by alcohol is enhanced with co-exposure to CE. We also show that sub-hepatotoxic levels of CE can enhance injury caused by the bacterial endotoxin, LPS. Another strength of this work is identifying a novel role of mTOR in VC-induced liver damage. The hypotheses that autophagy and the inflammasome are involved in VC-induced liver injury are novel and this work lays a foundation for ongoing research with those mechanisms.

The use of whole animal models in this work is also a strength. All of the experiments here were performed *in vivo*. Liver injury and its mediating factors is a complex process and as such is best observed at the level of the whole organism.

PITFALLS AND FUTURE DIRECTIONS

Further work is needed in order to identify more detailed mechanisms involved in mTOR's inhibition in VC-metabolite induced liver injury. A major limitation of this study is the 24 hour time point used. Future studies will be conducted in order to analyze mRNA expression at 4 hours post LPS administration, in addition to the 24 hour time point from this study. The time point used yielded necessary insight for establishing this model and determining pathology. However, an earlier time point will aid in gaining mechanistic insight to injury progression. For example, it is foreseeable that we will see more pronounced changes in mTOR and LC-3 protein levels via Western Blot analysis at an earlier time point. Moreover, at the 4 hour time point, we expect to see more significant differences with the rapamycin administered mice versus CE + LPS in both mRNA and protein expression of inflammatory and autophagy markers.

Another limitation of this study was the use of the VC-metabolite, CE. Although CE is a primary metabolite of VC, it is not the parent compound and therefore may not completely recapitulate the effects seen with VC gas.

Future directions include expanding this model to exposing mice to VC gas via an inhalation chamber and a high fat diet (HFD) to induce NAFLD. This will better mimic human circumstances and exposures in the environment. We also aim to examine the effects on mitochondria in response to VC and other factors in

greater depth. Lastly, we are interested in employing rapamycin in the VC-HFD model of liver injury to validate that mTOR is directly involved in injury progression and that autophagy indeed plays a crucial role in damage.

CONCLUSIONS

A paradigm shift has occurred in the past years has been investigating the impact of low, chronic exposures in contrast to high occupational acute exposures. This approach attempts to take into account multiple factors and damaging agents that may contribute to disease status. In this context, the impact of underlying disorders that may modify risk is critical. Liver disease is not contributed to one factor, rather is modified by other mitigating conditions, such as other environmental or genetic factors. This study establishes that indeed, VC-metabolite exposure can have influence over severity of liver injury when co-exposure occurs with other modifying factors.

REFERENCES

1. U.S.Department of Health and Human Services PHS. Agency for Toxic Substances and Disease Registry (ATSDR): Toxicological profile for Vinyl Chloride. 2006.
2. Sass JB, Castleman B, Wallinga D. Vinyl chloride: a case study of data suppression and misrepresentation. *Environ Health Perspect* 2005 Jul;113(7):809-812.
3. Kielhorn J, Melber C, Wahnschaffe U, Aitio A, Mangelsdorf I. Vinyl chloride: still a cause for concern. *Environ Health Perspect* 2000 Jul;108(7):579-588.
4. 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):282-286.
5. 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.
6. Tamburro CH, Makk L, Popper H. Early hepatic histologic alterations among chemical (vinyl monomer) workers. *Hepatology* 1984 May;4(3):413-418.
7. Wahlang B, Beier JI, Clair HB, Bellis-Jones HJ, Falkner KC, McClain CJ, et al. Toxicant-associated steatohepatitis. *Toxicol Pathol* 2013 Feb;41(2):343-360.
8. Cave M, Falkner KC, Ray M, Joshi-Barve S, Brock G, Khan R, et al. Toxicant-associated steatohepatitis in vinyl chloride workers. *Hepatology* 2010 Feb;51(2):474-481.
9. Rinella ME. Nonalcoholic fatty liver disease: a systematic review. *JAMA* 2015 Jun 9;313(22):2263-2273.
10. Day CP. Non-alcoholic fatty liver disease: current concepts and management strategies. *Clin Med* 2006 Jan;6(1):19-25.

11. Bugianesi E, Leone N, Vanni E, Marchesini G, Brunello F, Carucci P, et al. Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. *Gastroenterology* 2002 Jul;123(1):134-140.
12. Ratziu V, Bonyhay L, Di M, V, Charlotte F, Cavallaro L, Sayegh-Tainturier MH, et al. Survival, liver failure, and hepatocellular carcinoma in obesity-related cryptogenic cirrhosis. *Hepatology* 2002 Jun;35(6):1485-1493.
13. Chang CY, Argo CK, Al Osaimi AM, Caldwell SH. Therapy of NAFLD: antioxidants and cytoprotective agents. *J Clin Gastroenterol* 2006 Mar;40(3 Suppl 1):S51-S60.
14. McCullough AJ. Pathophysiology of nonalcoholic steatohepatitis. *J Clin Gastroenterol* 2006 Mar;40(3 Suppl 1):S17-S29.
15. Diehl AM, Li ZP, Lin HZ, Yang SQ. Cytokines and the pathogenesis of non-alcoholic steatohepatitis. *Gut* 2005 Feb;54(2):303-306.
16. Choi S, Diehl AM. Role of inflammation in nonalcoholic steatohepatitis. *Curr Opin Gastroenterol* 2005 Nov;21(6):702-707.
17. Hassan K, Bhalla V, El Regal ME, Kader HH. Nonalcoholic fatty liver disease: a comprehensive review of a growing epidemic. *World J Gastroenterol* 2014 Sep 14;20(34):12082-12101.
18. Gao B, Bataller R. Alcoholic liver disease: pathogenesis and new therapeutic targets. *Gastroenterology* 2011 Nov;141(5):1572-1585.
19. Fabbrini E, Sullivan S, Klein S. Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. *Hepatology* 2010 Feb;51(2):679-689.
20. Day CP, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology* 1998 Apr;114(4):842-845.
21. 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;94:2557-2562.
22. Anders LC, Lang AL, Anwar-Mohamed A, Douglas AN, Bushau AM, Falkner KC, et al. Vinyl Chloride Metabolites Potentiate Inflammatory Liver Injury caused by LPS in Mice. *Toxicol Sci* 2016 Mar 8.
23. World Health Organization. Alcohol. 2016.
24. Mann RE, Smart RG, Govoni R. The epidemiology of alcoholic liver disease. 2016.

25. Beier JI, McClain CJ. Mechanisms and cell signaling in alcoholic liver disease. *Biol Chem* 2010 Nov;391(11):1249-1264.
26. Nagy LE. The Role of Innate Immunity in Alcoholic Liver Disease. *Alcohol Res* 2015;37(2):237-250.
27. Flegal KM, Carroll MD, Ogden CL, Curtin LR. Prevalence and trends in obesity among US adults, 1999-2008. *JAMA* 2010 Jan;303(3):235-241.
28. 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):433-438.
29. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global Epidemiology of Non-Alcoholic Fatty Liver Disease-Meta-Analytic Assessment of Prevalence, Incidence and Outcomes. *Hepatology* 2015 Dec 28.
30. Dowman JK, Tomlinson JW, Newsome PN. Pathogenesis of non-alcoholic fatty liver disease. *QJM* 2010 Feb;103(2):71-83.
31. Fabbrini E, Magkos F. Hepatic Steatosis as a Marker of Metabolic Dysfunction. *Nutrients* 2015 Jun;7(6):4995-5019.
32. Greenberg AS, Obin MS. Obesity and the role of adipose tissue in inflammation and metabolism. *Am J Clin Nutr* 2006 Feb;83(2):461S-465S.
33. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 2003 Dec;112(12):1796-1808.
34. Gregor MF, Hotamisligil GS. Inflammatory mechanisms in obesity. *Annu Rev Immunol* 2011;29:415-445.
35. Shoelson SE, Herrero L, Naaz A. Obesity, inflammation, and insulin resistance. *Gastroenterology* 2007 May;132(6):2169-2180.
36. Alberto Rahde. Vinyl chloride. 1992.
37. Whysner J, Conaway CC, Verna L, Williams GM. Vinyl chloride mechanistic data and risk assessment: DNA reactivity and cross-species quantitative risk extrapolation. *Pharmacol Ther* 1996;71(1-2):7-28.
38. Sood C, O'Brien PJ. Chloroacetaldehyde-induced hepatocyte cytotoxicity. Mechanisms for cytoprotection. *Biochem Pharmacol* 1994 Aug 30;48(5):1025-1032.

39. Bruggemann SK, Radike K, Braasch K, Hinrichs J, Kisro J, Hagenah W, et al. Chloroacetaldehyde: mode of antitumor action of the ifosfamide metabolite. *Cancer Chemother Pharmacol* 2006 Feb;57(3):349-356.
40. Klionsky DJ, Cregg JM, Dunn WA, Jr., Emr SD, Sakai Y, Sandoval IV, et al. A unified nomenclature for yeast autophagy-related genes. *Dev Cell* 2003 Oct;5(4):539-545.
41. Ravikumar B, Sarkar S, Davies JE, Futter M, Garcia-Arencibia M, Green-Thompson ZW, et al. Regulation of mammalian autophagy in physiology and pathophysiology. *Physiol Rev* 2010 Oct;90(4):1383-1435.
42. Hayashi-Nishino M, Fujita N, Noda T, Yamaguchi A, Yoshimori T, Yamamoto A. A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation. *Nat Cell Biol* 2009 Dec;11(12):1433-1437.
43. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. *J Pathol* 2010 May;221(1):3-12.
44. Marzban H, Del Bigio MR, Alizadeh J, Ghavami S, Zachariah RM, Rastegar M. Cellular commitment in the developing cerebellum. *Front Cell Neurosci* 2014;8:450.
45. Amir M, Zhao E, Fontana L, Rosenberg H, Tanaka K, Gao G, et al. Inhibition of hepatocyte autophagy increases tumor necrosis factor-dependent liver injury by promoting caspase-8 activation. *Cell Death Differ* 2013 Jul;20(7):878-887.
46. Han W, Fu X, Xie J, Meng Z, Gu Y, Wang X, et al. MiR-26a enhances autophagy to protect against ethanol-induced acute liver injury. *J Mol Med (Berl)* 2015 Sep;93(9):1045-1055.
47. Nakadera E, Yamashina S, Izumi K, Inami Y, Sato T, Fukushima H, et al. Inhibition of mTOR improves the impairment of acidification in autophagic vesicles caused by hepatic steatosis. *Biochem Biophys Res Commun* 2016 Jan 22;469(4):1104-1110.
48. Yang L, Li P, Fu S, Calay ES, Hotamisligil GS. Defective hepatic autophagy in obesity promotes ER stress and causes insulin resistance. *Cell Metab* 2010 Jun 9;11(6):467-478.
49. Poso AR, Hirsimaki P. Inhibition of proteolysis in the liver by chronic ethanol feeding. *Biochem J* 1991 Jan 1;273(Pt 1):149-152.
50. Ding WX, Yin XM. Mitophagy: mechanisms, pathophysiological roles, and analysis. *Biol Chem* 2012 Jul;393(7):547-564.

51. Kim I, Rodriguez-Enriquez S, Lemasters JJ. Selective degradation of mitochondria by mitophagy. *Arch Biochem Biophys* 2007 Jun 15;462(2):245-253.
52. Laplante M, Sabatini DM. mTOR signaling at a glance. *J Cell Sci* 2009 Oct 15;122(Pt 20):3589-3594.
53. Bertola A, Mathews S, Ki SH, Wang H, Gao B. Mouse model of chronic and binge ethanol feeding (the NIAAA model). *Nat Protoc* 2013 Mar;8(3):627-637.
54. Kaphalia BS, Ansari GA. Hepatic fatty acid conjugation of 2-chloroethanol and 2-bromoethanol in rats. *J Biochem Toxicol* 1989;4(3):183-188.
55. Beier JI, von MC, Sies H, Klotz LO. Activation of ErbB2 by 2-methyl-1,4-naphthoquinone (menadione) in human keratinocytes: role of EGFR and protein tyrosine phosphatases. *FEBS Lett* 2006 Mar 20;580(7):1859-1864.
56. Arteel GE. New role of plasminogen activator inhibitor-1 in alcohol-induced liver injury. *J Gastroenterol Hepatol* 2008 Mar;23:S54-S59.
57. Smolen JS, Maini RN. Interleukin-6: a new therapeutic target. *Arthritis Res Ther* 2006;8 Suppl 2:S5.
58. Ouyang W, Rutz S, Crellin NK, Valdez PA, Hymowitz SG. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu Rev Immunol* 2011;29:71-109.
59. Zamora R, Vodovotz Y, Billiar TR. Inducible nitric oxide synthase and inflammatory diseases. *Mol Med* 2000 May;6(5):347-373.
60. Mizushima N, Sugita H, Yoshimori T, Ohsumi Y. A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy. *J Biol Chem* 1998 Dec 18;273(51):33889-33892.
61. Tanida I, Ueno T, Kominami E. LC3 conjugation system in mammalian autophagy. *Int J Biochem Cell Biol* 2004 Dec;36(12):2503-2518.
62. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosomal membranes after processing. *EMBO J* 2000 Nov 1;19(21):5720-5728.
63. Occupational Safety and Health Standards. 2016.
64. Borders TF, Booth BM. Rural, suburban, and urban variations in alcohol consumption in the United States: findings from the National Epidemiologic

- Survey on Alcohol and Related Conditions. *J Rural Health* 2007;23(4):314-321.
65. Jin M, Ande A, Kumar A, Kumar S. Regulation of cytochrome P450 2e1 expression by ethanol: role of oxidative stress-mediated pkc/jnk/sp1 pathway. *Cell Death Dis* 2013;4:e554.
 66. Riordan SM, Williams R. Alcohol exposure and paracetamol-induced hepatotoxicity. *Addict Biol* 2002 Apr;7(2):191-206.
 67. Botros M, Sikaris KA. The de ritis ratio: the test of time. *Clin Biochem Rev* 2013 Nov;34(3):117-130.
 68. Nyblom H, Berggren U, Balldin J, Olsson R. High AST/ALT ratio may indicate advanced alcoholic liver disease rather than heavy drinking. *Alcohol Alcohol* 2004 Jul;39(4):336-339.
 69. Vroon DH, Israili Z. Aminotransferases. 1990.
 70. Toshikuni N, Tsutsumi M, Arisawa T. Clinical differences between alcoholic liver disease and nonalcoholic fatty liver disease. *World J Gastroenterol* 2014 Jul 14;20(26):8393-8406.
 71. Alkhouri N, Dixon LJ, Feldstein AE. Lipotoxicity in nonalcoholic fatty liver disease: not all lipids are created equal. *Expert Rev Gastroenterol Hepatol* 2009 Aug;3(4):445-451.
 72. Tandra S, Yeh MM, Brunt EM, Vuppalanchi R, Cummings OW, Unalp-Arida A, et al. Presence and significance of microvesicular steatosis in nonalcoholic fatty liver disease. *J Hepatol* 2011 Sep;55(3):654-659.
 73. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell* 2012 Apr 13;149(2):274-293.
 74. Lu Y, Cederbaum AI. Autophagy Protects against CYP2E1/Chronic Ethanol-Induced Hepatotoxicity. *Biomolecules* 2015;5(4):2659-2674.
 75. Ni HM, Bockus A, Boggess N, Jaeschke H, Ding WX. Activation of autophagy protects against acetaminophen-induced hepatotoxicity. *Hepatology* 2012 Jan;55(1):222-232.
 76. Li J, Kim SG, Blenis J. Rapamycin: one drug, many effects. *Cell Metab* 2014 Mar 4;19(3):373-379.
 77. Benjamin D, Colombi M, Moroni C, Hall MN. Rapamycin passes the torch: a new generation of mTOR inhibitors. *Nat Rev Drug Discov* 2011 Nov;10(11):868-880.

78. Singh R, Kaushik S, Wang Y, Xiang Y, Novak I, Komatsu M, et al. Autophagy regulates lipid metabolism. *Nature* 2009 Apr 30;458(7242):1131-1135.
79. Wang CW. Lipid droplets, lipophagy, and beyond. *Biochim Biophys Acta* 2015 Dec 20.
80. Lin CW, Zhang H, Li M, Xiong X, Chen X, Chen X, et al. Pharmacological promotion of autophagy alleviates steatosis and injury in alcoholic and non-alcoholic fatty liver conditions in mice. *J Hepatol* 2013 May;58(5):993-999.
81. Malhi H, Gores GJ. Molecular mechanisms of lipotoxicity in nonalcoholic fatty liver disease. *Semin Liver Dis* 2008 Nov;28(4):360-369.
82. Feldstein AE, Canbay A, Guicciardi ME, Higuchi H, Bronk SF, Gores GJ. Diet associated hepatic steatosis sensitizes to Fas mediated liver injury in mice. *J Hepatol* 2003 Dec;39(6):978-983.
83. Malhi H, Barreyro FJ, Isomoto H, Bronk SF, Gores GJ. Free fatty acids sensitise hepatocytes to TRAIL mediated cytotoxicity. *Gut* 2007 Aug;56(8):1124-1131.
84. Chen R, Wang Q, Song S, Liu F, He B, Gao X. Protective role of autophagy in methionine-choline deficient diet-induced advanced nonalcoholic steatohepatitis in mice. *Eur J Pharmacol* 2016 Jan 5;770:126-133.
85. Williams JA, Ding WX. A Mechanistic Review of Mitophagy and Its Role in Protection against Alcoholic Liver Disease. *Biomolecules* 2015;5(4):2619-2642.
86. Shi CS, Shenderov K, Huang NN, Kabat J, Abu-Asab M, Fitzgerald KA, et al. Activation of autophagy by inflammatory signals limits IL-1beta production by targeting ubiquitinated inflammasomes for destruction. *Nat Immunol* 2012 Mar;13(3):255-263.
87. Yuk JM, Jo EK. Crosstalk between autophagy and inflammasomes. *Mol Cells* 2013 Nov;36(5):393-399.
88. Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. *Nat Rev Immunol* 2013 Jun;13(6):397-411.
89. Tschopp J, Schroder K. NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? *Nat Rev Immunol* 2010 Mar;10(3):210-215.
90. Tang D, Kang R, Coyne CB, Zeh HJ, Lotze MT. PAMPs and DAMPs: signal 0s that spur autophagy and immunity. *Immunol Rev* 2012 Sep;249(1):158-175.

91. Abais JM, Xia M, Zhang Y, Boini KM, Li PL. Redox regulation of NLRP3 inflammasomes: ROS as trigger or effector? *Antioxid Redox Signal* 2015 May 1;22(13):1111-1129.

ABBREVIATIONS

| | |
|--------------|---|
| VC | Vinyl chloride |
| ATSDR | Agency for toxic substances and disease registry |
| PVC | Polyvinyl chloride |
| TASH | Toxicant associated steatohepatitis |
| FLD | Fatty liver disease |
| ALD | Alcoholic liver disease |
| NAFLD | Non-alcoholic liver disease |
| LPS | Lipopolysaccharide |
| CE | Chloroethanol |
| FFA | Free fatty acids |
| TNF α | Tumor necrosis factor alpha |
| IL-6 | Interleukin-6 |
| IL-10 | Interleukin-10 |
| CAA | Chloroacetaldehyde |
| ROS/RNS | Reactive oxygen species, nitrogen species |
| Atg | Autophagy related |
| mTOR | Mechanism target of rapamycin |
| ALT | Alanine transaminase |
| AST | Aspartate transaminase |
| H&E | Hematoxylin and Eosin |
| CAE | Chloroacetate esterase |
| TUNEL | Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling |
| ORO | Oil-Red O |
| PAS | Periodic Acid Schiff |
| NLRP3 | NLR family pyrin domain containing 3 |
| LC-3 | Microtubule associated protein-1 light chain 3 |
| HMGB-1 | High mobility group box-1 |
| DAMP | Damage associated molecular pattern |

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Professional Memberships and Activities

2011-2013 SOT Undergraduate Student Affiliate

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Honors and Awards

2009-2013 David and Ruth B. Iler Scholarship

2012 First Place in Best Poster Presentation: Undergraduate Student.
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2013 Graduation B.S. (Cum Laude), Northern Kentucky University, KY

Abstracts and 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

Posters

1. Poster, 09/2012 “Impaired motor function in *Cyp1a1_Cyp1a2(-/-)* double knockout mice” Ohio Valley Society of Toxicology, Columbus, OH.
2. Poster, 03/2013 “Impaired motor function in *Cyp1a1_Cyp1a2(-/-)* double knockout mice” Society of Toxicology (SOT), San Antonio, TX. (Pfizer Undergraduate Travel Award)
3. Poster, 06/2015 “Effect of vinyl chloride metabolites on alcohol-induced liver injury” Ohio Valley of Toxicology Summer Meeting, Cincinnati, OH.
4. Poster, 10/2015 “Inhibiting mammalian target of rapamycin (mTOR) via rapamycin blunts liver damage caused by VC metabolites in mice” Research! Louisville, Louisville, KY
5. Poster, 03/2016 “Inhibiting mammalian target of rapamycin (mTOR) via rapamycin blunts liver damage caused by VC metabolites in mice”, Society of Toxicology, New Orleans, LA

Abstracts

1. 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 annual meeting, Louisville, KY.
2. 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 annual meeting, Louisville, KY. (Basic Science Research Faculty Award)
3. 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.
4. 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.

5. 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.
6. 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.
7. 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).
8. 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.
9. **Lang AL**, Bushau AM, Falkner KC, Cave MC, McClain MJ, Arteel GE and Beier JI (2015) Effect of vinyl chloride metabolites on alcohol-induced liver injury. OVSOT Summer Meeting, Cincinnati, OH.
10. **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.
11. 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.
12. Yeo H, Anders LC, Bushau AM, Kaelin BR, **Lang AL**, Arteel GE, Cave MC, McClain MJ and Beier JI (2015) Exploring Energy Metabolism Changes In Vinyl Chloride Induced Non-Alcoholic Fatty Liver Disease (NAFLD). Research! Louisville annual meeting, Louisville, KY.
13. 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
14. 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*, in press. (Resident Research Award)
15. **Lang AL**, Kaelin BR, Yeo H, Hudson SV, Poole LG, McKenzie CM, Sharp CN, Arteel GE, Beier JI (2016) Critical role of mammalian target of

rapamycin (mTOR) in liver damage caused by VC metabolites in mice.
SOT Annual Meeting, New Orleans, LA.

Publications

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).

Anders LC, Lang AL, Anwar-Mohamed A, Douglas AN, Bushau AM, Falkner KC, et al. Vinyl Chloride Metabolites Potentiate Inflammatory Liver Injury caused by LPS in Mice. *Toxicol Sci* 2016 Mar 8.