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Role of Dietary Fatty Acids in Liver Injury Caused by Vinyl Chloride Metabolites in Mice

By

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for Graduation *summa cum laude*

and

for Graduation with Honors from the Department of Biology

University of Louisville

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Lay summary

The goal of Beier lab is to investigate the effect of gaseous toxin vinyl chloride often found in surrounding residential areas of industrial complexes. While occupational exposure to this compound has been regulated, there are indications that low-level exposure to this compound can cause increased liver injury in residents that have underlying liver conditions due to their life style. For this project, we examine the interactions between vinyl chloride and fatty diet that involves either saturated or unsaturated fat.

Abstract

Background. Vinyl chloride (VC) is an environmental pollutant found in many industrial sites and it ranks 4th on the 2013 Hazardous Substances Priority List published by Agency for Toxic Substances and Disease Registry's (ATSDR). We have previously reported increased hepatocellular necrosis with high occupational VC exposure in human and in *in vitro* models; however, the effect of subtoxic VC exposure on liver with underlying conditions is unknown. The purpose of the current study is to investigate the hepatic injury caused by chloroethanol (CE; VC metabolite) and inflammatory response in two experimental models of high-fat diet (HFD) induced obesity.

Methods. Mice were administered a bolus dose of CE or vehicle after 10 weeks of high saturated fatty acid diet (HSFA; 42% milk fat), high polyunsaturated fatty acid diet (HPUFA; 42% corn oil) or low fat control diet (LFD; 13% milk fat LSFA or 13% corn oil LPUFA). Samples were harvested 24 hours after CE exposure for determination of liver damage, inflammation and inflammasome activation.

Results. In LFD-fed control mice, CE did not cause any significant changes to the liver. Chloroethanol induced significant liver damage and inflammation in HSFA fed mice, as well as HPUFA fed mice but to a lesser degree. Moreover, steatosis, hepatocyte ballooning, infiltrating

inflammatory cells and hepatic expression of proinflammatory cytokines that were observed with CE after HSFA-feeding was blunted by HPUFA-feeding.

Conclusions. Chloroethanol (as a surrogate VC exposure) exacerbated liver injury in a '2-hit' paradigm through inflammasome activation and endoplasmic reticulum (ER) stress. This serves as proof-of-concept that VC hepatotoxicity may be modified by diet-induced obesity and non-alcoholic fatty liver disease (NAFLD). These data implicate exposure to VC interact uniquely with saturated and polyunsaturated fatty acids to exacerbate NAFLD.

Introduction

Vinyl chloride is an organochlorine used to create the polymer, polyvinyl chloride (PVC). VC is ranked as the 4th most important toxic chemical on the Hazardous Substance Priority List, and its annual production was recently estimated at 27 million metric tons (1). In addition to direct production, VC is a degradation product at many Superfund sites, and is present in landfill leachate and groundwater near military installations. Occupational exposure is estimated to encompass more than 80,000 American workers (2). VC gas readily suffuses from water and is found in significant concentrations in the ambient air surrounding manufacturing complexes. Therefore, exposure to VC is widespread and potentially affects a large portion of the population.

VC is a known human hepatotoxicant that causes a spectrum of both benign and malignant diseases including hepatocellular carcinoma (HCC), hemangiosarcoma and toxicant associated steatohepatitis (TASH) (3), however, these direct effects of VC exposure require high occupational exposures and have limited relevance with existing VC safety regulations. Although environmental VC exposures in the areas around manufacturing complexes are subhepatotoxic, its interactions with underlying liver conditions that modify risk are largely unknown.

In the liver, the concept of multiple factors contributing to disease is well known. Indeed, liver disease is not based solely on one factor, but rather is modified by other mitigating conditions, such as genetic or environmental factors. Numerous studies have now established that physiological/biochemical changes to the liver that are pathologically inconsequential can become hepatotoxic in response to a second agent. This multiple 'hit' paradigm has been best exemplified in alcoholic (ALD) (4) and non-alcoholic fatty liver diseases (NAFLD) (5). The similarity of VC metabolism to that of other known hepatotoxicants such as ethanol (6), suggests that VC may also cause fatty liver. VC is metabolized via cytochrome p450 2E1 (CYP2E1) and aldehyde dehydrogenase dependent pathways to produce the corresponding

alcohol (chloroethanol) and aldehyde (chloroacetaldehyde); indeed, previous studies have suggested that VC oxidation is a bioactivation process (7). We hypothesize that concentrations of VC that are not overtly hepatotoxic may serve as a factor in a 'multiple hit' paradigm of liver disease.

It has become clear that the type of dietary fat plays an important role in the pathogenesis of liver disease. For example, in NAFLD, dietary saturated fatty acid (SFA) appears to be more detrimental than dietary polyunsaturated fatty acid (PUFA) (8-10). The potential interaction between dietary fat type and organochlorine toxicants to promote liver injury has not been studied.

In the field of chronic liver diseases there has been a recent focus on inflammasomes and their role in the pathomechanisms of chronic liver disease (11). Inflammasomes are large multiprotein complexes that not only function as intracellular sensors but also mediate inflammatory damage via multiple pathways. Inflammation is induced by pathogen-associated molecular patterns ("PAMPs"), such as lipopolysaccharide (LPS), as well as by molecules released from dead or dying cells (damage-associated molecular patterns; "DAMPs"). These danger signals interact with the Toll-Like Receptor (TLR) family of pattern recognition receptors, which thereby activate the Nod-like receptors (NLRs) to subsequently activate the inflammasome complex (12, 13). The net result is proteolytic activation and release of IL-1β, which induces the innate immune response and vicious cycle of inflammation and tissue injury (14, 15). We postulate that VC-metabolites stimulate and increase inflammasome activation and its effectors and thus enhance NAFLD. Overall, we hypothesize that the metabolic stress caused by different fatty acids sensitizes the liver to further injury by low dose VC via molecular, organelle, and cellular mechanisms that are dependent on types of fatty acids.

Methods

All of the following experiments were performed by the thesis writer (Biochemical Analyses, Histology and Immunohistochemistry; Immunoblots; RNA Isolation and Real-Time RT-PCR; and Statistical analyses) unless otherwise noted within the sections below.

Animals and Treatments

A graduate student performed all of the work in this section, Animals and treatments (prior to thesis writer's arrival). 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 University of Louisville Institutional Animal Care and Use Committee. Mice were fed low fat control or high fat diets (see below) for 10 weeks. At the end of the 10 weeks the animals were administered chloroethanol (CE; 50 mg/kg i.g.) or vehicle (water) 24 hours prior to sacrifice. The concentration of chloroethanol was determined by others to not directly cause liver damage (16) and was validated in previous experiments (17). At sacrifice, animals were anesthetized with ketamine/xylazine (100/15 mg/kg, i.m.). 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.

Low saturated fat diet, [13% fat in calories; 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 50.0 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 0.01 g/kg; (Harlan Laboratories, Madison, WI)]. *High saturated fat diet,* [42% fat in calories; 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,

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Biochemical Analyses, Histology and Immunohistochemistry

Plasma alanine transaminase (ALT) and aspartate transaminase (AST) were determined using standard kits (Thermotrace, Melbourne, Australia). Livers were stained with hematoxylin & eosin (H&E). Neutrophil accumulation was assessed by chloroacetate esterase stain (CAE; Sigma, St. Louis MO). Pathology was scored (steatosis, inflammation, necrosis) in a blinded manner by a trained pathologist as described elsewhere (18); the number of necrotic or inflammatory foci (involving >5 cells) was determined in 10 400× fields. Hepatic lipids (TG and NEFA) were extracted and determined as described previously (19). A graduate student evaluated oral glucose tolerance after the diet during animal treatment. Mice were fasted for 6 hours, then blood was sampled immediately after fasting, then at every 15 minute interval after oral administration of 2 mg/kg D-/(+)-glucose (Sigma, St. Louis, MO) in 4 ml/kg of sterile saline solution. Glucose concentrations were measured using Accu-Chek® Aviva Plus glucometer and glucose test strips (Toche Diagnostics Corp., Indianapolis, IN).

Immunoblots

Liver samples were homogenized in RIPA buffer (20), 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 *HMGB1, Caspase 1, and Nlrp3* were used and compared to *GAPDH* (Cell Signaling Technology; Beverly, MA). Densitometric analysis was performed using UN-SCAN-IT gel (Silk Scientific Inc., Orem, UT) software.

RNA Isolation and Real-Time RT-PCR

RNA was extracted immediately following sacrifice from fresh liver samples using RNA Stat60 and chloroform. 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).

Statistical Analyses

Sample size calculations were based on the ANOVA model, testing a planned comparison (contrast), e.g. HFD+ vs. HFD- with CE exposure for liver injury using plasma ALT levels. The total sample of 48 subjects (n=6 per group) achieves 85% power to detect a non-zero contrast of the means versus the alternative that the contrast is zero using an F test with a 0.05 significance level; we have increased to n=8 per group to account for any possible attrition during the feeding protocol. Plasma activity (IU/L) minimum difference of detection was set to 50; the common standard deviation within a group was assumed to be 20 (IU/l), based on historical data.

Results

High fat diet causes metabolic syndrome

Figure 1 shows the results of a glucose tolerance test (GTT) and the amount of food consumed by each group over the 10-week period. All diet groups showed a similar pattern of a sharp increase followed by a gradual decrease in blood glucose level when gavaged a known amount of glucose after starving. It is important to note that while both of the low fat diet control groups peaked in blood glucose level no higher than 340mg/dL, HPUFA and HSFA groups reached higher blood glucose level at 455mg/dL and 410mg/dL, respectively (Figure 1A). Moreover, 90 minutes after glucose gavage, only the LFD control groups (LSFA and LPUFA) returned to basal blood glucose levels. Figure 1B shows that all groups consumed similar amounts of food throughout the 10-week period.

Effect of CE on liver damage caused by high fat diet

Figure 2 shows representative photomicrographs depicting liver pathology (H&E) and neutrophil accumulation (CAE) 24 hours after CE. No pathological changes were observed in the two LFD control groups (Figure 2A; H&E) and this effect was unchanged by CE (not shown). Compared to LFD groups, there was a significant increase in neutrophil recruitment in both the HPUFA and the HSFA group. Interestingly, CE significantly enhanced this effect after HSFA feeding, while only minor differences were seen in the HPUFA group (Figure 2B). In line with these results, plasma levels of transaminases were within normal range in low fat-fed mice (Figure 3A) and CE did not significantly alter the result in either the LSFA or the LPUFA group. In the HSFA group, however, plasma transaminases were significantly elevated, which was further increased by CE. While HPUFA-feeding alone caused no increase in transaminase levels, CE markedly increased both ALT and AST levels, albeit less significant as after HSFA-feeding (Figure 3A). Furthermore, hepatic lipid contents were measured. CE did not affect the hepatic content of triglycerides and cholesterol in low fat-fed mice (Figure 3B). While triglyceride levels were not affected in either HFD groups, CE significantly increased triglycerides in both

HFD groups albeit much attenuated in the HPUFA group. Cholesterol levels were also increased significantly in either HFD group, however, while CE significantly increased cholesterol level, after HSFA-feeding, it significantly blunted cholesterol levels after HPUFA-feeding (Figure 3B).

Effect of CE on the induction of gene expression caused by high fat diet

As CE enhanced recruitment of inflammatory cells caused by fatty diet (Figure 2B), its effect on hepatic expression of key pro-inflammatory and anti-inflammatory genes was determined (Figure 4). CE caused no significant changes in expression of any genes studied in control groups. *Pai-1* expression was significantly increased by both HFD groups, but *Tnf-α* expression was unaffected by either of the HFDs. While *Pai-1* expression was increased significantly in both the HSFA+CE group and the HPUFA+CE group, the effect was far less accentuated in the HPUFA+CE group. *Tnf-α* expression was also increased significantly in the HSFA+CE group, but was unaffected in the HPUFA+CE group. Expression of ER stress marker *Atf3* and *Chop* was unaffected by CE in the control groups. Moreover, expression was also unaffected by either of the HFD. However, CE significantly increased both *Atf3* and *Chop* in the HSFA group. While CE also increased *Chop* expression in the HPUFA group, the effect was blunted when compared to HSFA-fed animals.

Effects of chloroethanol on inflammasome activation

To further analyze the hepatic inflammatory response, markers of inflammasome activation were analyzed. In Figure 5A, inflammasome activating and suppressing gene expressions are shown. In *Nlrp3, Nlrc4, Asc,* and *Il-1β* expressions that mark for inflammasome activation, no significant changes were noted in HSFA group with the exception of *Il-1β*, which was slightly increased. The addition of CE significantly increased expression of all four genes in the HSFA group. While HPUFA diet alone increases *Nlrc4* and *Il-1β* expression, CE significantly blunted this effect. *Sirt1* and *Ppary* gene expressions associated with inflammasome suppression showed the opposite effect to genes associated with activation (Figure 5B).

HSFA+CE significantly blunts expression of *Sirt1 and Pparγ.* Similarly, HPUFA+CE also decreased the expression of *Pparγ* but not of *Sirt1.* Proteins associated with inflammasome activation were measured via western blot analysis (Figure 5C). Protein expressions of Caspase1, and NLRC3, which are components of the assembled inflammasome, as well as HMGB1 (a DAMP) increased in the HSFA+CE group. Similarly, plasma IL-1β, a major component of the inflammasome, determined by ELISA analysis showed significant changes in active IL-1β responsible for inflammatory response (Figure 5D). Active protein increased in HSFA+CE group compared to HSFA group while it decreased in HPUFA+CE group compared to HPUFA group.

Discussion

Subhepatotoxic doses of chloroethanol enhance liver damage caused by high fat diet.

The role that chronic exposure to VC and/or its metabolites plays in disease is a major health concern in the US, because there are many areas with elevated VC in the ground water due to close proximity to contaminated sites (2). As mentioned in the *Introduction*, the liver is a known target organ of VC exposure, but its effects are observed only with high concentrations. Current safety restrictions in today's industry lessen such high exposure concerns. We have previously shown in a proof-of-concept study that CE sensitizes the liver to a second insult, LPS (17). Here, we tested the hypothesis that the consequences of HFD consumption may also serve as a sensitizing factor to CE.

The purpose of this project was therefore to investigate the effect of CE on HFD-induced liver damage caused by HFD enriched in different dietary fatty acids. It has been shown previously that while dietary SFA cause more severe liver damage than dietary PUFA in non-alcoholic fatty liver disease, it is the opposite in alcoholic fatty liver disease (8-10). Here, we observed that liver damage caused by CE was enhanced by both HFD, but this effect was significantly stronger in animals fed saturated fatty acids (Figure 2 and 3). Interestingly, while the animals consumed equal amounts of food, HPUFA consumption leads to a significant decrease in glucose tolerance (Figure 1) compared to HSFA consumption. In contrast, inflammation (neutrophil accumulation, Figure 2 and hepatic expression of genes involved in cytokine production, and ER stress, Figure 4) was significantly lower in the HPUFA+CE group than in the HSFA+CE group. In some cases, gene expression in HPUFA+CE group decreased compared to HPUFA group.

A previous study by this group (17) identified a new impact of CE exposure to dysregulate carbohydrate and lipid metabolism, causing glycogen depletion and steatosis in the liver. Indeed, here CE exacerbated both, hepatic TGs and cholesterol due to HSFA much more strongly than due to HPUFA. Continuous lipid accumulation in the liver (steatosis) can result in

non-alcoholic fatty liver disease (NAFLD), which is tightly linked to liver inflammation (steatohepatitis). Indeed, markers of inflammation such as neutrophil infiltration (CAE, Figure 2B) and expression of pro-inflammatory cytokines (*Tnf-α*/*Pai-1,* Figure 4A) were increased after HSFA+CE. Moreover, inflammasome activation has been shown to be a hallmark in inflammatory liver injury (14, 15). Previously, we have shown that VC-metabolite induced cell death favors necrosis due to mitochondrial dysfunction and ATP depletion (17). DAMPs derived from dying cells and ATP released by necrosing hepatocytes have been shown to directly activate the inflammasome (21). Indeed, here we showed increases in markers of inflammasome activation (mRNA and protein) due to CE only in animals fed HSFA but not in animals fed HPUFA (Figure 5). This is also in line with the gene expression data of *Pparγ* and *Sirt1* that function to decrease inflammasome activation (22, 23). Both *Pparγ* and *Sirt1* gene expressions were blunted in HSFA+CE compared to HSFA. The significant increase seen in plasma IL-1β, a key marker of inflammasome activation, measured by ELISA in HSFA+CE compared to decreased level of the same protein in HPUFA+CE shows that PUFA might be protective against VC metabolite damage. Western blot analysis of inflammasome-associated proteins depicts a similar result. There was a significant increase in active HMGB1, Caspase1, and Nlrp3 proteins in HSFA+CE group (Figure 5). The significant increase only in the HSFA+CE group but not HSFA alone indicates that CE exacerbates the damage due to fatty diet in a multi-hit fashion. Moreover, a recent study demonstrates that ER stress enhances inflammasome activity through C/EBP-Homologous protein (CHOP), which acts as a critical signaling node connecting ER stress and inflammasome activation. Reactive oxygen species (ROS), which are byproducts of VC metabolism, have also been linked to increase in ER stress. Indeed, here we have demonstrated markers of ER stress (Figure 4B) to be enhanced in both HFD+CE groups. However, ER stress markers were significantly lower in HPUFA+CE group compared to HSFA+CE (Figure 4A). This suggests that here ER stress may indeed be responsible for the observed increase in inflammasome activation in the HSFA+CE group. Furthermore, as ROS

are known to enhance Nlrp3 inflammasome activation, amplifying the effects of ER stress and inflammasome activation, results of our previous study (17), showing increased production of ROS supports this hypothesis.

Summary and Conclusions

The results of this study suggest that VC metabolite chloroethanol causes increased inflammation, increased inflammasome activation and oxidative ER stress when combined with a diet enriched in saturated fatty acids, while a diet enriched in PUFA largely blunts this effect. This suggests that saturated fatty acid sensitizes the liver much more strongly to CE than unsaturated fatty acids. This is very interesting because PUFA is known to exacerbate liver damage caused by alcohol while SFA protects against it and VC takes a very similar pathway as ethanol. On the other hand, PUFA are known to have protective effects in the heart and many other organs, making its opposite effect in the liver a unique one (9, 10, 18).

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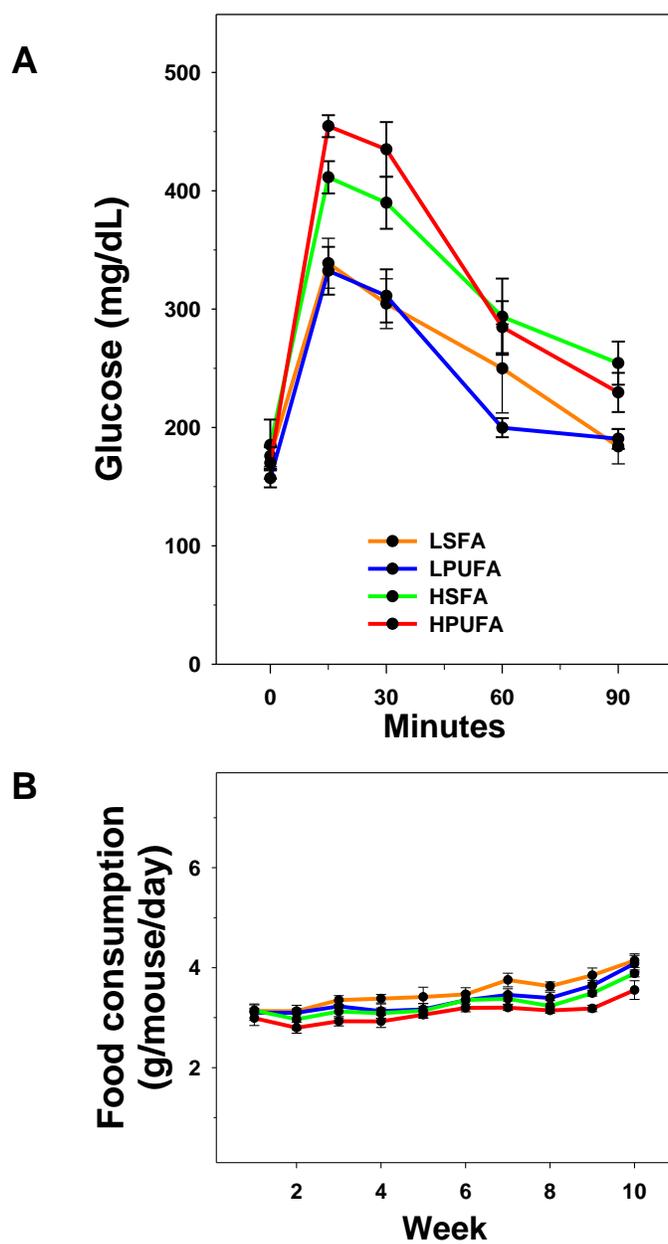
Figure legends:

Figure 1: High fat diet induces glucose intolerance.

A: Glucose tolerance test was performed for all groups to determine presence of metabolic syndrome. **B:** Food consumption of all groups during the 10 week diet.

Figure 2: Chloroethanol cause lipid accumulation and neutrophil recruitment.

A: Representative photomicrographs of H&E stains at 200x magnification are shown. **B:** Representative photomicrographs of CAE stains at 200x magnification are shown

Figure 3: Chloroethanol causes increased in hepatic damage markers and changes hepatic lipid content.

A: ALT/AST were determined in plasma samples collected 24 hours after injection of CE. **B:** Hepatic lipid contents were determined by the Piccolo Lipid Panel Plus Reagent Disc and Piccolo Xpress Chemistry Analyzer (Abaxis, Inc., Union City, CA) according to the manufacturer's instructions.

Figure 4: Chloroethanol alters expression of inflammatory genes.

Real-Time RT-PCR for markers of the inflammatory response was performed. $p < 0.05$ compared to the LFD group; $p < 0.05$ compared to the absence of chloroethanol. **A:** genes associated with cytokine expression **B:** genes associated with ER stress

Figure 5: Chloroethanol induces inflammasome activation in HSFA+CE group.

A: RT-PCR for genes associated with inflammasome activation **B:** RT-PCR for genes associated with inflammasome suppression **C:** Representative Western blots for HMGB1, Caspase1, and NLRP3 densitometric analyses of proteins are shown. [a], $p < 0.05$ compared to the absence of LPS; [b], $p < 0.05$ compared to the absence of CE. **D:** Serum IL-1β measured with ELISA.

Figure 6: Working Hypothesis

Currently working hypothesis is that diet-induced obesity impairs the elimination process of reactive intermediates formed from VC, which leads to ER stress and thus production and

release of proinflammatory cytokines and mitochondrial damage. This ultimately prevents oxidative phosphorylation resulting in compensatory anaerobic glycolysis, depleting hepatic glycogen reserve and causing a 'pseudo-fasted' state. In addition, lipid biosynthesis occurs rather than β-oxidation even in abundance of ATP, contributing to the 'pseudo-fasted' state. The combination of metabolic effects, cytokines and inflammasome activation leads to liponecrosis. Moreover, ROS and ER stress can directly cause inflammasome activation.

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A: Glucose tolerance test was performed for all groups to determine presence of metabolic syndrome. **B:** Food consumption of all groups during the 10 week diet.
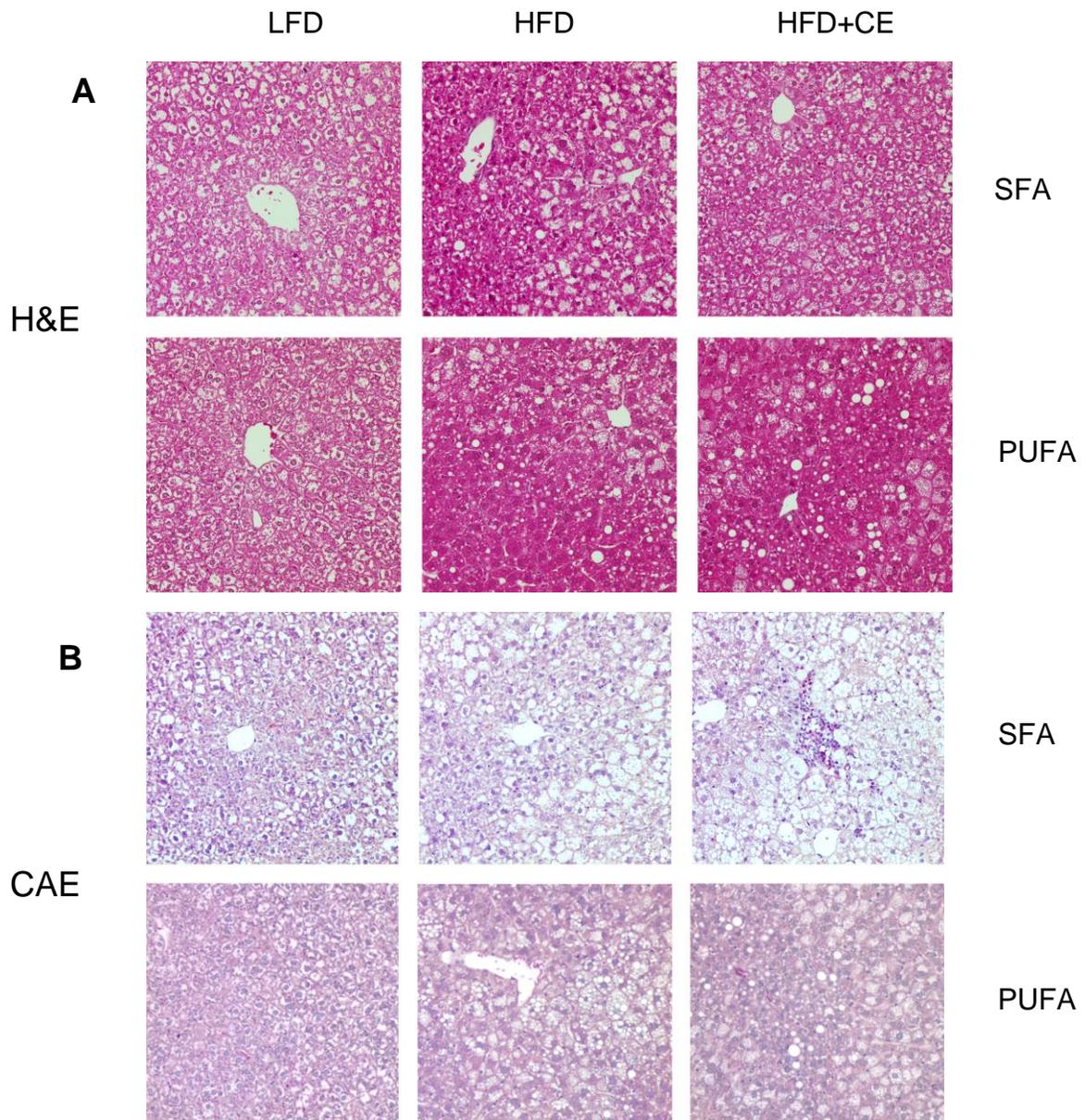
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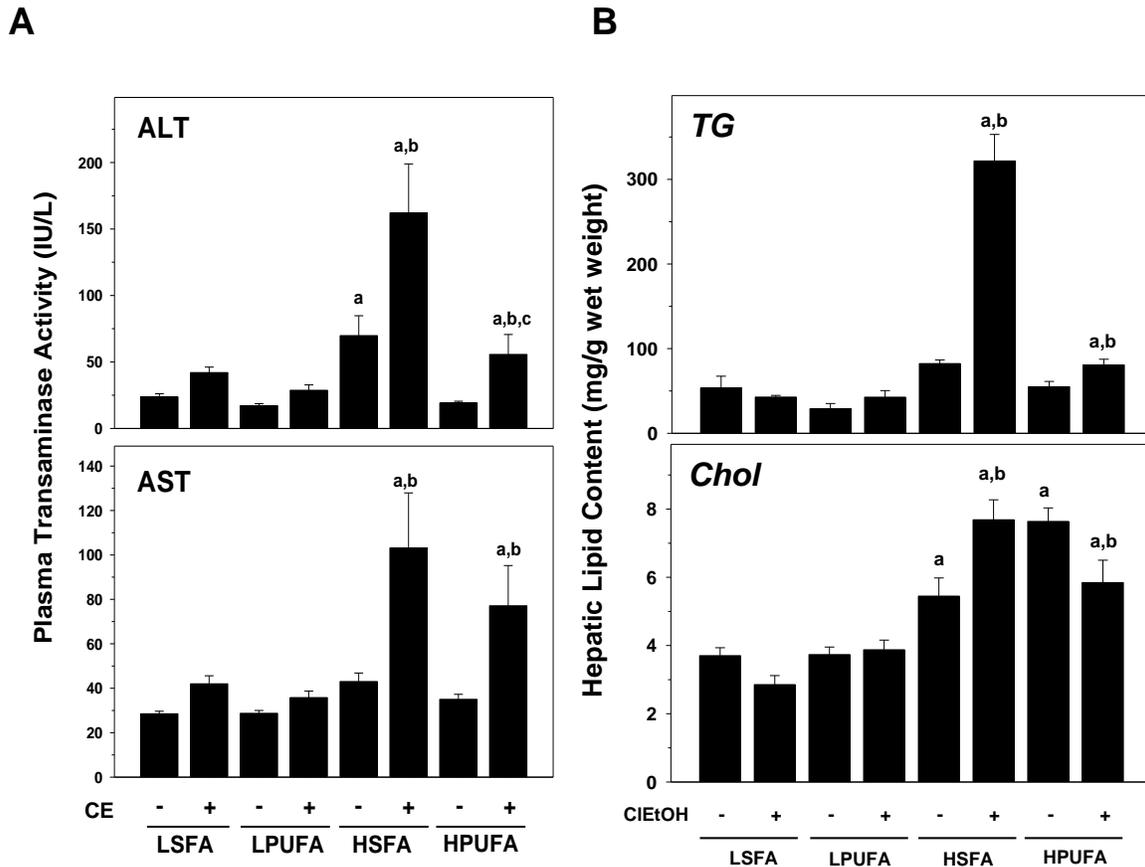
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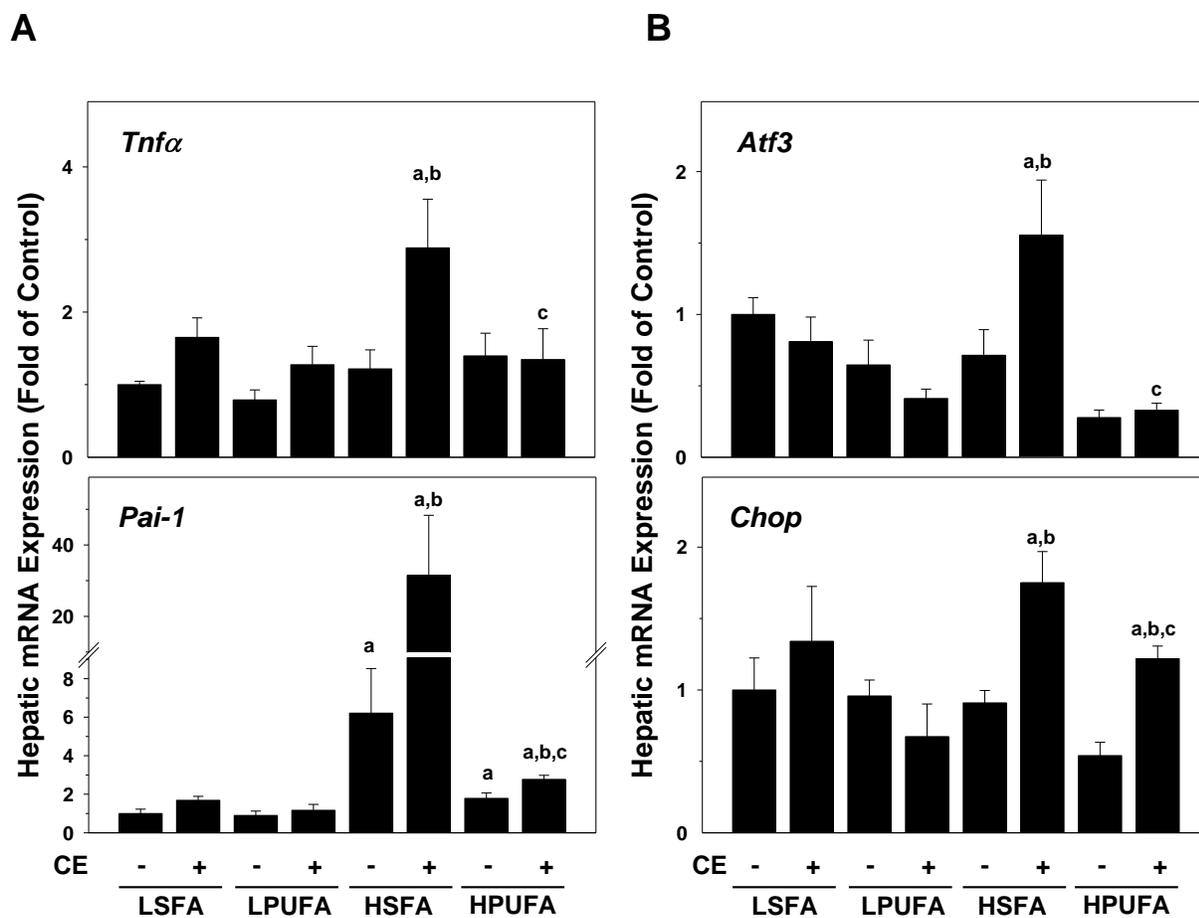
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B

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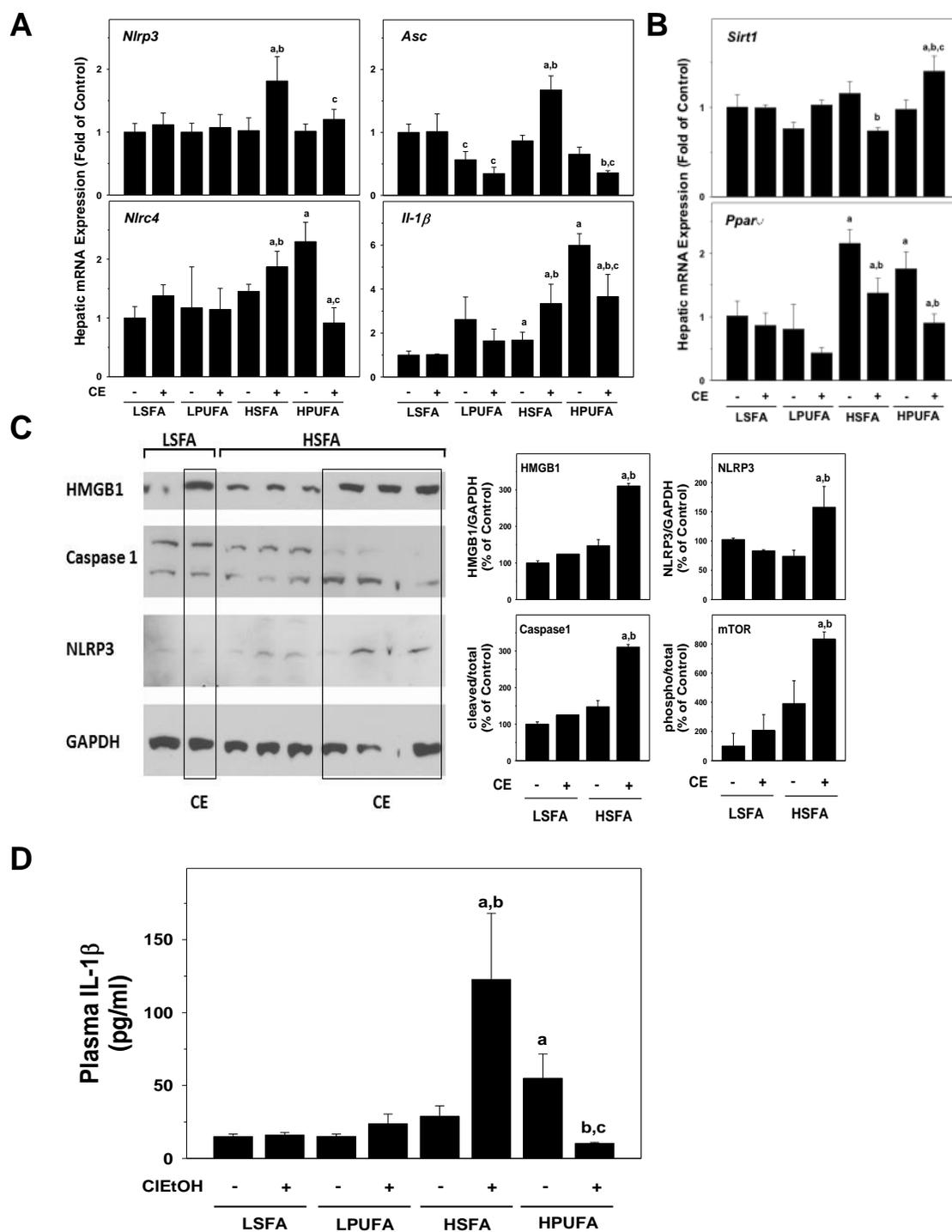
Figure 5: Chloroethanol induces inflammasome activation in HSFA+CE group.
A: RT-PCR for genes associated with inflammasome activation **B:** RT-PCR for genes associated with inflammasome suppression **C:** Representative Western blots for HMGB1, Caspase1, and NLRP3 densitometric analyses of proteins are shown. [a], $p < 0.05$ compared to the absence of LPS; [b], $p < 0.05$ compared to the absence of CE. **D:** Serum IL-1β measured with ELISA.
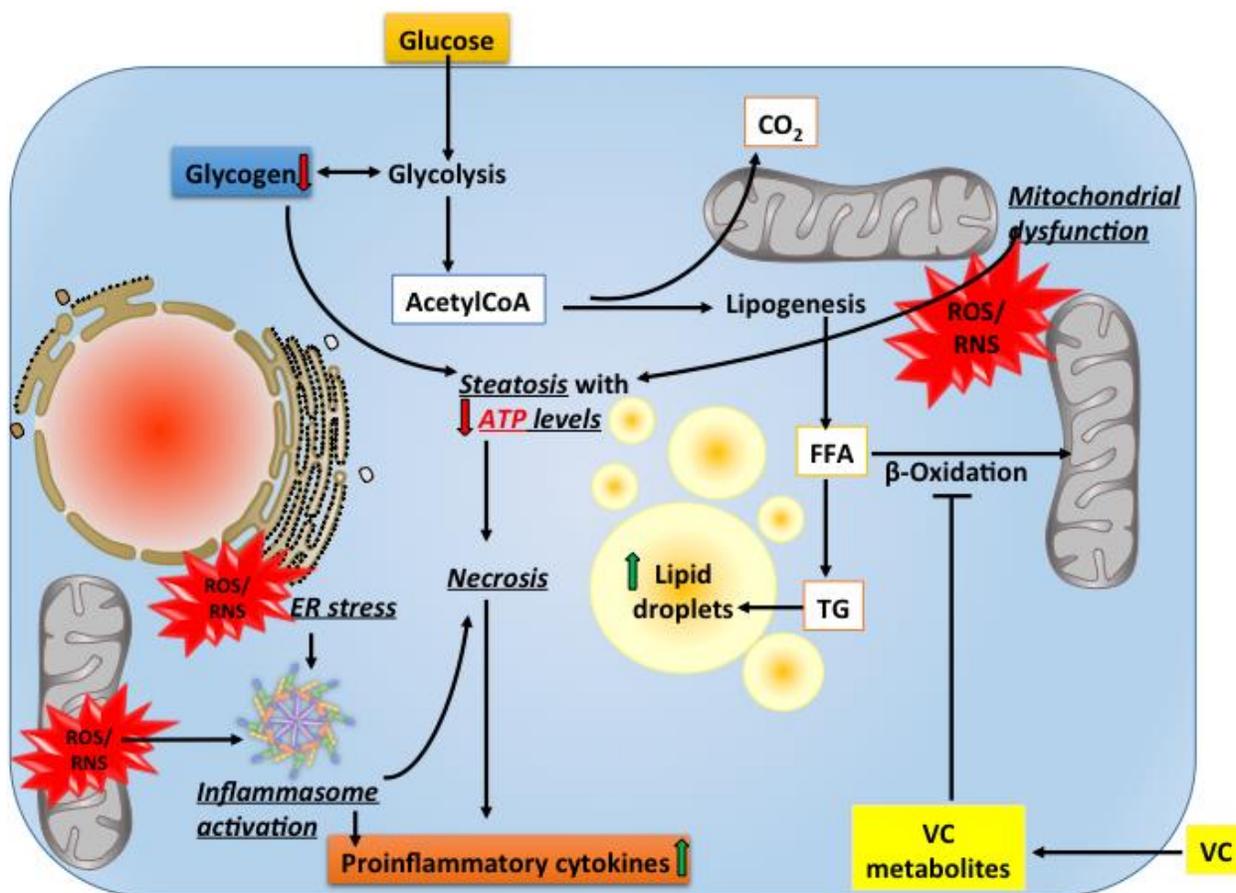
Figure 6: Working Hypothesis
Currently working hypothesis: 1) diet-induced obesity impairs the elimination process of reactive intermediates formed from VC, which leads to ER stress and thus production and release of proinflammatory cytokines and mitochondrial damage. This ultimately prevents oxidative phosphorylation resulting in compensatory anaerobic glycolysis, depleting hepatic glycogen reserve and causing a 'pseudo-fasted' state 2) increased lipid biosynthesis rather than β-oxidation in abundance of ATP, contributing to the 'pseudo-fasted' state 3) combination of metabolic effects, cytokines and inflammasome activation leads to liponecrosis.