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# The role of aldehyde dehydrogenase 2 in liver injury caused by vinyl chloride and high-fat diet.

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<https://doi.org/10.18297/etd/3120>

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THE ROLE OF ALDEHYDE DEHYDROGENASE 2 IN LIVER INJURY CAUSED  
BY VINYL CHLORIDE AND HIGH-FAT DIET

By

Liya Chen

B.S Wenzhou Medical University, 2014

M.D Wenzhou Medical University, 2016

A dissertation

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

Doctor of Philosophy in Pharmacology and Toxicology

Department of Pharmacology and Toxicology  
University of Louisville  
Louisville, KY

December 2018



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## DEDICATION

This dissertation is dedicated to my parents

Mr. Kangshi Chen

And

Mrs. Chunwei Zhi

who have given me educational opportunities.

## ACKNOWLEDGEMENTS

First and foremost, I would like to thank my mentor, Dr. Juliane Beier, for always believing in my scientific abilities, for her guidance, patience and support in my research. I would also like to thank my co-mentor, Dr. Gavin Arteel, for always pushing me to be my best and think critically. I would also like to thank my committees, Drs. Matt Cave, Wenke Feng, Jonathan Freedman, and Gary Hoyle, for their comments and assistance in my dissertation.

I must thank my parents and friends who have always understood and encourage me. Without your love, I would not stick with my study. Dr. Chang Xu, thank you for your patience and being there when I need you. To my loving Linkai Wang, your passion and optimism makes me strong and support me seek my highest potential.

## ABSTRACT

### THE ROLE OF ALDEHYDE DEHYDROGENASE 2 IN LIVER INJURY CAUSED BY VINYL CHLORIDE AND HIGH-FAT DIET

Liya Chen

November 16, 2018

**Background.** Vinyl chloride (VC) is an abundant environmental contaminant that causes steatohepatitis at high exposure levels. We have shown previously that low concentrations of VC exacerbate high fat diet (HFD)-induced liver injury in mice. The mechanisms involved in the progression of liver injury caused by VC and HFD include oxidative stress, inflammation, metabolic and mitochondrial dysfunction. Mitochondrial aldehyde dehydrogenase 2 (ALDH2) serves as a key line of defense against most reactive aldehydes, including lipid aldehydes (e.g., 4-HNE) and chloroacetaldehyde (VC metabolite). We hypothesize that this defense may play a key role in the interaction between HFD and VC in fatty liver disease.

**Methods.** Mice were exposed to VC via inhalation at concentrations below the current OSHA limit (<1 ppm), or room air for 6 hours per day, 5 days per week for 12 weeks. Mice were fed HFD or a low-fat control diet. Some mice were administered ALDH2 agonist Alda-1 (i.p. 20 mg/kg, 3 times/week) for 3

weeks prior to sacrifice. Metabolic phenotyping, biochemical and histological assessment of liver injury, indices for oxidative stress, inflammation and mitochondrial function were examined. **Results.** ALDH2 is a direct target of VC metabolite toxicity. Moreover, we demonstrated that ALDH2 activation by Alda-1 pre-treatment decreased lipid accumulation, oxidative stress, and prevented liver injury induced by acute exposure to VC metabolites. Chapter IV describes a chronic model of low-dose VC exposure with HFD. The interaction of VC and HFD decreased ALDH2 expression and activity in mitochondria. Liver injury was characterized by enhanced steatosis, inflammation and oxidative stress. This interaction correlated with mitochondrial dysfunction and metabolic stress. Administration of ALDH2 agonist Alda-1 prevented the decrease in ALDH2 activity and conferred profound protection against these changes caused by HFD+VC. **Conclusion:** Taken together, these results support the hypothesis that ALDH2 appears to be a direct target of VC exposure, which likely contributes to the enhancement of liver injury under these conditions. Importantly, ALDH2 plays a critical role in liver injury caused low-level VC exposure and HFD and may therefore be a potential target for future therapies.



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## CHAPTER I

### INTRODUCTION

#### **A. Background and the rationale for this study**

##### **1. Liver structure and function**

Liver, the largest and heaviest internal organ in the human body, is structurally and functionally complex. Unlike most organs, the liver has two distinct blood supplies. The portal vein supplies about 75-80% of blood flow while hepatic artery supplies 25% of the flow (1). The blood vessels divide into small capillaries known as liver sinusoids, which then leads to lobule. Lobules are the functional units of the liver and consist of millions of cells. Hepatocytes are most numerous and comprise 80% of the volume of liver. Being the workforce of liver, hepatocytes contain thousands of vital functions. Hepatocytes contain abundant organelles including endoplasmic reticulum (ER), lysosomes, peroxisomes and mitochondria, as well as cytoplasmic lipid and glycogen. Due to the variety of functional components, hepatocytes play a role in protein synthesis, lipid/carbohydrate metabolism and detoxification (2). In addition to hepatocytes there are also several other cell types in the liver which are vital to its overall health and function. Kupffer cells represents

of 15% of the liver cells and derived from monocytes. They are the major producer of cytokines contributing to inflammation. Hepatic stellate cells (HSC) comprise 5% of liver cells and are known to play a role in regeneration and hepatic fibrogenesis and cirrhosis (2).

The hepatic portal blood drains from gastric, splenic and pancreatic veins and travels to liver with various nutrients, toxins and chemicals. Liver, as the target of toxicant exposure, initiates detoxification in response to accumulative harmful substances. Cytochrome P450 (CYP) family is a group of enzymes highly expressed in the liver and linked to metabolism of endogenous and exogenous chemicals. They convert toxic chemicals into less harmful chemicals, this can be achieved by various reactions including oxidation, reduction and hydrolysis. However, free radicals are also generated during this process and lead to cell damage (3). Normally, antioxidants such as vitamin C and E protect the cell from these free radicals. When the balance between antioxidants and toxicants is disrupted, toxic chemicals become far more dangerous. Impaired cytochrome P450 system by toxic compounds results in a high level of free radical generation and leads to liver injury. Additionally, hepatocytes have the capacity to regenerate for compensating damaged cells to preserve healthy function of the entire organ. This protective capacity is limited, if the damage is too severe or if the injury is chronic, hepatic cell death and irreversible injury may occur.

Multiples risk factors including host genetics (e.g. gender, ethnicity), primary risk factors (e.g. obesity, alcohol) and comorbidities (e.g. viral

hepatitis, nutrition) can cause liver damage. According to different etiology or manifestation, liver diseases are diagnosed as different types. In this study, we mainly focused on the non-alcoholic fatty liver disease (NAFLD).

## **2. Non-alcoholic fatty liver disease and risk factors**

Non-alcoholic fatty liver disease (NAFLD) is a clinical diagnosis that include the presence of 5% or more hepatic steatosis in the absence of excessive alcohol use as determined by liver imaging or biopsy in the absence of secondary causes of hepatic fat accumulation. It ranges from simple steatosis to non-alcoholic steatohepatitis (NASH), liver fibrosis and cirrhosis, with its associated complications such as hepatocellular carcinoma (HCC). The prevalence of NAFLD in the United States has risen from 18% since 1988-1991 to 31% in 2011-2012 (4). Recent epidemic research has shown that NAFLD is the most prominent cause of liver diseases, representing over 75% of chronic liver disease (5). It has been reported that up to 70% of patients with NAFLD are also affected by NASH and about 25% of patients with NASH develop to cirrhosis (6). NASH is also a leading third indication for liver transplantation in the United States. That contributes to a major burden to mortality and morbidity, and it is expected to be the number one in 2020 (7). Moreover, NAFLD not only affects liver function, but is also related to the metabolic disorders such as diabetes (8). Therefore, it is significant and necessary to understand the physiopathologic mechanisms of NAFLD. NASH is a histological term that characterized by the presence of necro-inflammatory process whereby hepatocytes become injured in a

background of steatosis (9). About 40% of NASH develop to progressive fibrosis, leading to cirrhosis in 10-27% and HCC in 4-27% of those cirrhosis (10).

As the most common type of NAFLD, NASH can be affected by many risk factors including host genetics (e.g. gender, ethnicity), life style (e.g. smoking, alcohol consumption) and comorbidities such as diabetes (11). Diet, especially a diet high in fats, has been thought as an independent risk factor of NASH. Obesity in adults is defined as body mass index (BMI) of greater or equal to 30. The prevalence of obesity in United States is increasing in the past few years. There are about 39.8% of adults and 18.5% of youth being obese in 2016 (12). Based on the cohort studies, obese individuals have a 3.5-fold increased risk of liver damage and a has been reported dose-dependent relationship between BMI with NAFLD progression (13). As the obesity rate increases, the burden of obesity on liver diseases is nonnegligible. The fat accumulated in liver varies with different degree of obesity and even subtle changes in body weight are associated steatosis (14). Thus, obesity can be considered as a predictor of NAFLD (15).

Environmental toxicants are another relevant cause that are thought to be a primary risk factor of liver disease development. Long term occupational and environmental exposures to industrial chemicals directly cause steatohepatitis, which is termed as toxicant-associated steatohepatitis (TASH). Increasing evidence suggests that exposure to elevated levels of various industrial chemicals including volatile organic compounds, persistent

organic pollutants and toxic metals can contribute to the development of TASH, eventually leading to liver failure (16, 17). Chlorinated hydrocarbons are the most prevalent form of organic contaminants that cause human health problems via groundwater or air inhalation. Vinyl chloride (VC) is a specific type of chloride compound that is greatly concerning since it is distributed in both liquid and gas phase. Additionally, it is known for its role as a human carcinogen capable of damaging DNA (18). VC is therefore identified as a risk factor that impacts human health, especially in liver (19). What is less known, is that VC may also cause hepatic steatosis (20). The degree of liver damage caused by VC may be correlated to dose exposure. The cases of high-dose (> 1000 ppm) of VC induced severe liver injury has been reported (20), the effect of low-dose of VC exposure on liver function is less studied.

### **3. Vinyl chloride and liver damage**

Vinyl chloride (VC) monomer is a colorless gas that is widely used in industrial chemical synthesis such as polyvinyl chloride (PVC). PVC is a polymerized form of VC that is extensively involved in plastic manufacture. VC doesn't occur naturally, and it is mostly found in the industries for PVC manufactures. Additionally, VC is the intermediate of some volatile organic compounds such as trichloroethene (TCE), tetrachloroethene (PCE) and dichloroethylene (DCE), which is globally applied as degreasing agent for automotive and metal industries (21). VC is even present in cigarette smoking, depending on the concentration of chloride in tobacco. VC is closely related to our daily life and it serves as the commercial production that has

been widely consumed over 70 years in the United States (22, 23). VC production was recently estimated at 27 million metric tons in the America annually (22). As a representative organochlorine toxicant, VC has been considered as a priority pollutant listed by the US Environment Protection Agency (EPA) (24) as well as the Centers for Disease Control and Protection's Agency for Toxic Substances and Disease Registry (ATSDR). Industrial discharges, landfill leaching, improper storage or disposal, as well as atmospheric transport and deposition are important ways to enter groundwater or air for VC. It is present in landfill leachates and in the groundwater near military installations such as Camp Lejeune. To date, over tens of thousands of American chemical workers have been exposed to organochlorine pollution. Such huge amount of VC emission load causes occupational diseases especially among the people working in chemical and plastic industries. VC is reported to be easily absorbed in human body through respiratory system and to affect multiple organ functions including bronchial irritation, central nerves system impairment and liver damage (25). The toxicity of VC was first reported in the 1970s associated with angiosarcoma of the liver (ASL) (26, 27). In the past few decades in Louisville, cohort studies also elucidated the relationship between VC exposure and liver cancer (28). Attarchi et al also reported the case of altered liver function was found in PVC workers compared with office workers in the same industries (29). These data suggested that liver is the main target to VC.

Dr. Cave's laboratory documented that workers exposed to high-level VC had steatosis, hepatomegaly and fibrosis, in association with insulin resistance, altered adipocytokines, and antioxidant depletion (16). It may not be surprising that VC exposure leads to steatohepatitis because of its similar metabolism pathway to ethanol. Concentrations of VC up to approximately 220 ppm, are metabolized through cytochrome P450 2E1 (CYP2E1) forming the highly reactive genotoxic epoxide and chloroethylene oxide (Scheme 1.1). Chloroethylene is either spontaneously or enzymatically converted into chloroethanol (CE) and chloroacetaldehyde (CAA) (18). Previous work by our lab has shown that individual treatment of CE or CAA alters cytokine production, causes mitochondrial dysfunction, leads to disruption of hepatic carbohydrate/lipid metabolism and exacerbates steatohepatitis (30). Those studies showed that high-level of VC exposure is acknowledged to be an inducer of liver injury and the effect can not be ignored. The Occupational Safety and Health Association (OSHA) lowered the workplace air standard for VC from 500 ppm to a safety limited dose of 1 ppm (2.5 mg/m<sup>3</sup>). The morbidity of liver diseases is lower than before, however there are some cases of liver injury that were still observed in the low VC exposure workers (31, 32). This phenomenon suggests that it is possible for another risk factor to enhance liver damage by inhaled VC.

#### **4. Liver injury caused by fat and VC**

It is demonstrated by many studies that VC toxicity is associated with the development of liver disease. It has been well documented that workers

exposed to high concentrations of VC are prone to have high incidence of liver injury, ranging from simple steatosis, steatohepatitis to fibrosis even HCC (19). Obesity is also a risk factor for developing steatohepatitis. Fat accumulation in the liver may mediate hepatocyte injury (33). Our recent studies have shown that mice exposed to VC inhalation below the OSHA limit concentration (<1 ppm), could exacerbate liver injury in combination with a HFD. Increased inflammation, oxidative stress and mitochondrial impairment were observed (34). Based on our previous research of the mechanisms involved in NAFLD progression by VC and dietary fat, we aim to seek for a novel target avenue of treating liver damage in this study.

## **5. Aldehyde dehydrogenases 2**

Aldehyde dehydrogenases (ALDH) are a group of enzymes that are responsible for the oxidation of aldehydes. These ALDH isoforms are encoded by nuclear genes. Nineteen different functional genes have been identified in the human genome and are widely expressed in multiple tissues at the highest concentration in the liver (35). ALDH isoforms are found in various subcellular organelles including nucleus, mitochondria, cytosol and ER (36). ALDH2 is a tetrameric enzyme located in mitochondrial matrix belonging to the ALDH family. It is ubiquitously expressed in all tissues but is most abundant in the liver. ALDH2 is the most efficient enzyme in ethanol metabolism, and it converts acetaldehyde to acetic acid. ALDH2 has the lowest  $K_m$  to this substrate. This  $K_m$  is 900-fold lower than that of the other members of ALDH family (37).



ALDH2 genetic polymorphism studies show that the *ALDH2\*2* allele is the most relevant form of ALDH2 variant. People with *ALDH2\*2* allele are susceptible to having reactions such as facial flushing and nausea after alcohol consumption, due to a lower catalytic activity. *ALDH2\*2* is thought to be the most common enzyme deficiency effect the 8% of world population including in 35-45% Asian people (38). In the United States, ALDH2 deficiency is mostly found in the American Indian and the Asian populations (39, 40). The role of ALDH2 in acetaldehyde detoxification and liver disease progression has been reported in human and experimental models. High blood acetaldehyde levels were observed in individuals with ALDH2 mutant alleles after alcohol consumption, suggesting that the ALDH2 deficit increased human susceptibility to alcohol-induced organ injury (41). ALDH2 knockout mice showed an increased acetaldehyde accumulation in the liver after alcohol exposure or acetaldehyde inhalation, along with exaggerated inflammation and fibrosis (42). In contrast, activation of ALDH2 reverses alcohol-induced hepatic steatosis and overexpression of ALDH2 significantly reduced acetaldehyde as well as apoptosis in mice fed with chronic alcohol ingestion (43). These findings provide a strong rationale that ALDH2 activation plays a protective role in alcoholic fatty liver disease (AFLD). However, the role of ALDH2 in liver injury caused by VC and dietary fat remains elusive.

It is now understood that substrates to mitochondrial ALDH2 expand well beyond the canonical acetaldehyde. Other aldehydes including 4-

hydroxynoneal (4-HNE) and malondialdehyde (MDA) that are derived from lipid peroxidation are also substrates of ALDH2 (44). Our lab has demonstrated that oxidative stress as well as mitochondrial impairment caused by 4-HNE can be reversed by activation of ALDH2 (45), indicating that ALDH2 plays a role as an antioxidant enzyme. Indeed, ALDH2's protective role has been highlighted by many studies.

ALDH2 impairment has a deleterious effect on cellular function via overburden of lipid adducts, oxidative or ER stress, increased mitophagy and DNA damage, developing hepatic fibrosis, cirrhosis and cancer (46-48). Epidemic research revealed that the prevalence of NAFLD tends to be higher in the *ALDH2*\*2 population (49). These researches suggest that ALDH2 dysfunction may be a part of the mechanisms contributing to fatty liver disease. We have determined that mitochondrial function was impaired by VC (34). We therefore hypothesize that ALDH2 dysfunction may play a role in liver damage caused by VC. Our previous work showed that multiple mechanisms were involved in the liver injury caused by VC and fat such as oxidative stress, inflammation, ER stress (34). ALDH2 has been demonstrated to be a protective enzyme in alcoholic liver injury via regulation of ER stress, inflammation, oxidative stress and autophagy (50). Moreover, ALDH2 is capable to catalyze CAA (knowns as VC metabolite) (51). We hypothesize that ALDH2 activation will protect the liver from dietary fat and VC exposure.

## **6. Statement of Goal**

In our previous work, we showed that low-level VC exposure exacerbates liver injury caused by dietary fat via multiple mechanisms including inflammatory damage, oxidative stress, ER stress and mitochondrial dysfunction. In this study, we aim to seek for a novel molecular factor as a liver protective target. As introduced throughout this chapter, we know that ALDH2 is an important enzyme that has been clarified to be a protective factor in liver function against alcohol abuse in many studies. VC shares a similar pathway for metabolism as ethanol. Therefore in this dissertation, we hypothesize that activation of ALDH2 may affect liver injury caused by VC and HFD, and we will investigate the role of ALDH2 in regulating liver function recovery.

**B. Specific aims in this study.**

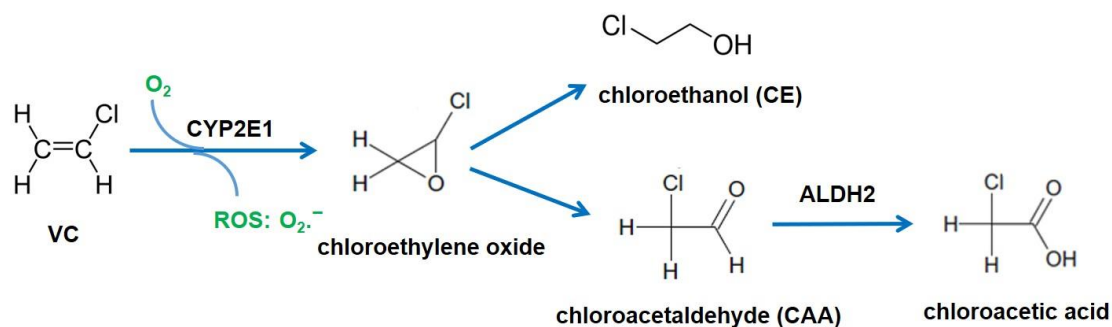
**1. Aim1: Evaluate the protective role of ALDH2 against VC metabolite exposure in mice**

This aim is mainly to investigate the role of ALDH2 in the CE or CAA exposure (metabolites of VC). Previous work by our lab showed that CE increased 4-HNE adducts and altered energy metabolism including disruption of carbohydrates/lipids, CAA treatment also caused toxicity in primary hepatocytes and HepG2 cells (30). ALDH2 is known to reduce 4-HNE induced oxidative stress and mitochondrial dysfunction (45). We hypothesize that ALDH2 plays a role in the protecting liver from VC metabolites. The goal of this aim is to study whether ALDH2 prevents co-induced liver damage. The

potential mechanisms that could be affected by ALDH2 are investigated. We also aim to explore the effect of CAA on ALDH2 activity.

**2. Aim2: Investigate the protective role of ALDH2 against liver injury caused by VC and HFD in mice**

Recent work from our group showed that the low-dose VC exposure enhanced HFD-induced liver injury. The role of ALDH2 in this animal model has not been studied before. The goals of this aim are therefore to (i) characterize whether ALDH2 function is impaired in the liver injury of our model; (ii) to determine whether activation of ALDH2 prevent liver damage from VC and HFD; (iii) and to explore the mechanisms involved in the effect of ALDH2 on liver injury.



### Scheme 1.1: VC metabolism

VC metabolism in the cell is represented. VC is metabolized through CYP2E1 and form chloroethylene oxide, which can be transformed into chloroethanol (CE) or chloroacetaldehyde (CAA). CAA can be degraded by ALDH2 into chloroacetic acid

## CHAPTER II

### EXPERIMENTAL PROCEDURES

#### **A. Animals and treatment**

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

##### **1. Acute mice model of CE exposure**

During the exposure period, mice were housed in pairs in shoebox cages in a room held at 75 °F. Food and tap water were allowed ad libitum. Mice were given control diet and randomly divided into 4 groups: **1)** control, **2)** Alda-1(ALDH2 activator), **3)** chloroethanol (CE, 50mg/kg, i.g.) and **4)** Alda-1+CE. The Alda-1 group was pretreated with ALDH2 activator (20mg/kg/d i.p.) for 3 days, on the fourth day, the mice were administered CE after 30 min- Alda-1 injection. Mice were anesthetized and sacrificed the after 24h CE treatment.

## **2. Chronic mice model of VC and HFD exposure**

Mice were fed low-fat control (LFD, 13% calories as fat) or high-fat diet (HFD, 42% calories as fat) for 12 weeks in bedding-free cages. The mice were exposed to VC (Kin-tek, La Marque, TX) via inhalation at the concentration below the current limit of OSHA ( $\sim 0.85 \pm 0.1$  ppm) for 12 weeks (6 hours per day, 5 days per week). Some mice were administered room air for the same pattern. At the end of 9<sup>th</sup> week, some mice were injected with a specific agonist of ALDH2, Alda-1 (20 mg/kg i.p., 3 days/week; EMD Chemicals, Gibbstown, NJ), which is dissolved in the vehicle of 50% dimethylsulfoxide and 50% polyethylene glycol. Body weight and food consumption were monitored weekly in modeling period. Mice were sacrificed at 12<sup>th</sup> week.

## **3. Oral glucose tolerance test**

Blood glucose level of chronic mice model of VC and HFD was measured at the 12<sup>th</sup> week by oral glucose tolerance test (OGTT). Transfer mice to cages without food and bedding 6 hours prior to experiment. After fasting period, blood was sampled from the tail cutting immediately after fasting to determine baseline. Following oral administration of 2 mg/kg glucose (Sigma, St Louis, MO) in 4 ml/kg of sterile saline solution, blood from tail vein was measured at 15, 30, 60, 90 and 120 minutes for glucose level. Glucose concentrations were tested using an Accu-Chek Aviva Plus glucometer and test strips (Roche Diagnosis Corp., Indianapolis, IN).

#### **4. Animals sacrifice, samples collection and storage**

At sacrifice, animals were fasted for 4 hours and anesthetized with ketamine/xylazine (100/15 mg/kg, i.p.). Blood was collected from the vena cava just prior to sacrifice and citrated plasma was stored at -80°C for further analysis. After washing with PBS buffer, liver tissue will be collected with portions of all tissues were snap-frozen in liquid nitrogen, embedded in frozen specimen medium (Sakura Finetek, Torrance, CA), or were fixed in 10% neutral buffered formalin for later histological staining. Total hepatic RNA will be extracted from additional portions using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) and chloroform: phenol extraction.

### **B. Histology**

#### **1. General morphology**

Liver tissues were fixed in formalin, paraffin embedded liver section 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). Pathology was scored (inflammation and necrosis) in a blinded manner as described before, the number of inflammatory or necrotic foci was determined ten 400x fields.



## **2. Neutrophil accumulation**

Neutrophil accumulation in liver tissue was measured using chloroacetate esterase (CAE) staining. Briefly, formalin fixed, paraffin embedded liver tissue was cut at 5  $\mu\text{m}$  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 ASD chloroacetate (1 mg/ml) in N,N-dimethylformamide, with 4% sodium nitrite and 4% new fuchsin for 25 min. The naphthol AS-D chloroacetate was enzymatically hydrolyzed by chloroacetate esterase in neutrophils, liberating the naphthol compound. Naphthol combined with a freshly formed diazonium salt, leaving bright pink color deposits at the site of enzymatic activity. The slides were counterstained in the hematoxylin for 15-30s and dipped in ammonium hydroxide solution for few seconds to stain nucleus in blue. After staining, check liver section for counter stained color and dehydrated them through graded ethanol, washed in Citrisolv, then mounted with Permount (Fisher, Waltham, MA). CAE positive cells were counted by Metamorph Imagen Analysis Software (Molecular Devices, Sunnyvale, CA) and expressed by the ration of positive staining cells in 100 hepatocytes.

## **3. Lipid and glycogen accumulation**

Lipid accumulation was detected via Oil Red-O (ORO) staining. This staining was based on the great solubility of oil-soluble dyes in lipid substances. Oil Red-O powder was dissolved in 99% 2-proponal (Fisher

A416-4) and make the stock solution at the concentration of 3mg/ml. Before staining, Oil Red-O must be made freshly by mix the stock with distilled water at the ration of 3:2. 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 rinsed in water and 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. Slides were washed in lukewarm water for 5 minutes, tissue section turned into dark pink color. Samples were then counterstained with hematoxylin for 45 seconds, washed in water, dehydrated through graded ethanol, washed in Citrisolv, and then mounted with Permount (Fisher, Waltham, MA).

#### **4. Immunohistochemistry**

4-HNE and MDA stained liver were performed by immunohistochemistry (IHC). Previous sectioned formalin-fixed, paraffin-embedded liver sections were deparaffinized in Citrisolv and hydrated through descending grades of ethanol to distilled water. Slides were incubated in target retrieving solution overnight at 72°C. Slides were placed in room temperature and then rinsed in

TBS. Following that, endogenous peroxidases were quenched in 3% hydrogen peroxide. Blocking for endogenous biotin was performed using a commercially available kit (Agilent Technologies, Santa Clara, CA). Prior to applying primary antibody, sections were blocked in 10% goat serum in TBS-0.01% Triton. Positive sections were incubated in 1:500 rabbit anti-MDA (Cell Signaling Technologies 2956S, Danvers, MA) and negative parts were incubated in TBS-0.01% Triton for 2 hours at room temperature. After washing several times in TBS, tissues were incubated in a biotinylated anti-rabbit IgG secondary antibody from the Vectastain Elite ABC kit detection (Vector Laboratories, Inc., Burlingame, CA) for 15 minutes at room temperature. Slides were rinsed in TBS and then incubated in a solution containing avidin-bound horseradish peroxidase (HRP) for 30 minutes. For 4-HNE staining, using peroxidase from DAKO kit to block deparaffinize and rehydrated tissues, slides were incubated in primary antibody of 4-HNE (Alpha diagnostics) which was diluted in PBS-T at 1:500 for 30 minutes at room temperature, second antibody was applied for 5 minutes. The HRP substrate 3, 3'-diaminobenzidine (DAB) (Agilent Technologies, Santa Clara, CA) was added to sections until positive (brown) staining was macroscopically visible. Slides were counterstained with hematoxylin for 1 minute, washed, dehydrated through graded ethanol and then mounted with Permount (Thermo Fisher, Waltham, MA). Each slide contained a negative tissue section that did not receive primary antibody. Slides were visualized

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

### **C. Biochemistry assay**

#### **1. AST, ALT assay**

Plasma activity levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined spectrophotometrically using standard kits (Thermo Fisher Scientific, Waltham, MA).

#### **2. Lipid, glycogen extraction and quantification**

A small piece of frozen liver was homogenized in tissue pulverizer, staying as cold as possible with the liquid nitrogen. Liver powder was transferred to a tared glass tube with chloroform and methanol addition using glass syringes. Appropriate volume of water was added in the samples followed by a complete vortex and sitting on ice for at least 30 minutes. Samples were mixed with chloroform and water, then vortexed completely and centrifuged down at a full speed. Appropriate volume of sample was taken out from the bottom phase and transferred to a new tube. N<sub>2</sub> drying apparatus was applied for drying samples. After that, dried lipids were resuspended with 200 µl 5% lipid free BSA. For triglyceride and cholesterol measurement, standard of L-DC cal solution in graded concentration were prepared with addition of triglyceride (TG)/cholesterol reagent as described in the protocol from Infinity kit (Thermo TR 13421). The plates were incubated in the plate reader for 5

minutes and read at wavelength of 500 nm. Non-essential fatty acid (NEFA) assay was measured as describe in instruction, then read absorbance at wavelength of 520 and 680 nm.

Glycogen determination in liver was modified from Seifter et al (52). 50-100 mg liver tissue was placed in 500  $\mu$ l of 30% KOH and incubated in 100°C for a complete digestion. After adding 625  $\mu$ l of 95% ethanol, samples were sitting at room temperature overnight. Samples were centrifuged at full speed and the pellets were resuspended in water. Samples or glucose standards were mixed with 0.2% anthrone solution by the ration of 1:2 and incubated at 100°C for 10 minutes. 150  $\mu$ l of vortex samples were transferred to 96-well plate. OD value was measured at 620 nm, glycogen was calculated by glucose standard curve and reported at  $\mu$ g/g tissue.

### **3. ALDH2 activity assay**

In the in vivo model, ALDH2 enzymatic activity was determined in mitochondrial extracts using a commercial kit (Abcam, ab115348). The enzyme is captured within wells of the microplates and activity is determined by the production of NADH in the ALDH2 catalyzed reaction. The generation of NADH is coupled to the 1:1 reduction of reporter dye to yield a colored reaction product whose concentration can be monitored by measuring the increase in absorbance at 450 nm. All the reagents were prepared as described in the protocol. Mitochondrial sample pellets were mixed with extraction buffer and centrifuged down for 20 minutes. Supernatants were diluted and placed in the well covered with acetaldehyde. After 3 hours of

incubation, wells were washed 3 times. The absorbance was recorded at 450 nm at 30 minutes and 120 minutes after addition of the activity solution.

Purified ALDH2 protein (10 µg) purchased from Sigma (St Louis, MO) were added to 50mM sodium phosphate buffer (pH 7.4) containing 1mM EDTA, 1 mM DTT and 1 mM NAD<sup>+</sup>. The reaction was initiated by the addition of CAA (Sigma, St Louis, MO) into the 1 ml cuvette. The ALDH2 activity was measured spectrophotometrically at 340 nm via the formation of NADH. For Alda-1 group, ALDH2 was incubated in Alda-1 (20 µM) for 2 minutes and measured activity immediately after CAA and NAD<sup>+</sup> addition. For CAA group, enzyme was exposed to CAA (40 µM) for 5 minutes, added NAD<sup>+</sup> and determined absorbance. To investigate the effect of Alda-1 intervention on CAA exposed enzymatic function, we pre-incubated enzyme to Alda-1 for 2 minutes, then exposed it to CAA for 5 minutes and measured activity after NAD<sup>+</sup> addition.

#### **4. TBARS assay**

The liver MDA level was quantified by determination of thiobarbituric acid reactive substances (TBARS) concentration using a commercial kit (R&D system, Minneapolis, MN). Free MDA is typically low, requiring release of MDA by acid treatment of proteins and breakdown of peroxides by heat and acid to facilitate color development in the TBARS assay. Liver homogenate was mixed with the same volume of TBARS acid reagent, incubated at room temperature for 15 minutes and centrifugated. TBARS standard and sample supernatants were placed in the 96-well plate with the addition of TBA

reagent. The OD value of each well was pre-read using a microplate reader at 532 nm. After incubation at 45°C for 2 hours, the OD was determined again. The pre-reading was subtracted from final the reading to correct for samples contribution to the final absorbance at 532 nm. A standard curve was created and MDA concentration was calculated. The MDA levels were reported at nmol/mg protein.

#### **D. Sample protein extraction**

Liver samples were homogenized in RIPA buffer [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (wt/vol) Triton X-100], containing protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO). Liver homogenates were centrifugated at full speed for 5 minutes, supernatants were collected and stored at -80°C. Mitochondria protein was isolated as describes by Wiekowski et al (53). Fresh liver tissues were washed in IB-1 (225 mM mannitol, 75 mM sucrose, 0.5% BSA, 0.5 mM EGTA and 30 mM Tris-HCl) immediately and then transferred to IB-3 (225 mM mannitol, 75 mM sucrose and 30 mM Tris-HCl). Liver homogenates were mixed with appropriate IB-1(4 ml/g of liver) and centrifuged at 740g for 5 minutes. Supernatants were collected and centrifuged by a higher speed at 9,000g for 10 minutes. The pellets were resuspended with IB-2 (225 mM mannitol, 75 mM sucrose, 0.5% BSA and 30 mM Tris-HCl) following by centrifuging at 10,000g for 10 minutes. The pellets were washed in IB-3 and centrifugated at the same speed. After that, the pellets were dissolved in MRB

(225 mM mannitol, 5 mM HEPES and 0.5 mM EGTA) and crude mitochondrial proteins were stored at -20°C.

#### **E. Immunoblots**

Samples were loaded onto SDS-polyacrylamide gels (4-12%) (Invitrogen, Thermo Fisher Scientific, Grand Island, NY), followed by electrophoresis and Western blotting onto PVDF membranes (Hybond P, GE Healthcare Bio-Sciences, Pittsburgh, PA). The PVDF membrane was washed in TBST buffer and blocked by TBST containing 5% milk. The membrane was incubated in the primary antibodies against Atg7, p62, LC-3 I/II, GAPDH (Cell Signaling Technology; Beverly, MA), PINK1 and PARKIN (Santa Cruz Biotechnology, Dallas, TX) of dilution in 1:1000 overnight at 4°C. Second antibodies were used at 1:5000. Target proteins were visualized using a chemiluminescence detection system. The expression of ALDH2 was determined using mitochondria extraction and primary antibodies of ALDH2 diluted in 1:1000, VDAC was performed as a loading control for mitochondria. Densitometric analysis was performed using UN-SCAN-IT gel (Silk Scientific Inc., Orem, UT) software.

#### **F. RNA Isolation and Real-Time qPCR**

RNA was extracted immediately following sacrifice from fresh liver samples using RNA Stat60 (Tel-Test, Ambion, Austin, TX) and chloroform.



Sample mixtures were centrifugated at 12,000g for 15 minutes at 4°C. The aqueous phase was transferred into a new tube, mixed with isopropanol and subsequently centrifugated completely. The pellets were washed in 75% ethanol. After removing supernatant by centrifuging, the pellets were resuspended with RNase free water in 65-70°C for 5 minutes. RNA concentrations were determined spectrophotometrically and 1µg of total RNA was reverse transcribed using a kit (Quanta Biosciences, Gaithersburg MD). Real-time qPCR was performed using a Step One 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 CT method was used to determine fold differences between the target genes and an endogenous reference (18S).

## **G. Hepatocytes and cellular function analysis**

### **1. Primary hepatocytes isolation and culture**

Mice were anesthetized with ketamine/xylazine (100/15 mg/kg, i.p.). Thread was set around inferior vena cava (IVC). Catheter was inserted into the vein in an appropriate position by visualizing a backflow of blood. EGTA solution was connected to catheter to perfuse liver (5 ml/min) for 6 minutes, then switch to collagenase buffer for 10 minutes. Liver was taken out and minced well in Waymouth media. The cells were filtered and centrifuge at a low speed for several times. After washing the cell pellet with HBSS buffer, the hepatocytes were seeded in the collagen covered plate at density of

10,000 cells per well. Cells were washed with HBSS after 90 minutes of incubation at 37°C.

## **2. Hepatocytes cytotoxicity measurement**

Primary hepatocytes isolated from 12-week HFD fed mice exposing to VC were then exposed to CAA (Sigma) at the different concentration for 1 hour in the incubator. Media containing Hoechst 33342 (1.5  $\mu$ M, nuclear fluorescence), TMRM (20  $\mu$ M, mitochondrial membrane potential indicator), TOTO-3 (1 $\mu$ M, index of cell membrane permeability), fluo-4 AM (1 $\mu$ M, intracellular ionized Ca) and MitoSox (5  $\mu$ M, mitochondrial superoxide indicator) dye was add to the wells after CAA treatment. Following 45 minutes incubation with mixed dye, the plate was placed into the Cellomics Assay San VTI HCS reader and analyzes as previously described by O'Brien et al (54). Ten fields per well at the 20 $\times$  objective was used to collect images for valid cell count (defined by Hoechst 33342 staining). The average of individual values for all fluorophores for each valid object was analyzed.

## **3. Hepatocytes oxygen consumption rates measurement**

Primary hepatocytes were placed at 10,000 cells per well on collagen-coated XF96 culture microplate (Seahorse Biosciences, Billerica, Massachusetts) and incubated at 37 °C to allow cell attachment. Cells were pre-exposed to Alda-1 at 20  $\mu$ M for 30min and changed to XF medium (Seahorse Biosciences) containing the same concentration of Alda-1. For mitochondrial respiration measurement, hepatocytes were injected with CAA

(40  $\mu\text{M}$ ), oligomycin (1  $\mu\text{g/ml}$ ), FCCP (4  $\mu\text{M}$ ), antimycin A (10  $\mu\text{M}$ ) and rotenone (10  $\mu\text{M}$ ). The injected compounds were diluted in XF medium. Oxygen consumption rates (OCRs) were measured using an XF96 Extracellular Flux Analyzer (Seahorse Biosciences, Billerica, Massachusetts).

#### **H. Statistic analysis**

Results were reported as means  $\pm$  SEM (N=4-7) and analyzed using Sigma Plot 11.0 (Systat Software, Inc; San Joes, CA). ANOVA with Bonferroni's post host test (for parametric data) or Mann-Whitney Rank sum test were used for determination of statistical significance among treatment groups, as appropriate. A p value  $< 0.05$  was selected before the study as the level of significance.

## CHAPTER III

### ALDH2 PROTECTS LIVER FROM VC METABOLITES

#### **A. Introduction**

The liver is the largest organ in the body and serves as the most complex organ in terms of metabolism. It plays a critical role in the metabolism of amino acids, biochemical oxidation and detoxification of drugs or environmental toxicants. Due to the role of liver functioning as the first line of defense, the liver is also the most common organ to be damaged by toxicants or chemicals. Industrial chemicals induced hepatocyte toxicity is depended on dose exposure (55). VC is an environmental toxicant ranked #4 on the ATSDR substances priority list that known to induce hepatotoxicity (56). High-levels of VC exposure have been demonstrated to be associated with occupational liver diseases such as steatohepatitis (TASH), liver cancer (30). Thus, we aim to find a potential target factor for protecting the liver from VC exposure. VC has a similar metabolic pathway to ethanol. VC is metabolized to CE and subsequently forms into CAA depend on aldehyde dehydrogenase pathways, therefore CE or CAA may be an important mediator in VC-induced liver injury.

Previous work by our lab has shown that CE slightly increases liver damage through elevating 4-HNE adducts, which is an index of oxidative stress, destroying energy metabolism including increased lipid droplets and depleted glycogen reserves in the liver (30). CAA damaged mitochondrial function and directly impaired cell viability in hepatocytes (30). These data demonstrated that VC metabolites are detrimental to the liver and could be the mediator in liver damage caused by VC inhalation. Mitochondrial dysfunction is known to be a part of mechanisms attributing to VC metabolites induced hepatocytes damage (30, 34). Protecting mitochondria may reduce CE or CAA caused cytotoxicity.

It is now accepted that substrates for ALDH2 go well beyond the canonical acetaldehyde, namely lipid aldehydes (e.g. malondialdehyde) are also reduced by ALDH2 (see also Chapter I). Indeed, we previously have found activation of ALDH2 protected mitochondrial function from 4-HNE cytotoxicity related mitochondrial membrane polarization and reduced oxidative stress in liver (45). The mitochondrion is the main organelle for cellular energy supply, which is especially important for carbohydrate and lipid metabolism. Thus, ALDH2 is proposed to protect mitochondria and to therefore maintain energy metabolism homeostasis. Whether ALDH2 plays a role in protecting liver of mice exposed to VC inhalation remains unclear. Therefore in this section, we aim to study the role of ALDH2 in the presence of VC metabolites instead of chronic VC inhalation.

Since CAA is also a substrate of ALDH2 (51), it is reasonable to speculate

that cell damage caused by CAA can be attenuated by ALDH2 and that effect may be via protecting mitochondrial function, normalizing energy homeostasis and oxidative balance. In this Chapter, we will investigate the impact of ALDH2 on liver phenotype and explore the mechanisms involved in.

## **B. Results**

### **1. ALDH2 regulates energy metabolism in liver of acute CE exposed mice**

Lipid accumulation and glycogen deposition are the important aspects that reflect fat and glucose metabolism in liver. Therefore, we performed analysis of lipids and glycogen by staining the quantitative level by hepatic extraction using commercial kits. ORO staining of neutral lipids showed that CE treatment caused an increase in lipid droplet accumulation. Pre-treatment of Alda-1 decreased ORO positive staining in the CE group (Figure 3.2A). This was also reflected in triglyceride (TG) levels in liver and plasma. CE or Alda-1 alone did not change NEFA and cholesterol levels in either liver or plasma (Figure 3.2B). Hepatic glycogen storage was visualized by PAS staining. CE caused an obvious depletion of glycogen deposition while Alda-1 did not blunt this effect (Figure 3.2A). Quantitation of PAS staining in liver also showed that CE significantly decreased glycogen stores. This was not prevented by Alda-1 pre-exposure (Figure 3.2B). Hepatic steatosis and glycogen depletion was mediated by alteration in expression of metabolic associated gene. To explore the regulation of ALDH2 in lipids and carbohydrates homeostasis, we

analyzed the expression of genes involved in the synthesis and catabolism of fat or glucose by RT-qPCR (Scheme 3.2).

Carnitine palmitoyltransferase 1a (CPT1) is the rate-limiting enzyme in fatty acid  $\beta$ -oxidation in the mitochondria. ATP citrate lyase (ACLY) is an enzyme representing an important step in fatty acid biosynthesis. It converts citrate to acetyl CoA, which is an intermediate link the metabolism of carbohydrates and production of fatty acids. Fatty acid synthase (FAS) is a multi-enzyme protein that catalyzes fatty acid synthesis. ALDH2 activation by Alda-1 significantly increased *Cpt1* expression suggesting that ALDH2 activation increased fatty acid  $\beta$ -oxidation in the mitochondria. There was no significant difference of *Fas* and *Acly* mRNA expression in each group (Figure 3.3).

Glucose transporter (GLUT4) permits glucose to enter the cell and increases glucose absorption, glucokinase (GCK) is a rate-limiting enzyme in glycolysis that facilitates phosphorylation of glucose to glucose-6-phosphate, it plays an important role in the regulation of carbohydrate metabolism. Glycogen synthase kinase 3b (GSK3b) increases the phosphorylation of the gluconeogenic enzymes, suppressing glycogen synthesis. Phosphoenolpyruvate carboxykinase (PCK) is a rate-limiting enzyme that catalyzes an irreversible step of gluconeogenesis and thought to be essential in glucose homeostasis. SIRT1 is a factor involving hepatic glucose and lipid hemostasis. However, by PCR data we found that none of these markers showed significantly different mRNA expression in each group (Figure 3.3).

## **2. ALDH2 activation inhibited CE-induced oxidative stress in liver**

4-HNE is a byproduct of lipid peroxidation and thought to be an indicator of oxidative stress. Previous work by our lab showed that CE increased oxidative stress in liver (30). For investigating whether ALDH2 functioning in protecting liver from CE, 4-HNE protein adduct accumulation was measured by staining and expressed by quantitation of positive staining. CE increased in 4-HNE positive staining comparing to control mice, pre-injection by Alda-1 decreased 4-HNE adducts level in the CE mice (Figure 3.4).

## **3. The effect of ALDH2 on proinflammatory cytokine gene expression in CE mice**

We also wanted to know how ALDH2 affected the inflammatory response in liver. Pai-1 is a plasminogen activator inhibitor that inhibits fibrinolysis. It is also known as a proinflammatory cytokine. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a pleiotropic cytokine produced by a variety of immune cells including macrophages/monocytes. TNF- $\alpha$  can trigger multiple signaling pathways involved in inflammation. Moreover, TNF- $\alpha$  has been shown to be an upstream regulator of PAI-1 (57). However, neither CE nor Alda-1 significantly changed mRNA expression of *Pai-1* and *Tnf- $\alpha$*  (Figure 3.5).

## **4. Pre-incubation of Alda-1 prevented reduction in ALDH2 activity by CAA**

ALDH2 is known to protect liver injury by decreasing 4-HNE caused oxidative stress or reducing lipid accumulation (45). There are multiple signal

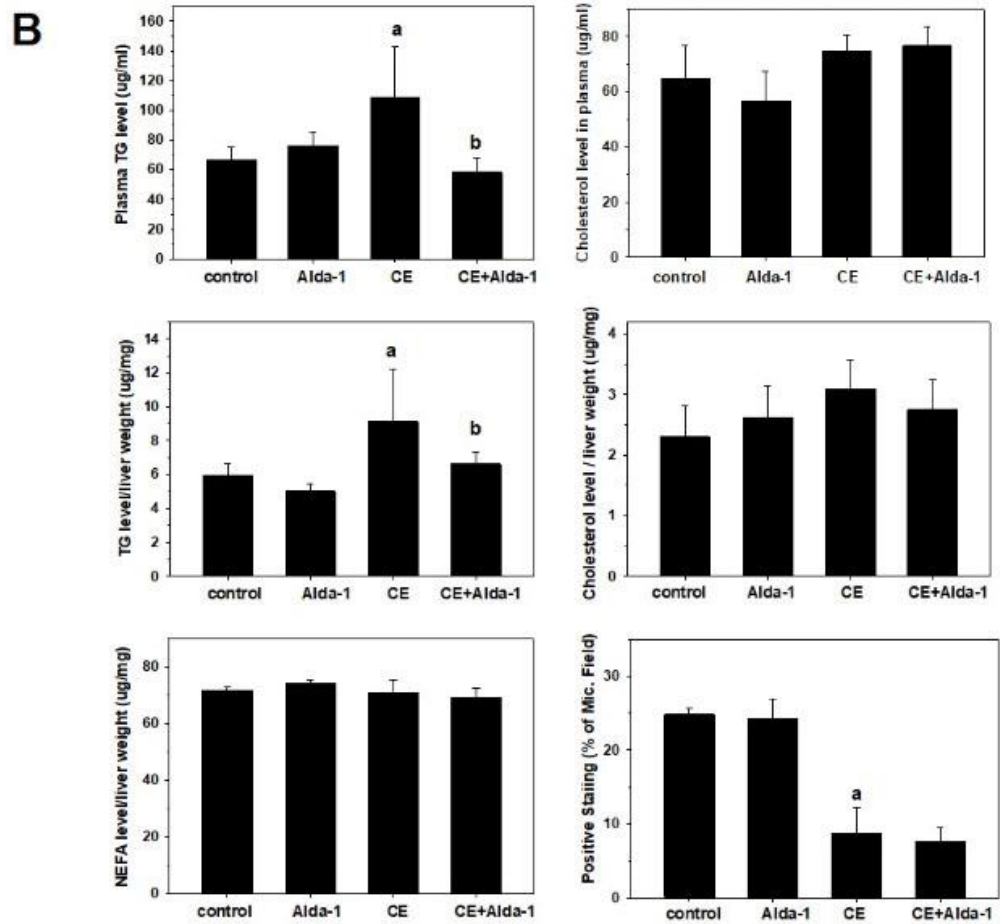
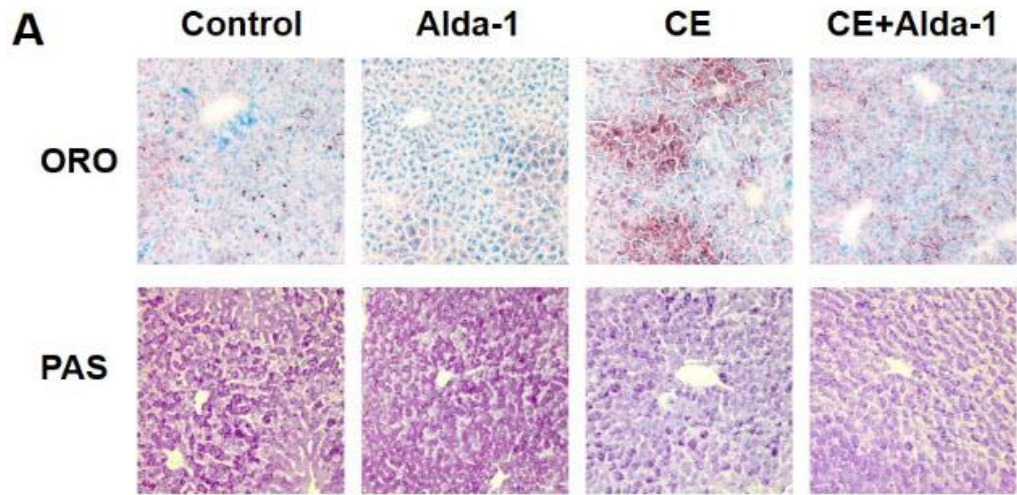


pathways involved in the animal model so that might be complex to figure out a predominant mechanism contributing to liver protection. ALDH2 utilizes aldehydes as substrate and  $\text{NAD}^+$  as cofactor to form into their corresponding acid. It is known that 4-HNE is one of the substrates to ALDH2 (58), activation of ALDH2 is demonstrated to play a critical role in aldehyde clearance thereby leading to cell protection (59). We want to know how ALDH2 performs in the exposure of CAA and explore a direct reaction between enzyme and toxic CAA. Purified ALDH2 were measured for enzyme characterization. We proved that CAA acted as the substrate for ALDH2, moreover, prolonged single CAA treatment blocked the utilization of  $\text{NAD}^+$  to form NADH and showed a significant inhibited ALDH2 activity. Alda-1 pre-treatment elevated enzymatic activity in both control and CAA group (Figure 3.6).

	Day 1	Day 2	Day 3	Day 4	Day 5
Control					sacrifice
Alda-1	Alda-1 (20mg/kg/d, <u>i.p.</u> )				
CE				CE (50mg/kg, <u>i.g.</u> )	
Alda-1+CE	Alda-1 (20mg/kg/d, <u>i.p.</u> )			Alda-1(30min) +CE	

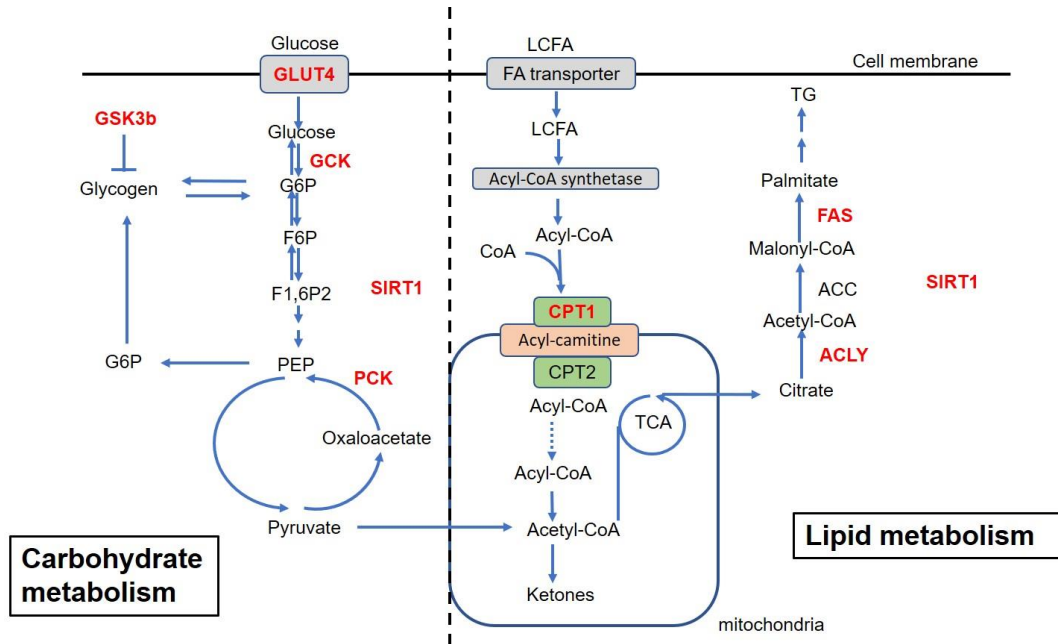
### Scheme 3.1: animal treatment by CE and Alda-1

Mice received Alda-1(20mg/kg) injection for 4 days and were gavaged with CE (50mg/kg) 24 hours prior to sacrifice. For mice of Alda-1+CE group, CE was given after 30 minutes Alda-1 treatment.



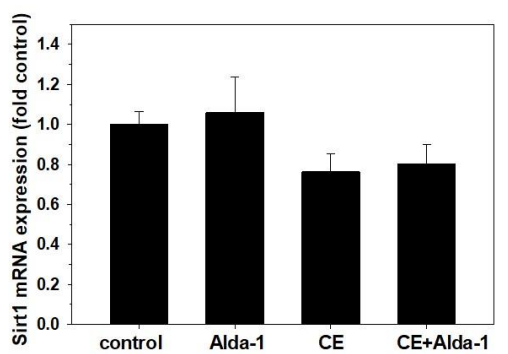
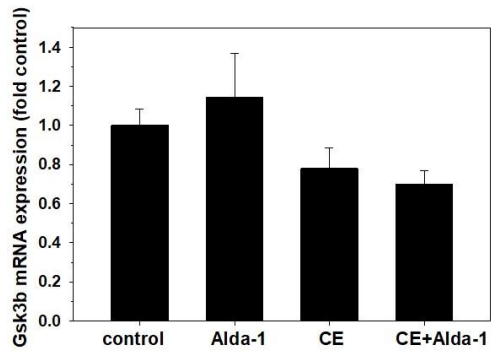
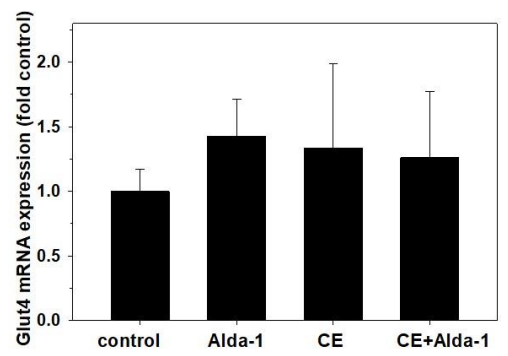
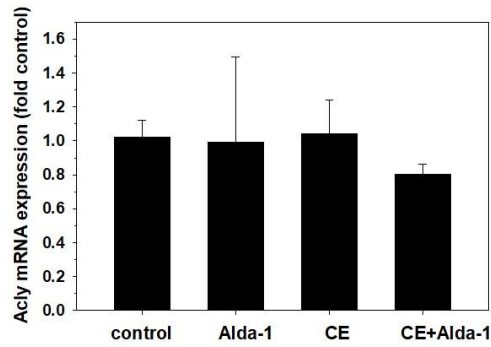
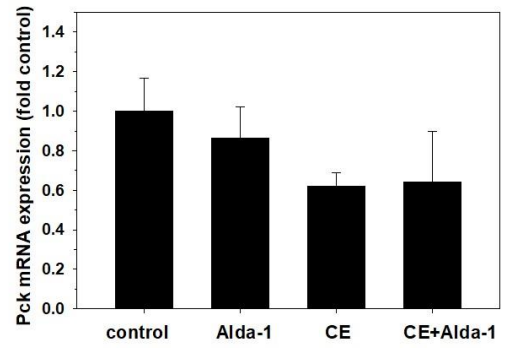
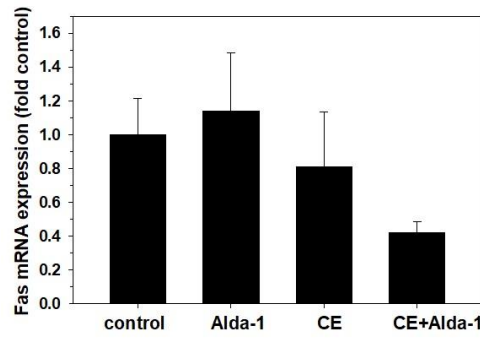
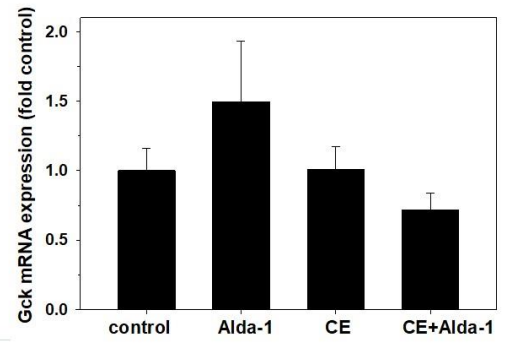
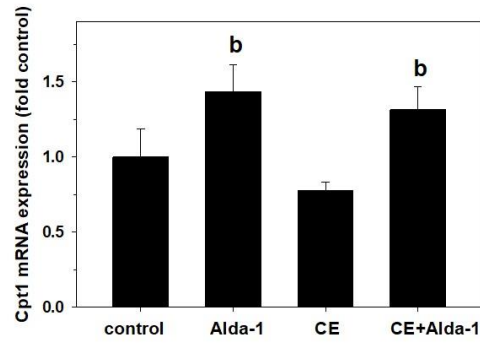
**Figure 3.2. The effect of ALDH2 on hepatic lipids and glycogen level in CE treated mice.**

A. Representative photomicrographs of ORO (neutral lipid, red) and PAS (glycogen, dark purple) staining are shown at 200x magnification. B. Hepatic and plasmatic TG, cholesterol and FFA level were measured as described in Chapter II, quantitative positive staining of glycogen storage in liver was expressed by Image Metamorph analysis software. Results are reported as means  $\pm$  standard error mean (SEM; n=4-6). <sup>a</sup>, p<0.05 compared to control group, <sup>b</sup>, p<0.05 compared to absence of Alda-1.



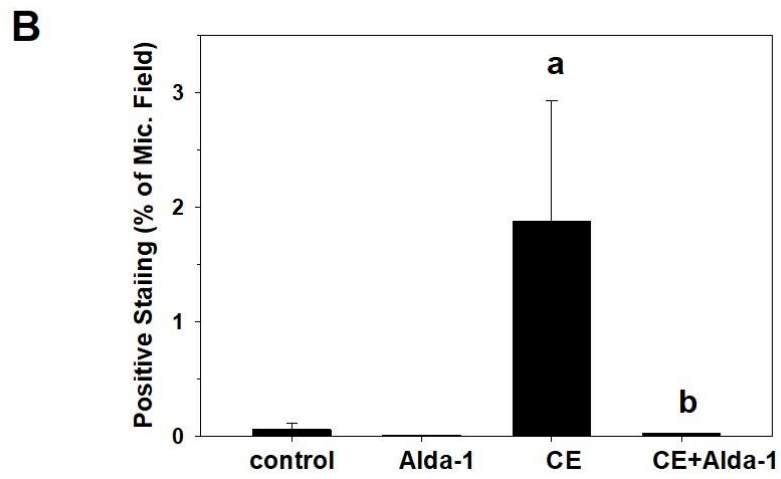
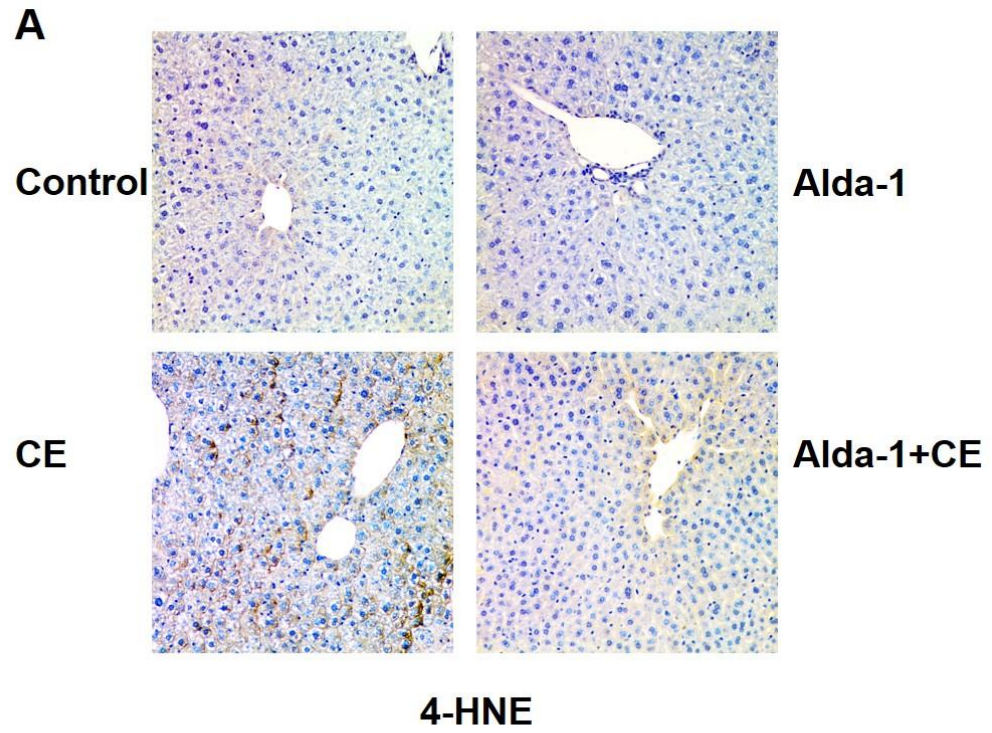
### Scheme 3.2: energy metabolism related gene regulation

Changes in glucose and lipid metabolism are represented. Carbohydrates metabolism related genes includes *Glut4*, *Gck*, *Pck*, *Gsk3b*. Lipid metabolism related gene includes *Cpt1*, *Acly*, *Fas*. *Sirt1* serves as the regulator in both glucose and lipid homeostasis.



**Figure 3.3. The effect of ALDH2 on energy metabolism related mRNA expression in CE mice**

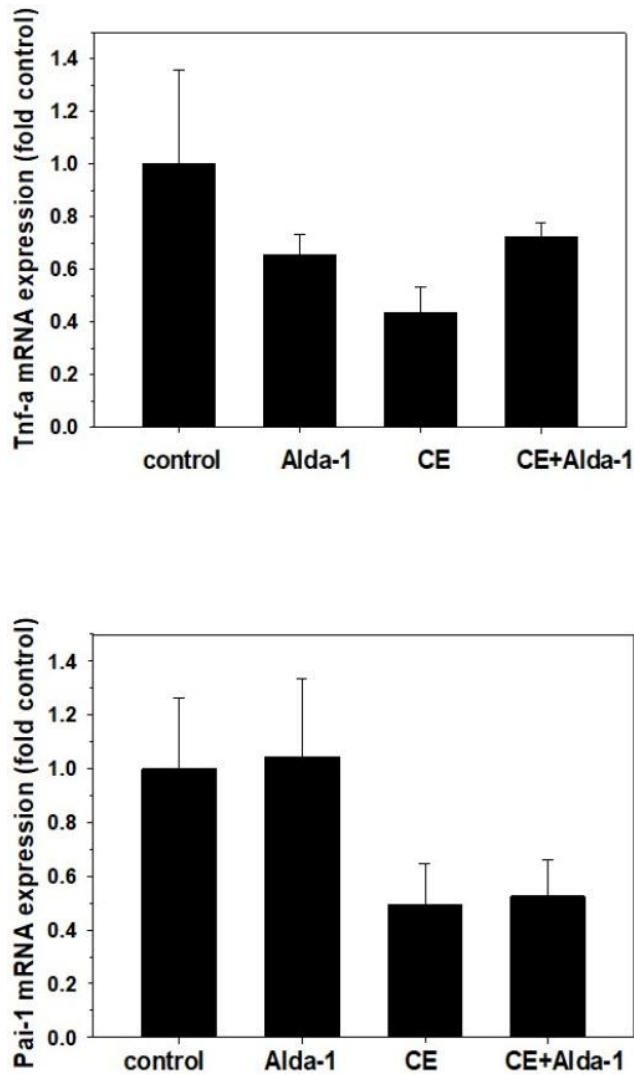
Lipid metabolism mRNA (*Cpt1*, *Fas*, *Acly*) and carbohydrate metabolism mRNA (*Gsk3b*, *Gck*, *Pck*, *Glut4*, *Sirt1*) expression in liver homogenate were measured by RT-qPCR in mice treated with CE or Alda-1. Results are reported as means  $\pm$  standard error mean (SEM; n= 4-6), <sup>a</sup>, p < 0.05 compared to control mice, <sup>b</sup>, p < 0.05 compared to the absence of Alda-1.





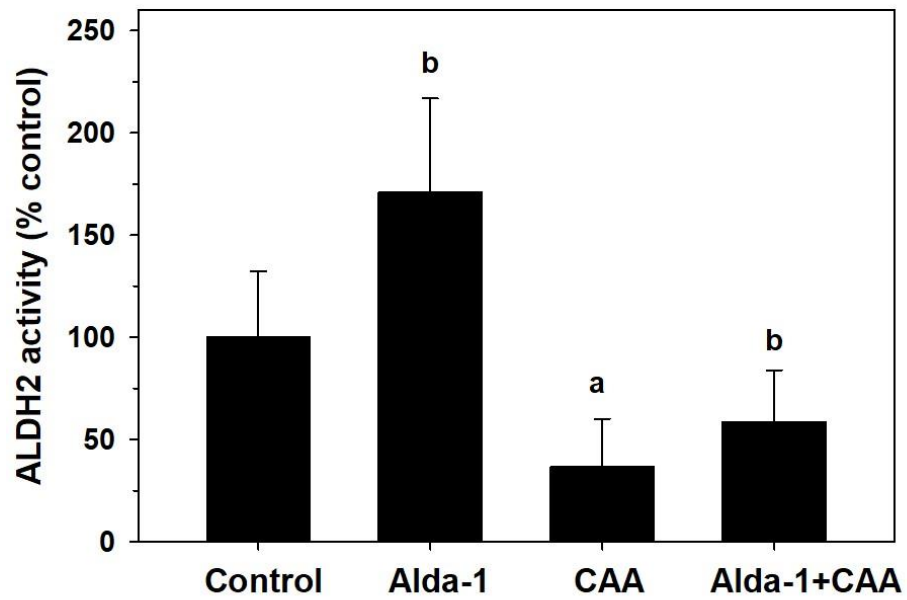
**Figure 3.4. The effect of ALDH2 on liver oxidative stress caused by CE**

A. Representative photomicrograph of 4-HNE staining (index of oxidative stress, brown) in liver are shown at 200x magnification. B. Quantitative positive staining of 4-HNE adducts in liver was expressed by Image Metamorph analysis software. Results are reported as means  $\pm$  standard error mean (SEM; n=4-6). <sup>a</sup>, p<0.05 compared to control group, <sup>b</sup>, p<0.05 compared to absence of Alda-1.



**Figure 3.5. The effect of ALDH2 in proinflammatory cytokines mRNA expression in the CE group**

Hepatic mRNA expression in liver homogenate of proinflammatory cytokines (*Tnf-α*, *Pai-1*) were measured by RT-qPCR in mice of all groups. Results are reported as means  $\pm$  standard error mean (SEM; n= 4-6), <sup>a</sup>, p < 0.05 compared to control mice, <sup>b</sup>, p < 0.05 compared to the absence of Alda-1.



**Figure 3.6. Purified ALDH2 characterization**

ALDH2 enzymatic activity was measured in purified protein after treating with CAA or Alda-1 as described in Chapter II. The activity was determined at 1 min time point after substrate addition. Results are reported as means  $\pm$  standard error mean (SEM; n= 4-6), <sup>a</sup>,  $p < 0.05$  compared to control group, <sup>b</sup>,  $p < 0.05$  compared to the absence of Alda-1.

### **C. Discussion**

As mentioned in the Introduction, NAFLD is composed of steatosis and steatohepatitis that potentially develops to hepatic fibrosis and cirrhosis. Steatosis or steatohepatitis are considered a hallmark for diagnosis of early-stage liver damage. Environmental toxicants such as VC cause hepatic steatohepatitis. Most clinical cases were found in workers with chronic occupational exposure to VC at high concentrations (20). CE and CAA, metabolites of VC, are important mediators in VC induced liver damage. Here, we exposed mice to acute CE at a concentration which equates to ~100 ppm of VC bolus rodents. The concentration of CE was determined by others to not directly cause liver damage (60) and was validated in our previous work (30). Activation of ALDH2 has been shown to be a protective, especially in alcoholic liver disease (43). The role of ALDH2 in VC exposed liver function has not been studied. Here, our first aim was to investigate the effect of ALDH2 activation in an animal model of short-term treatment of CE. We determined by ORO staining that CE increased lipid accumulation in liver. The main form of lipid storage in hepatocytes are TG. Other lipids that can accumulate are FFA or free cholesterol (61). Elevated TG levels in both liver tissue and plasma were observed in CE-treated mice. Alda-1 pre-treatment attenuated lipid droplet formation in the liver, paralleled with an decrease in TG levels (Figure 3.2). Hepatic TG accumulation is mainly synthesized from FFA. We speculated that the effect of CE or Alda-1 on TG showed a consistent trend with FFA level. However, neither CE nor ALDH2 changed

FFA level (Figure 3.2). FFA content is depended on the lipolysis, lipid synthesis and the destination such as FFA oxidation in mitochondria (7). We measured some key gene in catalyzing FFA synthesis (*Acly*, *Fasn*) by PCR, however there was no significant difference found in each group. CE didn't alter *Cpt1* mRNA expression while Alda-1 caused significantly upregulated expression levels (Figure 3.3). This suggested that CE did not affect FFA lipogenesis or oxidation. The effect of ALDH2 in lipids metabolism might be attributed to the increased FFA oxidation in mitochondria. Overall, these data indicate that ALDH2 activation is sufficient to inhibit CE mediated steatosis. Additionally, previous data in our lab showed that CE decreased hepatic glycogen storage due to an increased demand of glucose compensation for ATP depletion which is derived from CE caused mitochondrial impairment (30, 34). The protective role of ALDH2 in mitochondria has been demonstrated previously (45). Here we measured the effect of ALDH2 on glycogen deposition in liver. Glycogen can be impacted by some key metabolic process such as glycolysis, glycogenolysis, glycogenesis and gluconeogenesis. In this study, CE did not affect mRNA expression of genes (*Glut4*, *Pck*, *Sirt1*, *Gsk3b*, *Gck*) involved in glucose homeostasis but showed a decrease in glycogen levels in the liver (Figure 3.3). However, this may be due to temporal differences in gene expression and appearance of an altered phenotype. The effect of ALDH2 on glycogen levels was parallel with the performance of gene expression (Figure 3.2). However, activation of ALDH2

does not seem to play a significant role in regulating glucose metabolism in the acute CE exposure model.

Excessive fat production has been shown to promote low grade inflammation by increasing expression of proinflammatory cytokines resulting in further damage and/or fibrogenesis (7). TNF- $\alpha$  is a key factor involved in the M1 response that mediates steatohepatitis (62). PAI-1 contributes to liver lipid transport in the early stages of liver injury leading to steatosis, in the later stages, PAI-1 acts as a key regulator in inflammation or fibrosis (63). By our study, the mRNA level of *Tnf- $\alpha$*  and *Pai-1* weren't altered by CE or Alda-1 significantly (Figure 3.5). This result indicates that these factors are not major players in the mechanism(s) of action in this model.

It is accepted that increased lipid accumulation and liver damage are linked with oxidative stress (64). increased lipid deposition is the source of endogenous aldehydes such as 4-HNE. ALDH2 is known for its role in aldehydes degradation and antioxidant. Indeed, we demonstrated ALDH2 activation reversed lipid accumulation and suppressed 4-HNE level (Figure 3.4). These results indicate that activation of ALDH2 protects the liver from CE-induced oxidative stress. The VC metabolite, CAA is a reactive aldehyde similar to 4-HNE that has been demonstrated to cause hepatocellular damage by our previous work (30). CAA has been demonstrated to be not only substrate to ALDH2, but also a direct inhibitor of ALDH2 enzymatic activity (Figure 3.6). It has been known that aldehydes inactivate ALDH2 by interfering with the correct binding of the substrate to the active site or

disrupting protein-substrate interaction (58). We hypothesize that CAA reacts with ALDH2 at the active site and affects the binding of  $\text{NAD}^+$  and therefore blocks the enzymatic activity. We showed that pre-incubation of ALDH2 to Alda-1 affected the decrease in enzymatic activity caused by CAA. As a selective ALDH2 agonist, Alda-1 binds to the entrance of the active site but does not interfere with catalytic residue; it permits substrate binding and facilitates catalytic product release, protecting enzymatic activity (38, 65). We proposed that pre-activation of ALDH2 prevented enzymatic activity from CAA inhibition and blunted decreased aldehydes clearance, this effect decreased the production of CAA and other endogenous aldehydes (e.g. 4-HNE), and therefore attenuated liver damage.

#### **D. Conclusion**

In this aim, we demonstrated that Alda-1 pre-treatment plays a role in protecting the liver from acute CE exposure. Alda-1 improved metabolism dyshomeostasis, especially on the lipids level and it decreased 4-HNE-associated oxidative stress. We suggested that this effect was initiated by activation of ALDH2-mediated clearance of toxic aldehydes. CAA inhibited ALDH2 activity and enhanced production of aldehydes, leading to liver injury. Alda-1 activates ALDH2 and prevents inhibition of enzymatic activity by CAA. As major metabolites of VC, CE and CAA are mediators of VC-induced liver injury. We therefore hypothesize that ALDH2 is impaired by inhalation of VC

proper and that ALDH2 activation may ameliorate liver injury caused by chronic VC exposure.



## CHAPTER IV

### ALDH2 FUNCTIONS IN PROTECTING LIVER AGAINST CHRONIC VC EXPOSURE CONCOMITANT WITH HIGH-FAT DIET

#### **A. Introduction**

VC was first used commercially in the 1920s, the techniques was not devised to polymerize VC into a stable PVC until 1930s. Over that period, workers were required to clean up the reaction tank because a film of PVC forms inside wall of reactor after the polymerization finished, so that the workers were exposed to high concentration of VC up to 1,000 ppm or higher peaks of exposure (21). This occupational exposure of VC caused multiple complications such as liver cancer. The cases of hepatic angiosarcoma (ASL) were diagnosed in 1974 among VC workers in Louisville, Kentucky (66). Additionally, the mortality risks of other liver cancers (e.g. HCC) were associated with VC exposure (19). Based on those epidemiological investigations, VC exposure is regarded as a carcinogen of liver as evaluated by IARC. ASL and HCC, as the typical VC-induced liver cancers, were normally observed in workers exposed to high concentrations of VC. Since 1975, OSHA has set stricter limitations for VC exposure (1 ppm, 8hrs/day), which efficiently decreases morbidity of occupational acute exposure of VC

related liver diseases. However, to this day it is unclear if low concentrations, that are currently considered safe, may enhance or alter liver injury caused by other risk factors. The risk for low-dose chronic exposure of VC therefore remains a concern for workers and residential populations surrounding to industrial or VC-contaminated sites.

As a terminal state of liver disease, liver cancer can be mediated by high-level VC exposure. We have shown that chronic low-dose VC exposure mediates low to moderate degrees of liver damage and we hypothesize that these concentrations may also potentially contribute to more severe and irreversible stages of liver injury. It is proposed that additional other risk factors synergistically lead to progression of liver damage, ultimately leading to fibrosis and liver cancer (67). Obesity is the most prevalent underlying disorder that impacts over 50% of the US population (see also Chapter I). NAFLD is the major hepatic manifestation associated with obesity. Previously, we have demonstrated that VC dysregulates metabolic homeostasis and enhances liver injury caused by high-fat diet (34). ALDH2 is accepted to be a protective enzyme for its pivotal role in metabolism regulation as well as clearance of aldehydes, which results in reduced oxidative stress. Indeed, we have data supporting a similar mechanism in our animal model of acute CE exposure (see Chapter III).

In this section, we focused on the effect of ALDH2 on liver function in the interaction of chronic low-dose of VC exposure and consumption of a high-fat diet. We have shown previously that the mechanisms involved in the chronic

low-level VC exposure induced liver injury in HFD fed mice is similar to that of single acute CE exposure (34). Here, we proposed that ALDH2 activation will decrease liver injury caused by VC and HFD. Although we will determine the role of ALDH2 in the interaction of VC exposure and HFD *in vivo*, it is extremely difficult to obtain complete toxicological data due to the various cell types and multiple signal pathways that are involved. For further investigations of the underlying mechanism(s), we will utilize cell culture models and document pathological changes on organelle and molecular level.

Mitochondria are key to maintaining cellular energy homeostasis. Previous work by our lab showed CAA causes cellular damage to HepG2 and primary hepatocytes via mitochondrial dysfunction, involving mitochondrial respiration impairment as well as decreased mitochondrial membrane potential (30). Furthermore, cellular toxicity induced by 4-HNE can be reversed by ALDH2-catalyzed aldehyde degradation (45), which is also supported by the data shown in Chapter III. Thus, we hypothesize that activation of ALDH2 protects hepatocytes from VC and HFD, and we aim to investigate the involved mechanisms as well.

## **B. Results**

### **1. ALDH2 dysfunction is involved in the interaction of VC and HFD in liver**

ALDH2 is a critical enzyme for aldehyde metabolism as well as a protective factor in liver injury. However, the role of ALDH2 function in exposure to VC has not been elucidated yet. For investigating ALDH2 function in this model, hepatic mitochondria were isolated from mice exposed to VC and HFD for 12 weeks. Western blot and enzymatic activity assays were performed for determination of protein expression levels and of aldehyde clearance rate, respectively. Figure 4.2 shows the effect of VC and HFD on ALDH2 protein expression and activity in vivo. VDAC was used as a loading control for mitochondrial protein. ALDH2 protein levels were quantitated by densitometric analysis. Western blot results showed a significant difference in ALDH2 levels between control and mice exposed to VC inhalation (Figure 4.2A). Quantified protein level was expressed as the ratio of intensity of ALDH2 to VDAC. HFD significantly increased ALDH2 protein expression compared to LFD feeding mice, VC exposure significantly decreased ALDH2 levels in both LFD and HFD groups. Interestingly, the ALDH2 enzymatic activity of HFD showed a different trend from the Western blot results. HFD feeding significantly decreased ALDH2 activity in the liver compared to LFD control, while the addition of VC enhanced this effect only in the presence of HFD (Figure 4.2B). This result supports the hypothesis that VC-enhanced liver injury in the HFD group might be via, at least in part, regulating ALDH2 function.

## **2. ALDH2 affected metabolic phenotype of mice in the interaction of VC and HFD**

For evaluating the metabolic phenotype of mice, body weights were measured once per week and food consumption was monitored twice per week. Blood glucose levels were determined at the 12-week time point. Figure 4.3 A shows that there was no difference in body weights between the LFD and LFD+VC groups. Body weights were significantly increased with HFD. VC did not further increase body weights. Mice injected with Alda-1 were significantly lighter in body weight compared to non-Alda-1 treated mice. Figure 4.3B shows the amount of food consumed by each dietary group throughout 10 weeks. All these groups consumed similar amounts of food and no significant difference was observed (Figure 4.3B). Figure 4.3C shows blood glucose levels at different time points after administration of glucose. None of the groups showed significantly different baseline glucose levels. At 15, 30, 60, 90, 120 min, blood glucose levels of HFD fed mice were significantly higher than that of LFD control. However, glucose level of neither LFD nor HFD mice was changed by VC addition. Alda-1 significantly decreased blood glucose levels compared to mice without Alda-1. The area under the curve (AUC) of OGTT, shown in Figure 4.3C, was not altered by either HFD or VC, while activation of ALDH2 with Alda-1 significantly decreased AUC, suggesting an increase in glucose tolerance.

### **3. Activation of ALDH2 attenuated liver injury caused by HFD and VC**

Figure 4.4A shows representative photomicrographs depicting general hepatic morphology (H&E). No obvious pathological changes were observed

in liver tissue of LFD groups and VC did not enhance this effect. HFD fed mice showed obvious steatosis while VC did not visibly change this effect. Alda-1 intervention, however decreased hepatic steatosis. Liver pathology scores for inflammation and necrosis are shown in Figure 4.4C. While HFD significantly increased liver pathology scores compared to LFD, VC did not change this effect. ALDH2 activation by Alda-1 decreased hepatic inflammation significantly in both HFD groups. The enzymatic activity of transaminases AST and ALT were determined in plasma as a marker of liver injury (Figure 4.4B). LFD control had normal plasma transaminase activity and VC did not significantly alter that. HFD alone significantly increased both ALT and AST activity. in the HFD group VC inhalation significantly enhanced this effect. Importantly, Alda-1 significantly decreased transaminase activity, both in the HFD and in the HFD+VC group.

#### **4. ALDH2 decreased neutrophil infiltration and oxidative stress in livers of VC and HFD**

Figure 4.5A shows representative photomicrographs depicting neutrophil accumulation (CAE) and oxidative stress (MDA) in livers of mice exposed to VC for 12-week. HFD alone slightly promoted inflammatory cell recruitments and the addition of VC enhanced this effect. Alda-1 significantly reduced these markers. Neutrophil infiltration was quantitated and expressed as the ratio of CAE positive cells to hepatocytes (Figure 4.5B). HFD significantly increased the amount of CAE positive cells comparing to LFD group, which was furtherly increased by VC. Alda-1 significantly alleviated inflammatory cell

accumulation in livers of mice exposed to HFD and VC. MDA is the main byproduct of lipid peroxidation and is typically used for evaluating cellular oxidative stress. LFD and LFD+VC showed mild positive staining of MDA in liver, HFD alone caused a stronger staining compared to the LFD controls (Figure 4.6A), but VC did not enhance this effect. However, Alda-1 suppressed lipid peroxidation in mice exposed to HFD+VC. These data were also reflected in a quantitative TBARS (Figure 4.6B).

CYP2E1, is known as the major enzyme in drug metabolism as well as in metabolism of xenobiotics (68). Also, CYP2E1 mediated ROS production is a part of cause of alcohol induced liver injury (69). In our study, whether ALDH2 affects CYP2E1 expression in the interaction of HFD and VC remains unknown. As Western blots show (Figure 4.7), HFD and HFD+VC significantly increased CYP2E1 protein expression comparing to the respective control groups, and these effects were reversed by Alda-1.

## **5. ALDH2 reversed metabolic disorders in the interaction of HFD and VC.**

Lipid accumulation and glycogen storage are associated with energy metabolism in liver, disruption of carbohydrate/lipid homeostasis can potentially impact hepatic regeneration and induce hepatotoxicity (70). Liver sections were stained for ORO and PAS to evaluate hepatic metabolism. Figure 4.8A shows representative photomicrographs depicting lipid and glycogen deposition and quantitative analysis of hepatic glycogen and lipid levels. LFD and LFD+VC showed normal fat accumulation in ORO stained

livers. As expected, more lipid droplets accumulated in HFD fed mice, and this was enhanced by VC. Quantitative lipids were measured by TG and NEFA analysis were expressed as the ratio of lipid amount to liver weight (Figure 4.8B). HFD significantly increased TG, NEFA and cholesterol production in liver comparing to LFD group, however VC enhanced this effect only on TG levels. Alda-1 intervention reduced both hepatic TG and NEFA levels significantly. However, cholesterol levels were further increased by ALDH2 activation. Hepatic glycogen content as visualized by PAS staining, was normal in the LFD and the LFD+VC group. HFD and HFD+VC decreased hepatic glycogen stores, which was restored by Alda-1 administration (Figure 4.9A). Quantitative analysis of hepatic glycogen level shows a similar trend (Figure 4.9B), albeit not significant.

## **6. ALDH2 decreased liver damage via participating in autophagy regulation**

Autophagy is considered an adaptive process that degrades unwanted, excess and/or damaged cytosolic components in response to multiple cellular stressors including oxidative stress and pharmacological insults. Ubiquitin-activating enzyme Atg7 is required for conversion of the soluble form of LC3I to autophagic vesicle associated form of LC3II which is associated with the autophagosome membrane and also serves as an index of autophagy. Sequestosome 1 (p62) interacts with LC3II and is subsequently degraded. Impaired autophagy causes p62 accumulation in the cell; thus, decreased p62 also reflects autophagic activity (71, 72). Our previous data showed that



mitochondrial function was impaired by VC (34). The cell removes damaged mitochondria to control mitochondrial quality, this process is mediated by mitophagy, a subtype of autophagy. The autophagic recognition of damaged mitochondria is mediated by the PINK/PARKIN signalling pathway (73). As the Western blot results show (Figure 4.10), HFD and HFD+VC increased Atg7 expression significantly compared to their respective control mice. Alda-1 didn't affect Atg7 level but caused a significant decrease in p62 protein expression. VC increased the ratio of LC3II to LC3I in LFD and HFD mice, while Alda-1 had no effect on LC3II expression. ALDH2 activation significantly suppressed PINK1/PARKIN level in the HFD and HFD+VC groups.

#### **7. The effect of ALDH2 in cytotoxicity of primary hepatocytes**

As the data above have shown (Figure 4.1) activation of ALDH2 protects the liver from damage caused by the combination of VC inhalation and HFD. There are multiple signalling pathways and cell types involved in animal models, making an in depth analysis complicated. Therefore, in addition we utilized cells for investigating the effect of ALDH2 on cellular function directly. Primary hepatocytes isolated from mice fed a HFD for 12-weeks in combination with VC inhalation  $\pm$  Alda-1, were used for measurement of cytotoxicity parameters as described in previous section. Furthermore, we investigated whether the hepatocytes were more susceptible to damage by the ex vivo addition of CAA.

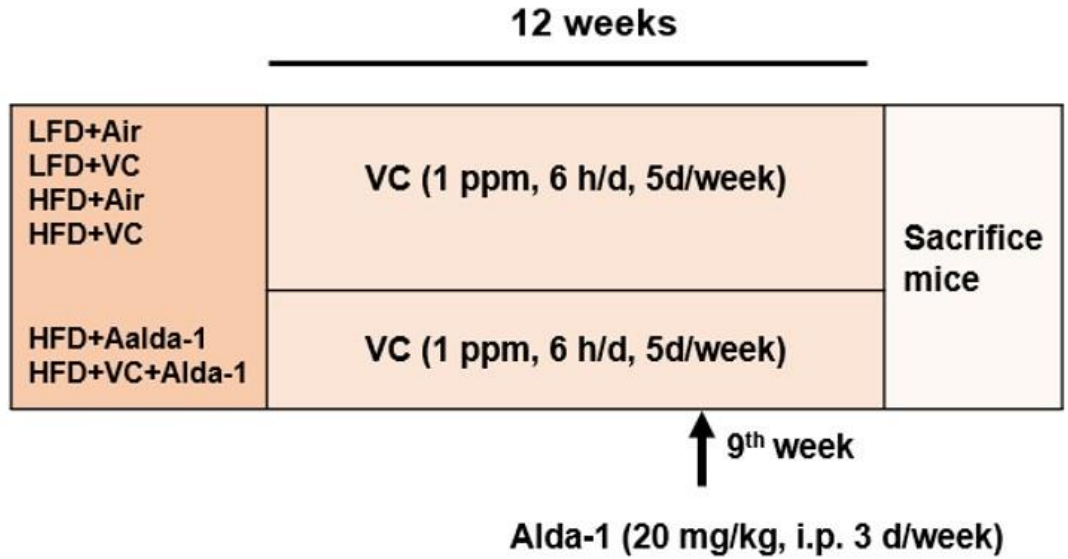
TMRM dye acts as an indicator of mitochondrial membrane potential. which is an essential component in the process of energy generation

reflecting oxidative phosphorylation. The cell maintains stable levels of mitochondrial membrane potential for normal cellular function, therefore changes to this factor may induce loss of cell viability and various pathologies (74). TOTO-3 is a nucleic acid dye that can not penetrate intact cells but stains permeabilized, damaged cells (75). Fluo-4 AM is a dye that has been widely used as an indicator of intracellular free calcium concentration. Large increases of intracellular calcium are thought to trigger apoptosis (76). MitoSox is a novel fluoroprobe for selective detection of superoxide production in mitochondria of living cell, indicating oxidative stress (77). Via HCS analysis of hepatocytes, there was no significant difference of these indices of cytotoxicity (TMRM, TOTO-3, Fluo-4, mitoSox) among the groups. Ex vivo exposure to CAA significantly decreased TMRM fluorescent intensity and increased mitoSox levels. This effect was not reversible by Alda-1 (Figure 4.11).

#### **8. ALDH2 protected mitochondrial function from VC metabolites in primary hepatocytes**

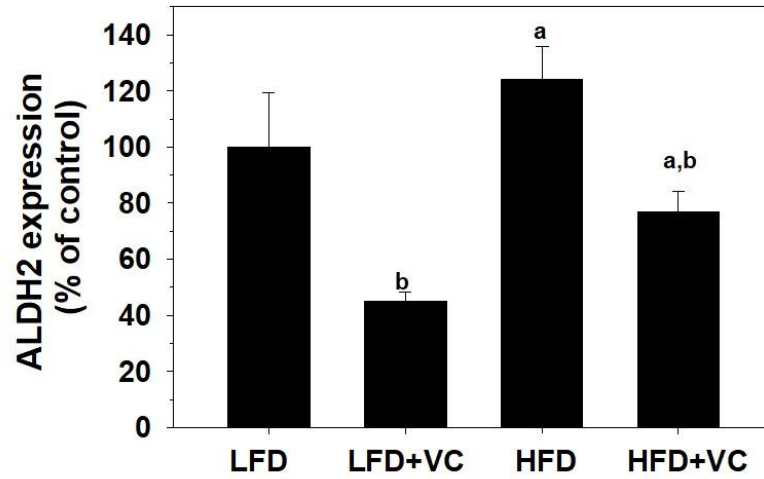
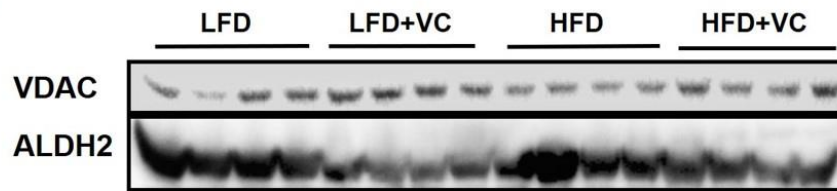
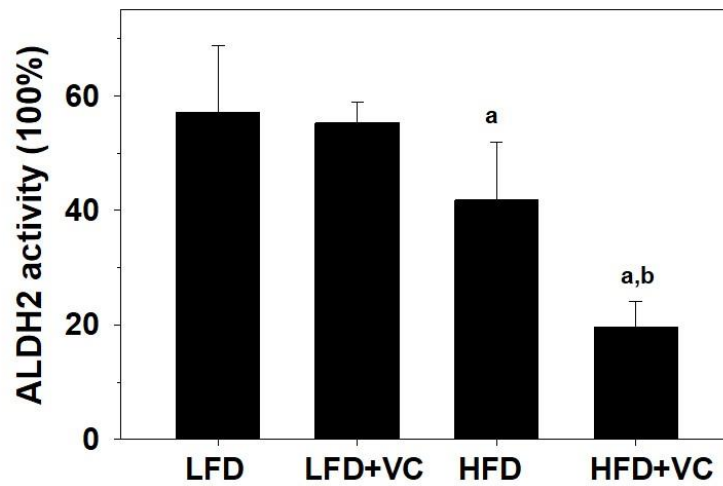
Mitochondrial function is indicative of altered mitochondrial respiration. Previous work by our lab demonstrated that VC metabolites (CAA) directly impaired mitochondrial membrane potential, decreased oxygen consumption rate (OCR) which represented mitochondrial reserve bioenergetic capacity (30). Our recent work also showed that ALDH2 reversed decreased mitochondrial membrane potential caused by 4-HNE exposure (45). We hypothesized that CAA causes mitochondrial dysfunction that can be rescued

by activation of ALDH2. To explore this hypothesis and the effect of ALDH2 activation on the impairment of mitochondrial respiration, Seahorse bioenergetic analysis for electron transport chain function was performed on murine primary hepatocytes. OCR at all time points is shown in Figure 4.12A. Delta OCR (Figure 4.12B) was calculated by subtracting the OCR at the first measured time point after CAA addition from the OCR baseline. CAA treatment significantly increased delta OCR, indicating that OCR was inhibited immediately after CAA addition. Importantly, pre-exposure cell to Alda-1 significantly attenuated this effect.



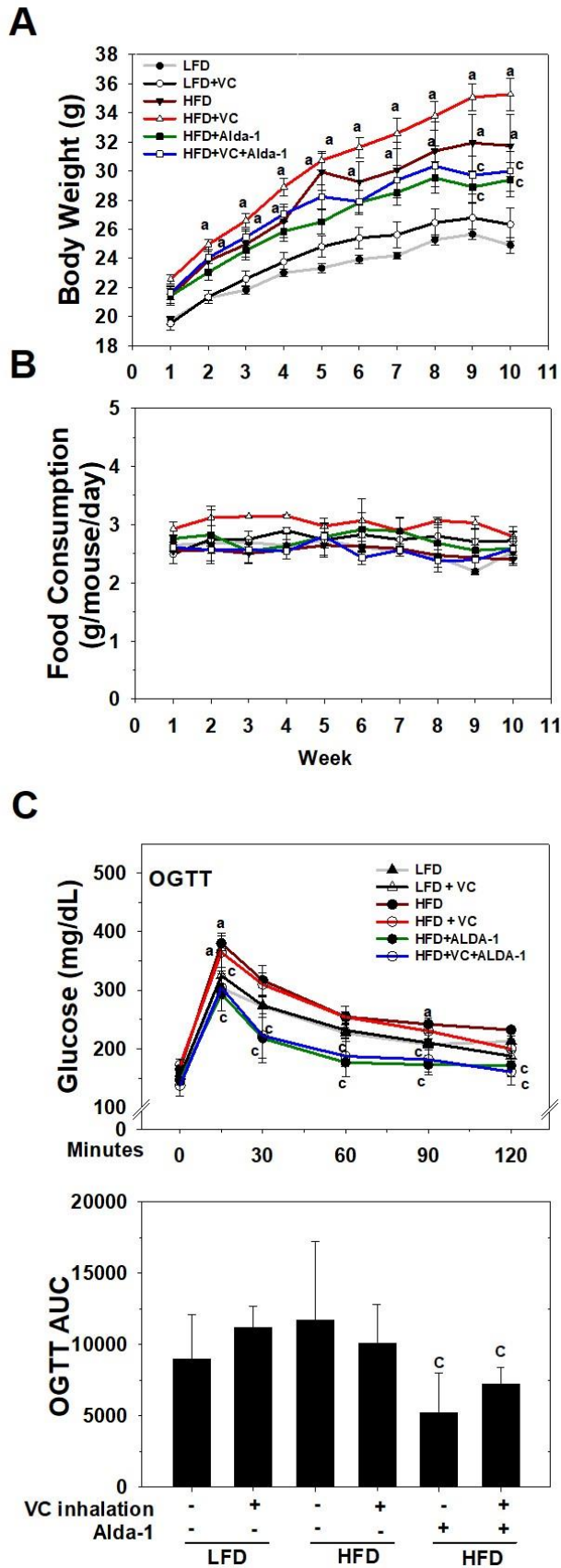
**Scheme 4.1: Chronic animal model of VC and HFD**

Mice were randomly divided into 6 groups and exposed to either VC (1ppm) or room air (control) in our inhalation facility as described above. Alda-1 treatment started 3 weeks prior to sacrificing.

**A****B**

**Figure 4.2. ALDH2 expression and enzymatic activity.**

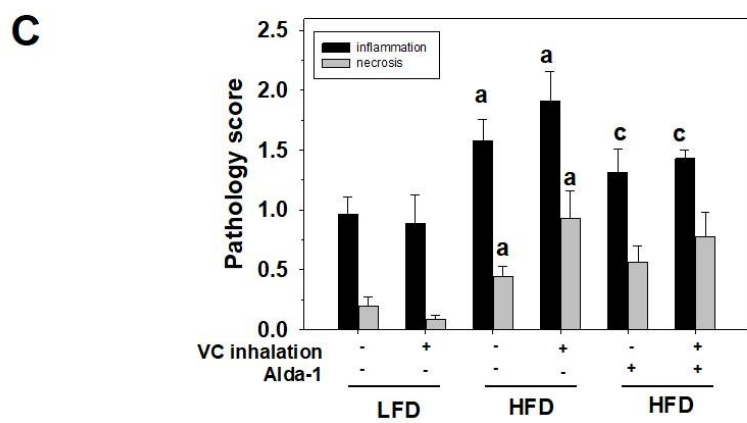
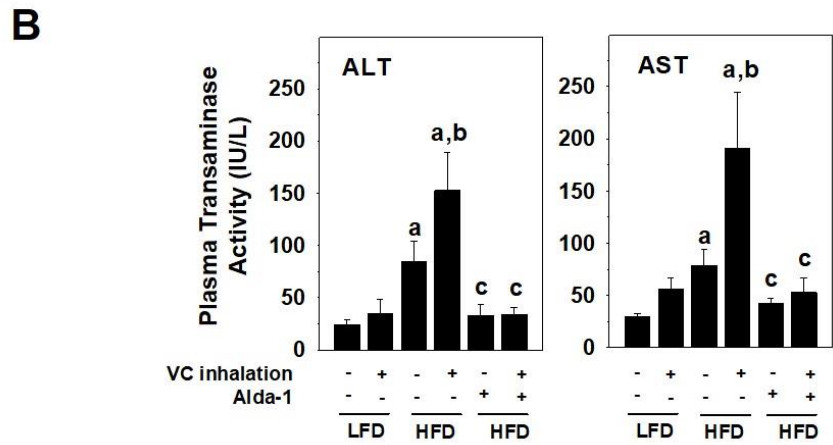
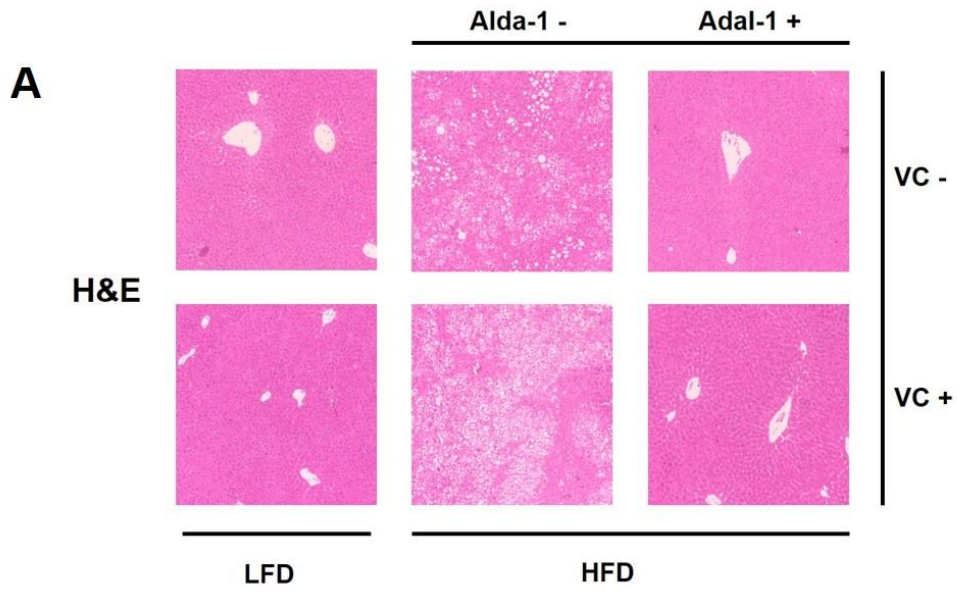
A: ALDH2 expression levels were measured in mouse liver mitochondria via Western blot analysis, as well as densitometric analysis of ALDH2. VDAC serves as a loading control for mitochondrial protein. B: ALDH2 activity was determined as described in Chapter II. Results are reported as means  $\pm$  standard error mean (SEM; n=4-6). <sup>a</sup>, p<0.05 compared to LFD control, <sup>b</sup>, p<0.05 compared to absence of VC.



### **Figure 4.3. Metabolic phenotype of 12-week mice**

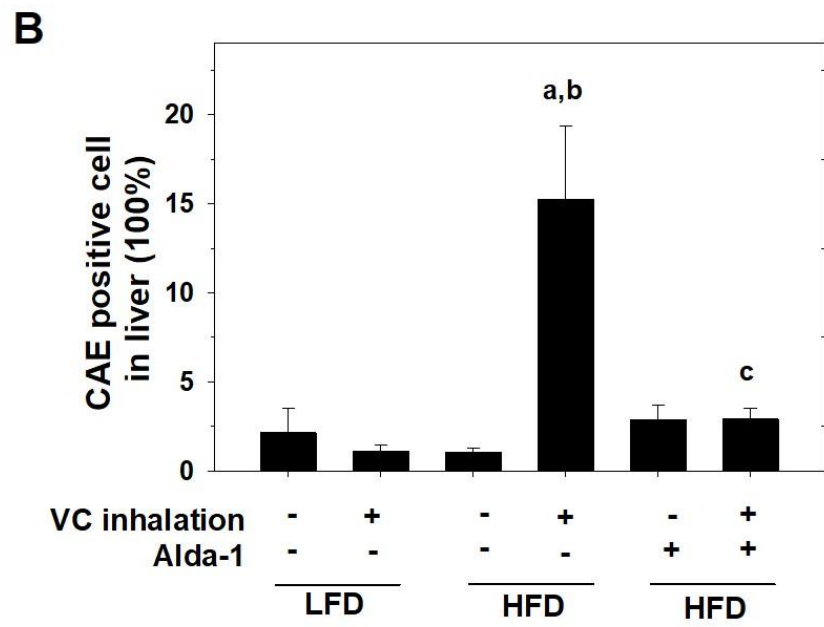
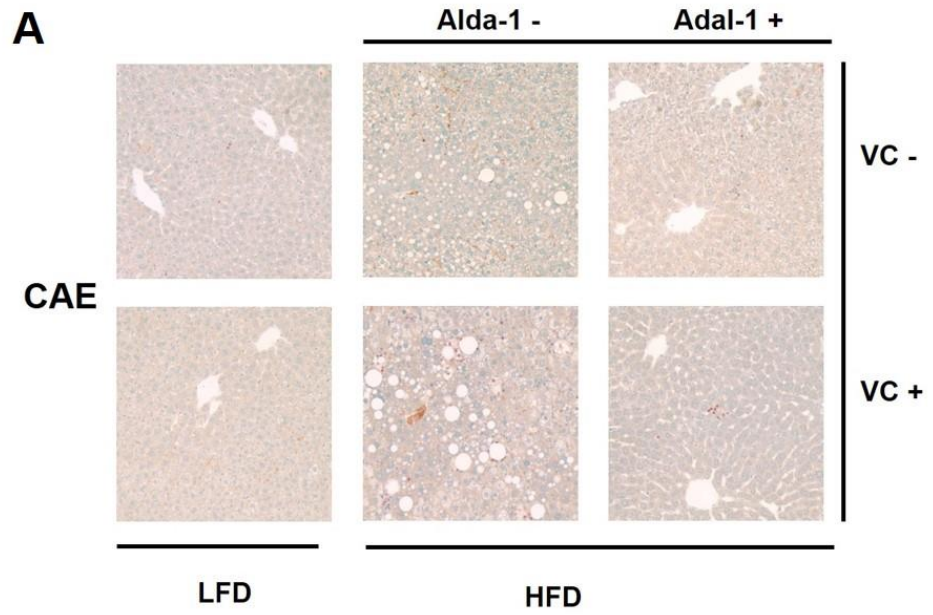
A: Body weights of mice for all groups were monitored once per week over the course of the 12-week exposure period B: Food consumption was measured twice per week for the 12 weeks exposure period. C: Blood glucose level at different time point was measured as describe in Chapter II, OCTT area under curve stand for glucose tolerance is performed. Results are reported as means  $\pm$  standard error mean (SEM; n=4-6). <sup>a</sup>, p<0.05 compared to LFD control, <sup>c</sup>, p<0.05 compared to absence of Alda-1.





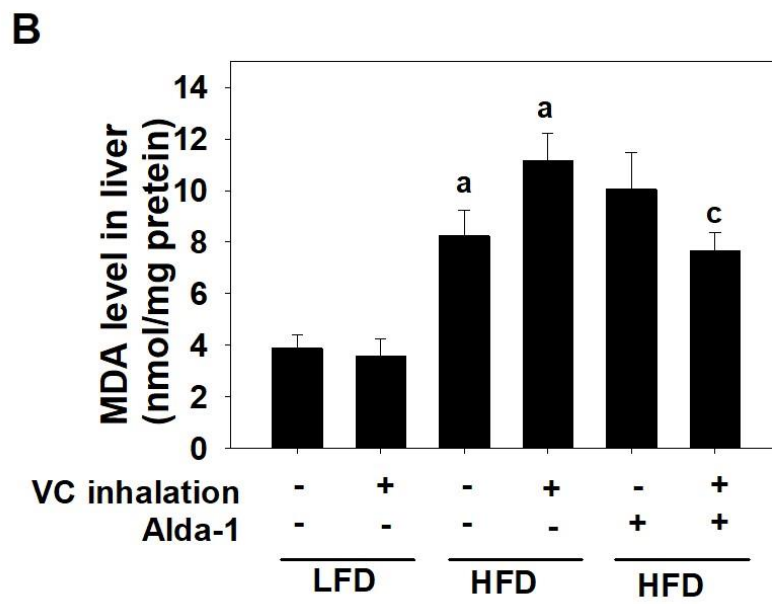
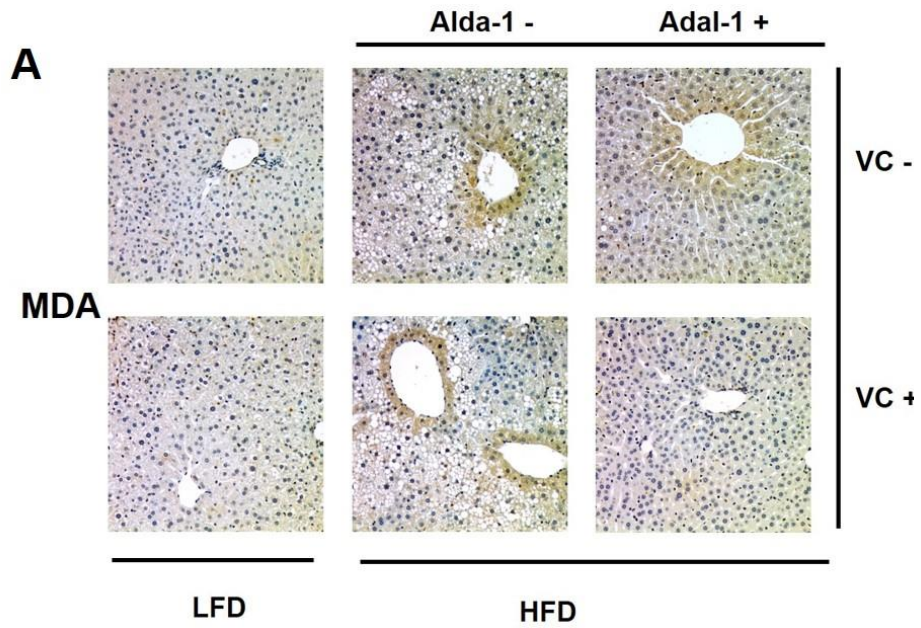
#### **Figure 4.4. ALDH2 decreased liver injury caused by HFD and VC**

A: Representative photomicrographs of H&E (general morphology, 200x) are shown at the 12-week time point. B: Plasma transaminase (ALT/AST) levels were determined for all the experimental groups at the 12-week time point. C: Pathological score including inflammation and necrosis of all groups was evaluated as described in Chapter II. Results are reported as means  $\pm$  standard error mean (SEM; n=4-6). <sup>a</sup>, p<0.05 compared to LFD control, <sup>b</sup>, p<0.05 compared to absence of VC, <sup>c</sup>, p<0.05 compared to absence of Alda-1.



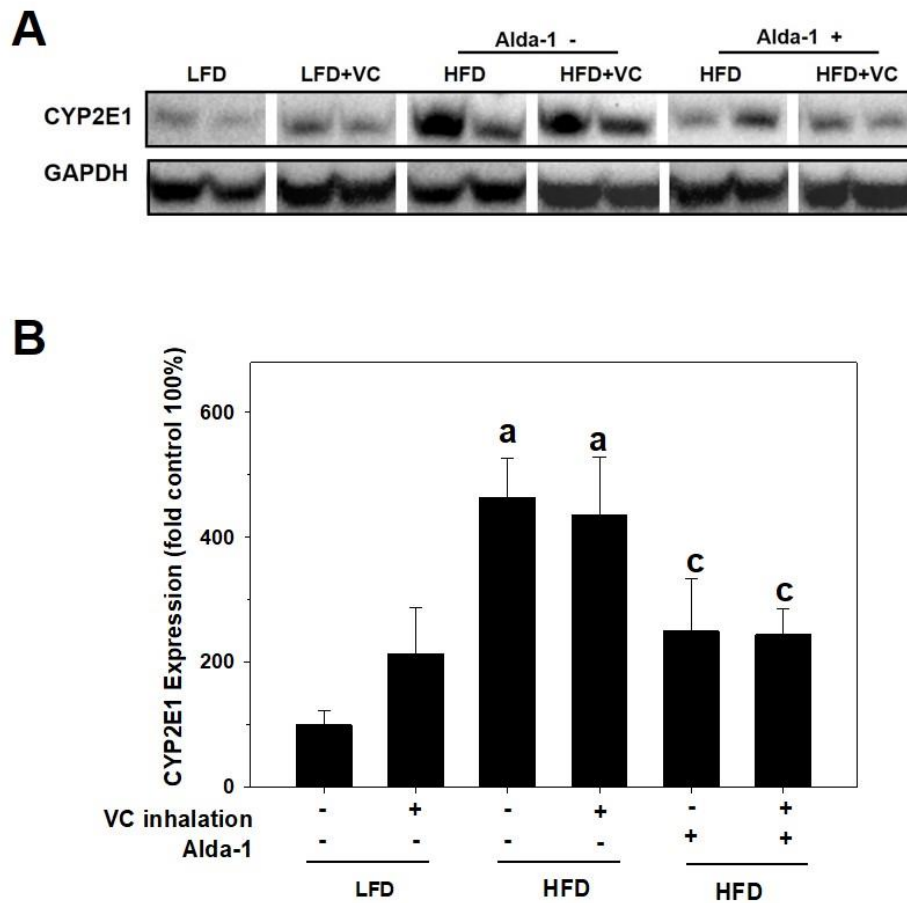
**Figure 4.5. ALDH2 reduced liver inflammation in HFD and VC**

A: Representative photomicrographs of CAE (neutrophils, purple) of all groups are shown at the 200x magnification. B: CAE-positive cells were counted and graphed as positive cells per 100 hepatocytes. Results are reported as means  $\pm$  standard error mean (SEM; n=4-6). <sup>a</sup>, p<0.05 compared to LFD control, <sup>b</sup>, p<0.05 compared to absence of VC, <sup>c</sup>, p<0.05 compared to absence of Alda-1.



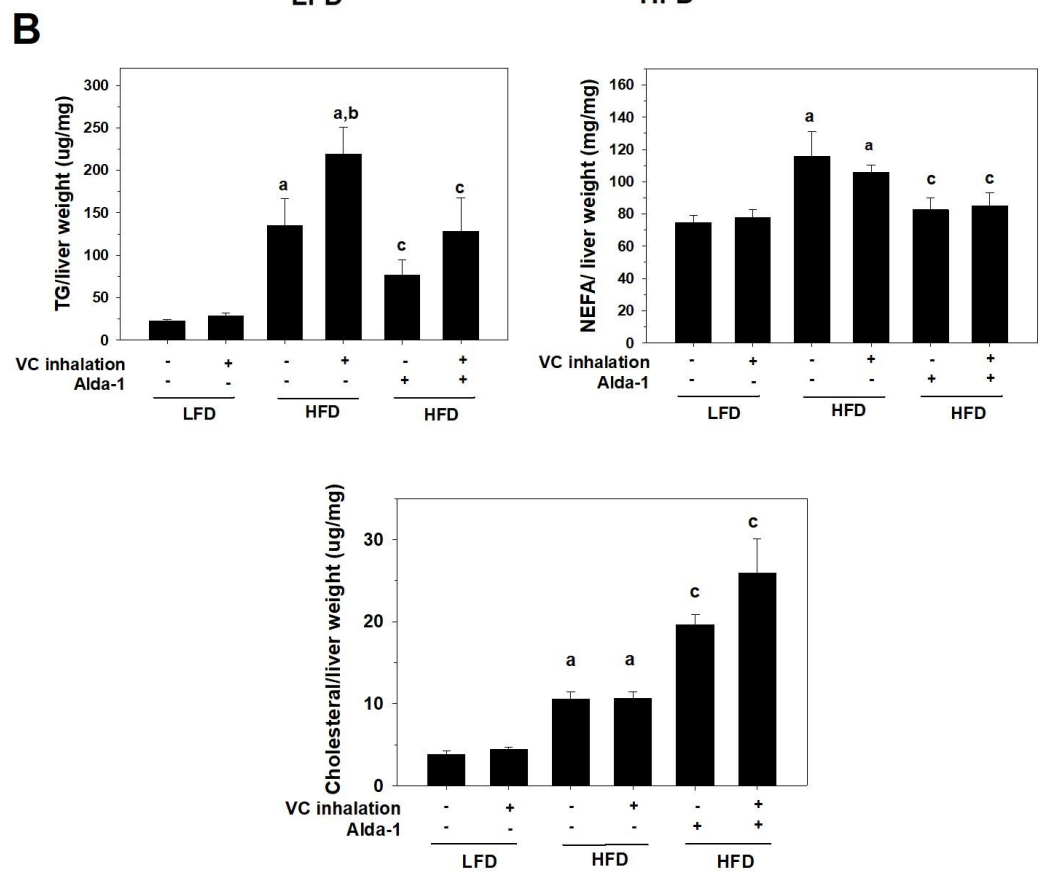
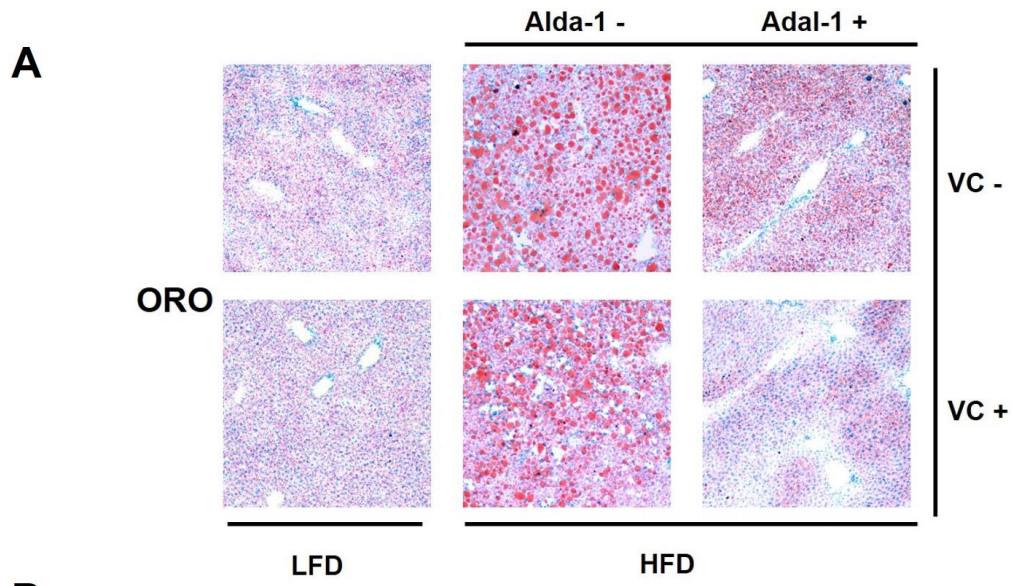
**Figure 4.6. ALDH2 reduced oxidative stress in HFD and VC**

A: Representative photomicrographs of MDA (index of oxidative stress, brown) of all groups are shown at the 200x magnification. B: MDA levels in liver were measured by TBARS assay as described in the previous section. Results are reported as means  $\pm$  standard error mean (SEM; n=4-6). <sup>a</sup>, p<0.05 compared to LFD control, <sup>b</sup>, p<0.05 compared to absence of VC, <sup>c</sup>, p<0.05 compared to absence of Alda-1.



**Figure 4.7. ALDH2 decreased CYP2E1 expression in liver of HFD+VC**

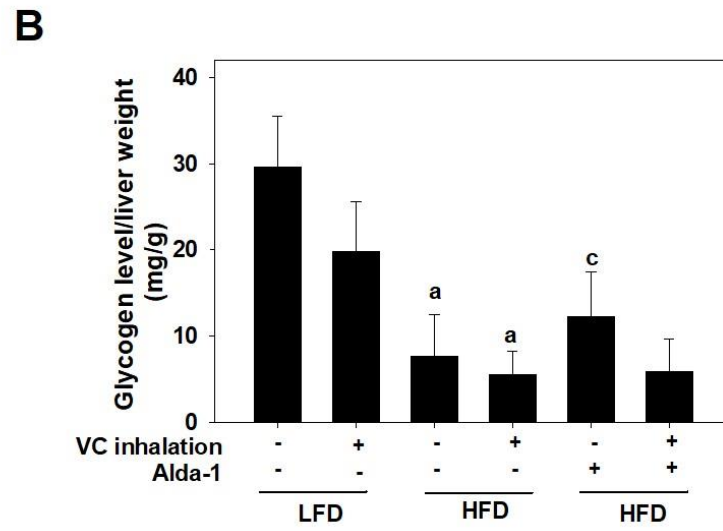
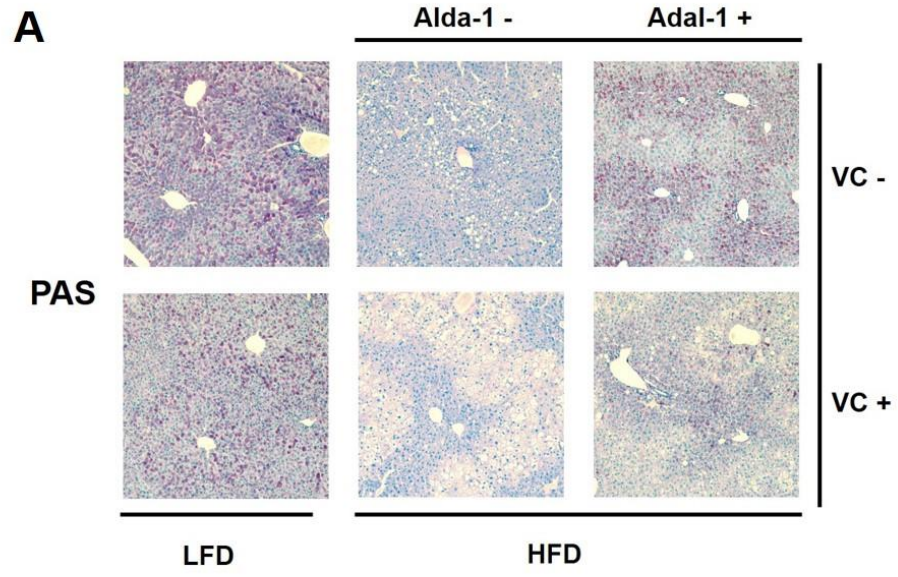
A: Representative Western blot results of CYP2E1 expression are shown for 12 weeks mice. B: Densitometric analysis of CYP2E1 expression is graphed. Results are reported as means  $\pm$  standard error mean (SEM; n=4-6). <sup>a</sup>, p<0.05 compared to LFD control, <sup>b</sup>, p<0.05 compared to absence of VC, <sup>c</sup>, p<0.05 compared to absence of Alda-1.





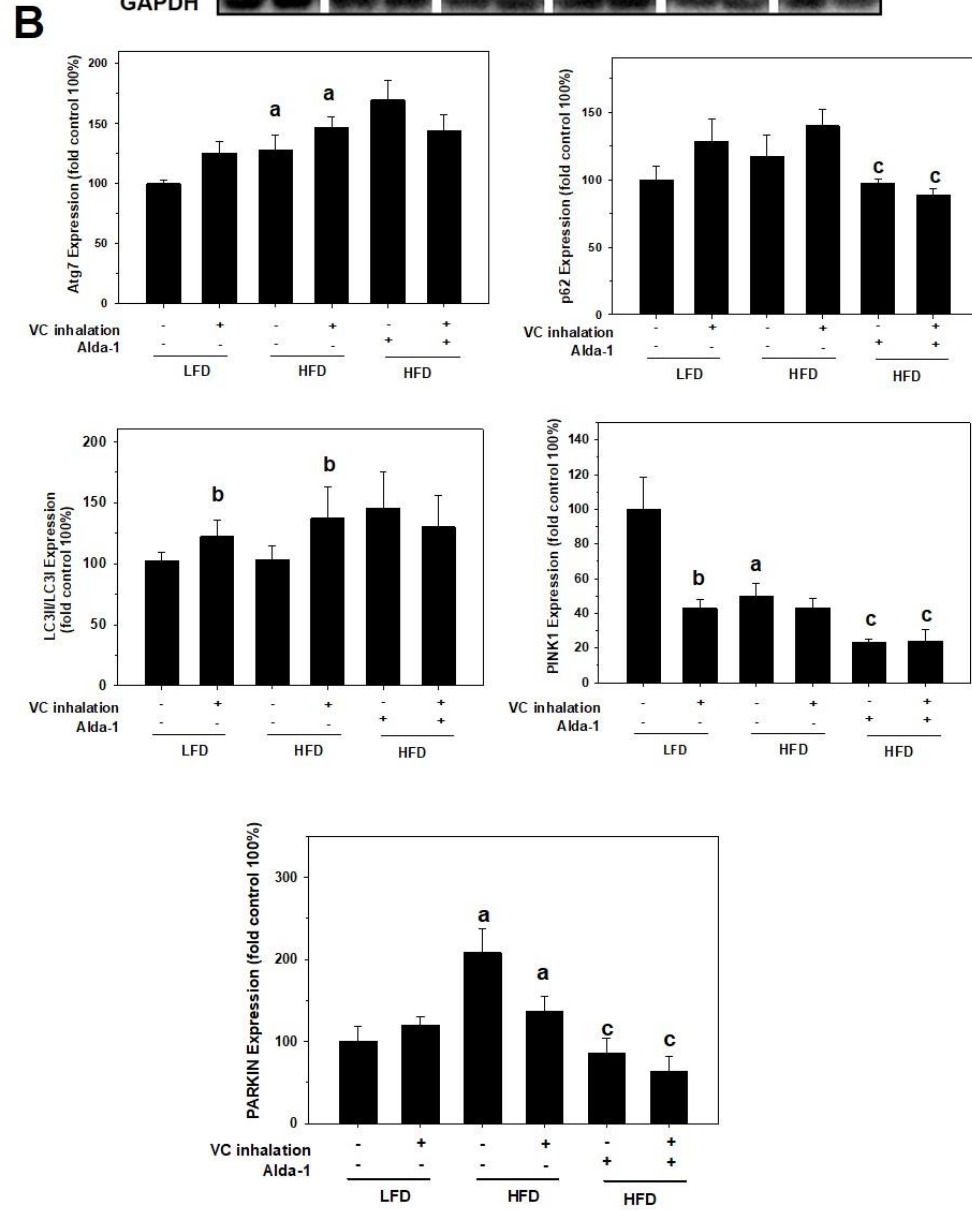
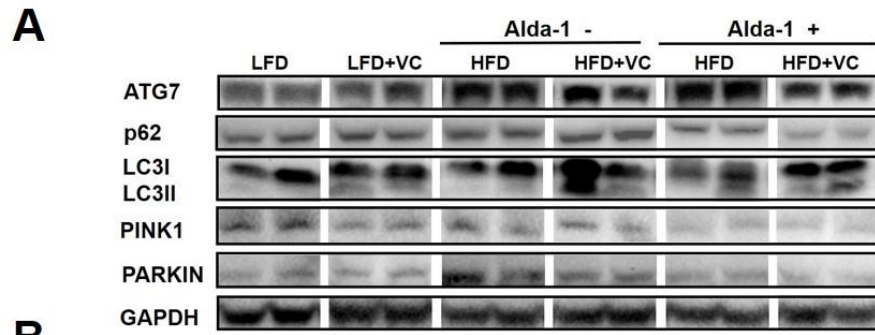
#### **Figure 4.8. The effect of ALDH2 in lipids level**

A: Representative photomicrographs of ORO (neutral lipid, red) are shown at 200x magnification. B: TG, FFA and cholesterol level of all groups were measured as described Chapter II. Results are reported as means  $\pm$  standard error mean (SEM; n= 4-6), <sup>a</sup>, p<0.05 compared to LFD control, <sup>b</sup>, p<0.05 compared to absence of VC, <sup>c</sup>, p<0.05 compared to absence of Alda-1.



#### **Figure 4.9. The effect of ALDH2 in hepatic glycogen deposition**

A: Representative photomicrographs of PAS (glycogen, dark purple) staining are shown at 200x magnification. B: Hepatic glycogen expression of all groups were measured as described in previous section. Results are reported as means  $\pm$  standard error mean (SEM; n= 4-6), <sup>a</sup>, p<0.05 compared to LFD control, <sup>b</sup>, p<0.05 compared to absence of VC, <sup>c</sup>, p<0.05 compared to absence of Alda-1.



**Figure 4.10. The effect of ALDH2 on autophagy regulation**

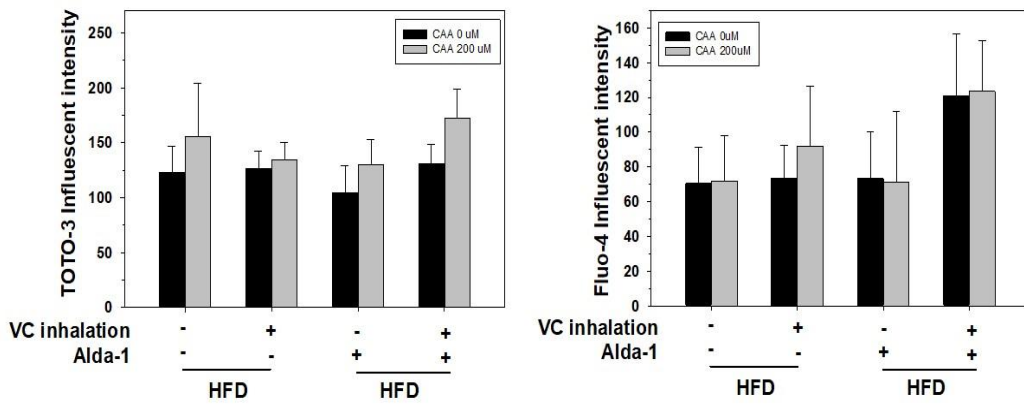
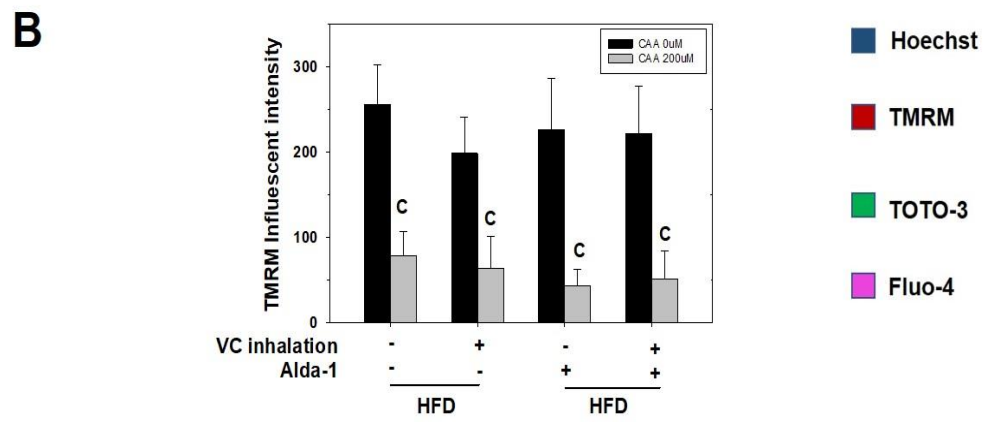
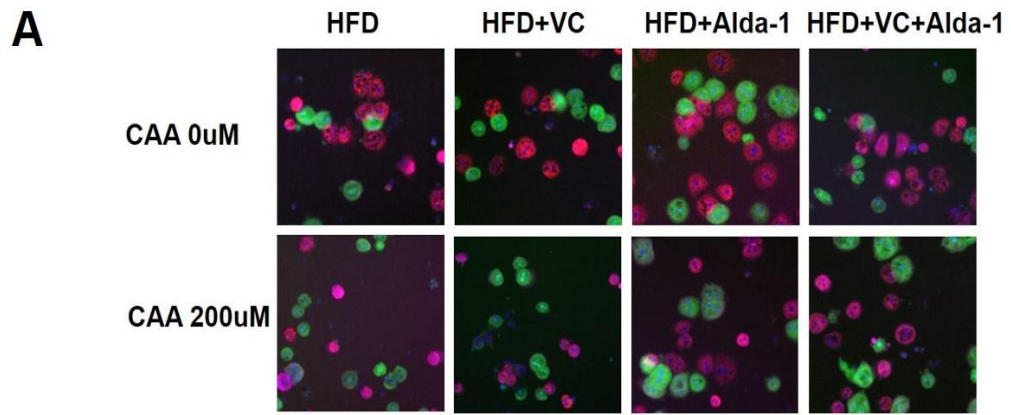
A: Representative western blot results for autophagy associated indices expression level in liver: Atg7, p62, LC3I/LC3II, PINK1 and PARKIN. B:

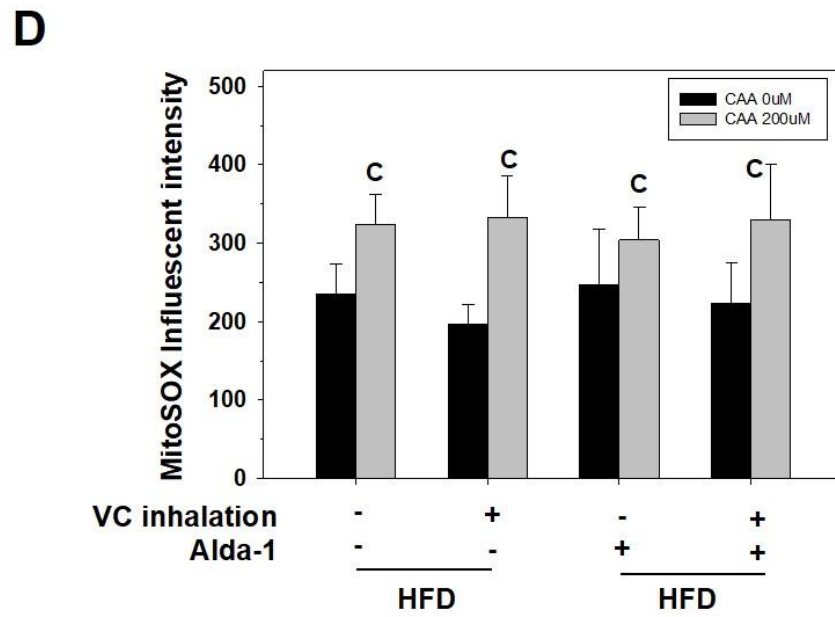
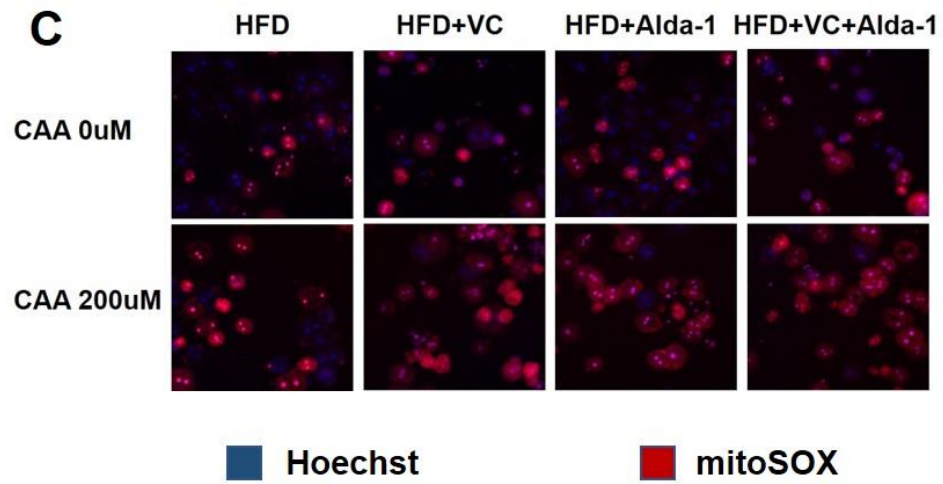
Densitometric analysis for western blot is calculated by the ratio of intensity of autophagy related protein to GAPDH. Results are reported as means  $\pm$

standard error mean (SEM; n= 4-6). <sup>a</sup>, p<0.05 compared to LFD control, <sup>b</sup>,

p<0.05 compared to absence of VC, <sup>c</sup>, p<0.05 compared to absence of Alda-

1.

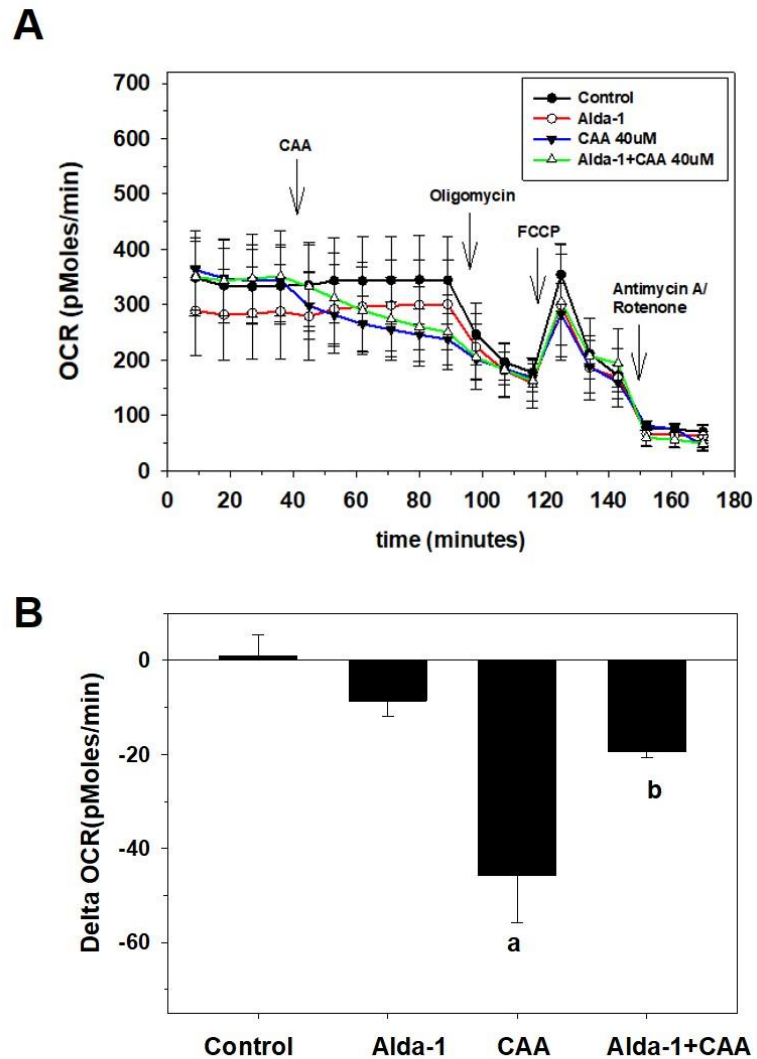




**Figure 4.11. The effect of ALDH2 on cytotoxicity in primary hepatocytes**

A: Representative photomicrographs depicting nuclear fluorescence (Hoechst; blue), mitochondrial membrane potential (TMRM; red), cell membrane permeability (TOTO-3; green) and intracellular calcium flux (Fluo-4; pink) were determined in primary hepatocytes from 12-week modeled mice using Cellomics HCS analysis. B: Fluorescence values analysis of TMRM, TOTO-3 and Fluo-4 were performed. C: Representative photomicrographs depicting nuclear fluorescence (Hoechst; blue) and mitochondrial superoxide (MitoSox; red) were determined. D: Fluorescence values analysis of MitoSox for each group are performed. <sup>a</sup>,  $p < 0.05$  compared to absence of VC, <sup>b</sup>,  $p < 0.05$  compared to absence of Alda-1, <sup>c</sup>,  $p < 0.05$  compared to absence of CAA treatment.





**Figure 4.12. ALDH2 protects mitochondrial respiration from CAA**

A: OCR at different time point is measured in primary hepatocytes exposed to CAA by Seahorse XF96 analyze as described in Chapter II. B: Delta OCR is calculated by the subtracting the OCR at the first measured time point after CAA addition from basal OCR value. <sup>a</sup>,  $p < 0.05$  compared to absence of CAA, <sup>b</sup>,  $p < 0.05$  compared to absence of Alda-1.

### **C. Discussion**

VC is an important hazard that causes many diseases. VC contaminated water or air are the major ways to enter the human body and increase the risk of multiple health problems, especially liver function. High concentrations of VC (>100 ppm) have been demonstrated to cause hepatotoxicity. However, the effects of low concentrations (< 1 ppm) of VC in combination with other risk factors are understudied. Another independent risk factor that promotes liver injury is obesity due to consumption of diets rich in fat. The pandemic of obesity is arguably the most prevalent underlying disorder that impact US population. Ingestion of large amounts of fat causes health complications such as NAFLD, which is closely associated with metabolic syndrome. Previous work in our lab also showed that low-dose exposure of VC exacerbated NAFLD in the interaction of HFD (34). This conclusion is a concern for occupational exposure and residential populations living in close proximity to VC-contaminated sites. VC directly impairs hepatic mitochondrial electron transport chain independent of diet (45, 78). In this section, we hypothesize that VC will also affect mitochondrial ALDH2, an enzyme that has previously shown to be protective. The aim was to explore the role of ALDH2 in the combination of low-dose VC and HFD, as well as the impact of ALDH2 in regulating cellular function using an in vitro model. For the mouse model of VC inhalation, we used a concentration below the OSHA standard (<1 ppm, 8 hour/day) for 12 weeks in combination with HFD feeding. We first determined ALDH2 function as well as expression levels in this model.

## **1. ALDH2 is impaired by VC and HFD.**

ALDH2 is an important mitochondrial enzyme involved in several cellular processes, notably in detoxification of alcohol-derived aldehyde. Ethanol is transformed to acetaldehyde which can further be converted to acetate by ALDH2. ALDH2 dysfunction causes aldehyde aggregation and increases ROS production (38). The active site of ALDH2 contains a thiol group which is sensitive to oxidative modification, making ALDH2 prone to oxidative inactivation (30). The results summarized here showed that VC enhanced the inhibition of ALDH2 activity in the interaction of HFD (Figure 4.2). Decreased ALDH2 activity can be result from covalent modifications of the pivotal amino acid residues of ALDH2 such as cysteine (79). Cysteine containing thiol groups are sensitive to redox modifications. Furthermore, cysteine residues can also be oxidized by endogenic aldehydes such as MDA and 4-HNE (80). MDA and 4-HNE are products derived from lipid peroxidation, which can be enhanced by ingestion of fatty acids (34); thus, feeding mice with HFD could oxidize cysteine residues, leading to ALDH2 inactivation. Indeed, MDA staining and quantitative TBARS levels were elevated by HFD feeding (Figure 4.6), which supports the previous observation of decreased ALDH2 activity. Moreover, CAA (VC metabolite) was found to impair ALDH2 function directly as shown in the previous chapter. In addition, Moon et al have reported that inhibition of ALDH2 activity is mediated by phosphorylation via JNK (79). Activated JNK is translocated to the mitochondria and phosphorylates serine residues on ALDH2, causing conformational changes and contributing to the

inhibition of ALDH2 activity (81). Chloroethanol (CE), as a metabolite of VC, has been shown to increase JNK phosphorylation (82). Therefore, the enhancing effect of VC on HFD-induced inhibition of ALDH2 activity, might be regulated, in part through activating JNK-mediated phosphorylation. However, the full mechanism of ALDH2 activity regulation by VC and HFD is still unclear.

One reason for a decrease in enzyme activity can be alterations in protein expression (83). We therefore expected a decrease in ALDH2 protein levels in mitochondria isolated from mice fed a HFD. However, HFD significantly increased ALDH2 expression (Figure 4.2). It has been shown that condensed mitochondria with increased cristae membrane caused by HFD, could be due to the overexpression of protein including ALDH2 (84). It is suggested that this is a compensated upregulation of protein expression in response to inhibition of the enzymatic activity caused by increased aldehyde toxicity. It is also known that  $\epsilon$ PKC translocation mediated by HFD-induced ROS production in mitochondria is a direct activator of ALDH2 expression (78). Upregulation of ALDH2 expression contributes to recovery of mitochondrial functional, improves metabolism of drugs and xenobiotic in liver (85). As shown by Western blot (Figure 4.2), a decreased ALDH2 activity by VC inhalation was only seen in HFD mice, however, VC decreased protein expression levels independent of diet. These data suggest that VC exposure inhibits ALDH2 enzymatic activity rather than affecting protein levels in the LFD mice, but enhances ALDH2 impairment in both two aspects during

additional risk factor exposure (HFD). Based on these findings, we confirmed that VC exacerbates ALDH2 impairment in the HFD mice. Hence, we next activated ALDH2 by Alda-1 in the HFD group to investigate the role of ALDH2 in liver function in the combination of HFD and VC.

## **2. ALDH2 reduced liver injury caused by HFD and VC**

Previous work by our lab has shown that low concentrations of VC increase the risk of liver damage in the present of HFD (34). Consistently, we showed VC promoted hepatic aminotransferase level including AST and ALT in the mice with HFD. AST and ALT are the representative and sensitive enzymes that can be elevated in response to liver injury in humans and animals. Activation of ALDH2 significantly blunted the increase in transaminase levels as well as pathological morphology (e.g. inflammation) in livers of the HFD mice (Figure 4.4). Indeed, the increased neutrophil infiltration in the HFD mice by VC inhalation was reversed by Adla-1 administration (Figure 4.5), indicating that hepatic inflammation and injury was attenuated by ALDH2. Neutrophil recruitment can be induced by chemokines such as lipopolysaccharide-induced CXC chemokine (LIX) which serves as the mediator of neutrophil recruitment that can be induced by oxidative stress (86). Decreased neutrophil accumulation by Alda-1 might therefore be due to the inhibition of oxidative stress-induced inflammatory chemokines. ALDH2 restores disruption of energy metabolism, reduces inflammation, decreases oxidative stress and protects liver function. Furthermore, increased hepatic MDA in the mice of HFD+VC was decreased

by Alda-1 treatment (Figure 4.6), supporting that ALDH2 reduced liver injury is via suppressed lipid mediated oxidative stress. Additionally, CYP2E1 expression was increased in the HFD groups and this was reversed by Alda-1 intervention (Figure 4.7). CYP2E1 is known to be a contributor to oxidative stress (87), decreased CYP2E1 level by Alda-1 demonstrated that ALDH2 activation protected liver from oxidative stress. These data demonstrated that ALDH2 is critical in protecting the liver against damage due to environmental toxicant exposure and dietary fat.

HFD and VC are risk factors that have been shown in our previous studies to cause disruption of hepatic metabolism (34). Energy metabolism includes lipid and carbohydrate homeostasis. Abnormal fat or glycogen storage in liver can cause cell damage. An overload of lipid accumulation is associated with cellular oxidative stress caused by aldehyde production, which result from lipid peroxidation. Metabolic phenotyping (Figure 4.3) showed that HFD increased body weight and blood glucose level of the mice, however VC addition did not enhance these effects. ALDH2 activation caused an opposite trend in the HFD mice. Consistent with the observations in body weight, HFD increased macrovesicular steatosis as depicted in ORO staining and quantitative hepatic TG levels, as well as microvesicular lipids (FFA and cholesterol) (Figure 4.7). TG levels were enhanced by VC exposure whereas FFA and cholesterol were not changed significantly. It suggested that VC has a stronger effect on regulating hepatic TG level rather than FFA and cholesterol level in the HFD group. We hypothesized that VC addition may

increase lipogenic gene expression involved in TG synthesis such as peroxisome proliferated-activated receptor  $\gamma$  (PPAR $\gamma$ ) and sterol regulatory element-binding protein-1c (SREBP-1c) in the HFD mice (88). All these markers were reduced by ALDH2 intervene except cholesterol. TG is thought to be the main component of fats, decreased lipids accumulation or steatosis by Alda-1 is predominantly attributed to ALDH2 mediated TG downregulation. Even though cholesterol was elevated after Alda-1 treatment, we believed the total effect of these forms of lipids in the HFD+VC was suppressed by ALDH2.

However, the mechanisms of ALDH2 involvement in lipid regulation remains unclear. It has been shown in mice that after Alda-1 treatment the most upregulated proteins were related to  $\beta$ -oxidation of fatty acid in mitochondria such as enoyl-Coenzyme A hydratase (ECHS1) and acetyl-Coenzyme A acyltransferase 2 (ACAA2) (81). These proteins assist with fatty acid degradation and ameliorate TG and FFA content in the liver. On the other hand, ALDH2 activity may also affect hepatic lipogenesis. You et al., found that acetaldehyde increased SREBP1 known as a mediator in lipid synthesis, inhibition of ALDH2 enhanced SREBP1 expression thereby leading to fatty liver (89). These studies may explain how ALDH2 regulates lipids storage in liver. Together with mice phenotype, these data affirmed that ALDH2 activation attenuated hepatic steatosis caused by HFD and VC.

Glucose homeostasis is also an important component of energy metabolism. HFD has been demonstrated to elevate blood glucose in our

study (Figure 4.3C). This was also previously demonstrated by increased insulin resistance as measured by ITT performed (34). Insulin resistance not only controls blood glucose but also is related to dyslipidemia (e.g. increased TG level) (90). Moreover, lipid peroxidation derived aldehydes could induce insulin resistance and result in metabolic syndrome (91). Alda-1 treatment was shown to decrease blood glucose in the HFD group, indicating a reversed effect of ALDH2 on glucose intolerance (Figure 4.3C). The role of ALDH2 in ameliorating insulin insensitivity has been demonstrated to act via regulating insulin signaling factors such as insulin receptor substrate (IRS) (50). We also found that decreased storage of glycogen in liver by HFD can be blunted by ALDH2 (Figure 4.9). HFD-induced FFA production inhibited glycogen synthesis (92), leading to energy depletion and lipids synthesis (causing steatosis) (34). ALDH2 has been reported for its role in regulating cellular glycogen synthesis related transcriptional factors including AKT, as well as its downstream target GSK3. It is known that inactivation and phosphorylation of GSK3 by AKT induction increases glycogen synthesis (93). Zhang and colleagues observed that Alda-1 increased activation of AKT/GSK3 signalling pathway leads to cardio-protection in an experimental diabetes model (94), suggesting that increased ALDH2 activity ablated cell damage via participating in AKT/GSK3 pathway and regulating glucose homeostasis. In the HFD+VC group, however, there was no significant difference in glycogen level after Alda-1 treatment. We hypothesize that VC impacts factors involving in the ALDH2 mediated glucose or glycogen



regulation. Taken together, these data suggest that ALDH2 plays a role in protecting impaired energy metabolism from HFD and VC.

### **3. The mechanisms involved in the role of ALDH2 in liver injury of HFD and VC**

We demonstrated that ALDH2 played a role in protecting the liver in the interaction of HFD and VC. However, the mechanisms of ALDH2 activation in hepato-protection remains elusive. We have shown that VC and HFD associated liver injury could be via increased inflammation, oxidative stress and metabolic homeostasis disruption. During the response to stress, cells initiate rapid changes to guarantee survival and to protect themselves against harmful conditions. Autophagy is one of the key pathways that mediate stress-induced adaption and damage control, allowing cells to eliminate damaged components such as organelles, proteins and portions of cytoplasm (71). LC3II is associated with the autophagic membrane and acts as an indicator of autophagy. However, here Alda-1 did not change LC3II expression (Figure 4.10). It is also known that p62 can be degraded by the autophagy-lysosome pathway, and an increase in p62 protein levels may result from impaired autophagy (60). Here, we found that Alda-1 significantly decreased p62 protein expression in the HFD and HFD+VC groups (Figure 4.10), possibly suggesting increased autophagy to protect from liver injury caused by HFD and VC. From our previous work, we know that VC promotes mitochondrial dysfunction in mice (34). Cells keep quality control by removing damaged mitochondria via mitophagy. This process is mediated by the

PINK1/PARKIN pathway. Here, Alda-1 was shown in the HFD+VC mice to decrease PINK1 and PARKIN expression, suggesting an inhibition of mitophagy (Figure 4.10). Ji et al., have also shown that ALDH2 activation had a protective effect on cells via suppressing PINK1/PARKIN mediated mitophagy (47). ROS-induced mitochondrial damage is an important upstream activator of mitophagy, and the PINK1/PARKIN pathway may be activated in response to oxidative stress induced cell damage (47). ALDH2 activation by Alda-1 reduced oxidative stress caused by HFD and VC, suggesting to play a role in mitochondrial protection. It seems paradoxical that ALDH2 activation protects mitochondria by inhibition of mitophagy. We hypothesize that under severe pathophysiologic conditions, such as oxidative stress, excessive mitophagy was triggered and caused mitochondrial fission and loss, which was detrimental to mitochondrial quality control (95). Activation of ALDH2 attenuated oxidative stress and suppressed PINK1/PARKIN-mediated mitophagy, resulting reduced mitochondrial injury. Thus, we proposed that ALDH2 has a dual regulatory role in autophagy to protect liver from HFD and VC.

Further, we studied ALDH2 function at the cellular level for cytotoxicity related indices, including mitochondrial membrane potential (TMRM), cell membrane permeability (TOTO-3), intracellular calcium ion (Fluo-4) and mitochondrial superoxide (mitoSox). As shown in the data above, ALDH2 ameliorated liver injury by reversing disruption of energy metabolism and oxidative stress, both of which are linked to mitochondria. Mitochondrial

membrane potential is a representative marker of mitochondrial function, serving as an intermediate involving in ATP synthase (74). Oxidative stress is an inducer of mitochondrial depolarization or Mitochondrial membrane potential loss, leading to mitochondrial dysfunction and deleterious effects (96). ALDH2 activation was shown to inhibit oxidative stress caused by HFD. However, there was no significant difference of TMRM fluorescent intensity found in Alda-1 groups compared to their controls (Figure 4.11). Intracellular calcium is known as one of the key events that causes mitochondrial injury via ROS generation, such as superoxide (97, 98). These markers were unchanged by ALDH2 activation as depicted by the cellomics assay. This suggests that the protective role of ALDH2 in hepatocytes of chronic HFD and VC model is not mainly controlled by regulation of calcium or superoxide related mitochondrial membrane potential. We additionally determined that Alda-1 treatment didn't alter TOTO-3 fluorescent intensity, which implied an unchanged cell membrane permeability during chronic HFD feeding. Previously, we have shown that ALDH2 increased mitochondrial membrane potential and decreased membrane permeability in the acute 4-HNE exposure (45). This paradoxical role of ALDH2 in these cytotoxicity indices, might be due to a cellular adaption in response to prolonged conditions of stress. When the cells are suffering from long-term harmful factors, mitochondrial membrane potential and cellular membrane permeability may be more compromised to the cellular stress and therefore not sensitive to ALDH2 mediated regulation.

Via Seahorse analysis, OCR was measured in primary mouse hepatocytes immediately after adding CAA. CAA decreased OCR and this effect was blunted by Alda-1 pre-incubation. However, this effect only occurred at the very beginning and disappeared as time progressed (Figure 4.12). We propose that ALDH2 activation protects mitochondrial respiration from short-term damage. CAA is a strong mitochondrial toxicant and the extent of the damage caused by CAA exceeds the protective properties of ALDH2 activation. ALDH2 catalyzes the conversion of CAA in the presence of  $\text{NAD}^+$  and forming NADH. In our Seahorse experiment, we did not supply extra  $\text{NAD}^+$ , while  $\text{NAD}^+$  was consumed as time progressed. Thus, it might be possible that the effect of ALDH2 in degrading CAA was decreased due to insufficient coenzyme. Additionally, it is proposed that Alda-1 has an inhibitory effect on ALDH2 when aldehyde levels are at a low concentration. Binding of Alda-1 to ALDH2 induces a change in the sequence of binding of substrate to enzyme. Alda-1 promotes a decrease of the  $pK_a$  of the catalytic cysteine, which may facilitate the nucleophilic attack to the aldehyde prior to  $\text{NAD}^+$ . Alternatively,  $\text{NAD}^+$  could bind to ALDH2 as the first substrate and form the non-productive complex (Alda-1-ALDH2- $\text{NAD}^+$ ). When aldehyde levels are low, the non-productive complex is predominantly formed and this complex can decrease catalytic reaction rate (99). In our study, CAA concentration was high at beginning, binding of Alda-1 to ALDH2 accelerated CAA catalysis. As the reaction continued, substrate of CAA was lower, the substrate binding sequence might be changed and resulted in the formation

of the non-productive complex, which inhibited ALDH2 function, leading to a decreased mitochondrial protection. These data might also explain the discrepant role of ALDH2 in mitochondrial dysfunction caused by different time point-dependent deleterious factors exposure.

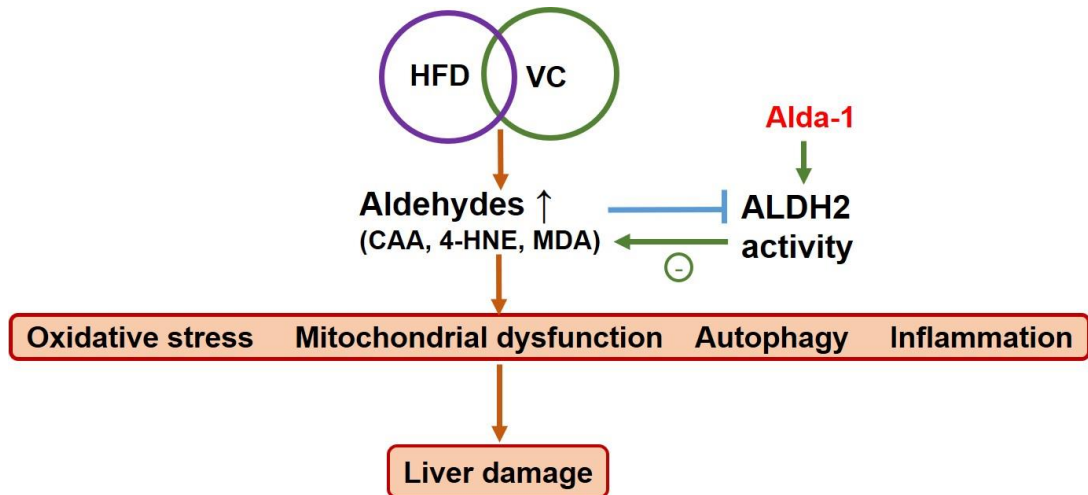
Mitochondrial membrane potential is known to not only play a role in energy supply but also participate in the process of elimination of damaged mitochondria (74). It has been demonstrated that mitophagy mediated by PINK1/PARKIN signal is triggered by depolarization of the Mitochondrial membrane potential (100). Interestingly, the mitochondrial membrane potential in ALDH2 was not consistent with PINK1/PARKIN expression. However, mitochondrial membrane potential is not the only factor in regulation of PINK1/PARKIN-mediated mitophagy. There are other signalling factors participating in this process. It has been found that Nrf2 is a novel transcriptional upregulator of *Pink1* genes via the activation of an antioxidant responsive elements (ARE) sequence in the promoter of PINK1. Nrf2 is normally in the cytosol and degraded by KEAP1. While under oxidative stress conditions, oxidants and electrophiles oxidize cysteine residues in KEAP1 and increase Nrf2 release, leading to PINK1 increase (101). Additionally, ATF4, known as the marker of ER stress involved in liver damage (102), is demonstrated to upregulate PARKIN by binding to a specific CREB/ATF site within PARKIN promoter (103). As discussed before, augmented mitophagy under severe condition could be deleterious to mitochondria. We hypothesize that excessive PINK1/PARKIN-mediated mitophagy might be triggered by

increased oxidative and ER stress. ALDH2 decreased oxidative stress may attenuated Nrf2 mediated PINK1 promotion. It has been shown that ATF4 upregulation is associated with ALDH2 impairment (104). We suggest that ALDH2 inhibited PINK1/PARKIN signal pathway might be due to the suppressed oxidative and ER stress. These data also suggest complex and mixed mechanisms of ALDH2 in protecting mitochondria. In the early state of liver injury, ALDH2 activation may have increased PINK1/PARKIN signalling via preserving mitochondrial membrane potential (45, 105) and plays a role in mitochondrial protection, partly to attenuate cell damage. As the time went on or under more severe conditions, more excessive mitophagy may have occurred and impaired mitochondrial homeostasis. The severe degree of mitochondrial membrane potential depolarization might not be rescued by ALDH2 regulation, thus, ALDH2 may shunt to regulate other factors (e.g. Nrf2, ATF4) and suppress PINK1/PARKIN-mediated mitophagy, protecting mitochondrial function.

#### **D. Conclusion**

In this aim, we employed a model of chronic low-dose VC exposure in mice fed with a HFD and studied the role of ALDH2 in this combination as well as the related mechanisms in vivo and in vitro. Both ALDH2 enzymatic activity and expression level were impaired in the interaction of HFD and VC. This impairment contributes to liver injury via increased aldehyde levels and related oxidative stress. Activation of ALDH2 accelerated aldehyde clearance

and provided a protective effect. Additionally, ALDH2 plays a role in reversing metabolic disorder including lipids/carbohydrates and inhibiting inflammation caused by HFD and VC. This protection by ALDH2 activation can be mediated by some signalling factors. ALDH2 decreased p62 expression, indicating increased autophagy resulting in the removal of damaged proteins or components in hepatocytes. Further, ALDH2 was demonstrated to inhibit PINK1/PARKIN-induced mitophagy, to decrease excessive mitophagy and to act a regulator of mitochondrial quality control. Impairment of the mitochondrial respiratory chain by CAA was reflected in a decrease in OCR of hepatocytes. This effect was attenuated by ALDH2 activation, suggesting that ALDH2 activation protects mitochondrial respiration from the toxic effects of CAA. Overall in this aim, we demonstrated that ALDH2 alleviated liver damage in the interaction of chronic VC exposure and HFD, via decreasing oxidative stress and inflammation, reversing metabolic dysfunction, regulating autophagy and protecting mitochondrial function (Scheme 4.2).



**Scheme 4.2: regulation of ALDH2 in liver injury caused by HFD and VC**

ALDH2 dysfunction is involved in the liver injury caused by HFD and VC, which is due to an increased aldehydes attack. Activation of ALDH2 plays a role in protecting liver function against HFD and VC by decreasing oxidative stress, inflammation, protecting mitochondrial function and regulating autophagy.



## CHAPTER V

### SUMMARY AND CONCLUSION

#### **A. Aims in this study**

VC and obesity are the independent risk factors that are known to induce liver injury. Our group has previously observed that low-dose of VC exposure exacerbated liver damage in HFD-fed mice and started to gain insight into the associated mechanisms (34). ALDH2 is known to play a protective role in the many diseases. However, whether it ameliorates liver injury caused by HFD and VC has not been studied. The overall goals in this dissertation were to investigate the role of ALDH2 in the interaction of VC and HFD. For that goal, we set two aims. The first aim was to determine the effect of ALDH2 in an acute setting of VC metabolite exposure. Part of that aim was also to study the catalytic activity of ALDH2 protein under exposure to CAA, as described in Chapter III. The second aim described in Chapter IV was to characterize the role of ALDH2 in liver injury caused by chronic low-dose VC exposure and HFD. Taken together, these studies provide the evidences that ALDH2 plays a role in protecting liver function by regulating complex mechanisms of liver injury.

## **B. Major findings in this dissertation**

### **1. Protection of ALDH2 enzymatic activity reduced liver injury against acute CE exposure**

Since CE is a major metabolite of VC, the role of ALDH2 on liver function after CE exposure serves as an indicator to evaluate its impact in mice exposed to a moderately high, acute dose of VC. Mice therefore received CE at concentrations equal to high-level VC inhalation that caused liver damage. In Chapter III, we first determined the effect of ALDH2 in the disease phenotype. CE was shown to disrupt hepatic carbohydrate and lipid metabolism as presented by histologic staining and quantitative measurements. ALDH2 activation reversed lipid (e.g. TG) accumulation in the CE group while it did not increase glycogen stores. When looking at the expression of metabolism related genes, ALDH2 increased *cpt1* expression supporting a promotion of FFA  $\beta$ -oxidation in the mitochondria. These data may explain the fact that ALDH2 decreased lipid storage. Previous work in our lab showed that CE increased 4-HNE adduct formation after LPS administration (30). It has also been shown previously that 4-HNE adduct formation can be inhibited by ALDH2 (45). Therefore, oxidative stress was also assessed in this aim. As expected, ALDH2 decreased 4-HNE adduct formation induced by CE, indicating that lipid peroxide levels were attenuated by ALDH2 activation. Overall, we demonstrated that ALDH2 prevented liver injury caused by CE exposure via reducing hepatic steatosis and oxidative stress.

The second aim in Chapter III was to explore a direct reaction between ALDH2 and CAA. ALDH2 catalyzes aldehydes into nontoxic acids with NAD<sup>+</sup> as a cofactor. We showed that ALDH2 degraded CAA, which was initiated immediately by addition of NAD<sup>+</sup>. ALDH2 pre-incubation with CAA showed lower catalytic activity after adding NAD<sup>+</sup>. This result suggests that CAA is not only the substrate but also an inhibitor of ALDH2. Activation of ALDH2 prior to CAA exposure rescued the inhibition of the enzymatic activity caused by CAA. These data demonstrated that ALDH2 activation by Alda-1 pre-treatment prevented enzyme function from CAA. The preserved ALDH2 catalytic activity accelerated elimination of toxic aldehydes such as 4-HNE and CAA, removed aldehyde-related oxidative stress, and therefore protecting the liver from the detrimental effects of VC metabolite exposure.

## **2. ALDH2 dysfunction is involved in the liver injury caused by HFD and VC**

As described in Chapter III, we found that ALDH2 activity was impaired by CAA. It is reasonable to speculate that ALDH2 function will also be inhibited by VC inhalation in HFD-fed mice. First, in Chapter IV, we measured ALDH2 expression as well as enzymatic activity in chronic low-level VC exposure in combination with HFD. HFD decreased ALDH2 activity but increased protein expression, likely as a compensatory reaction. VC however, decreased both. These data supported our hypothesis that ALDH2 dysfunction is critically involved in the interaction of VC and HFD. In the *in vitro* experiment as described in Chapter III, we demonstrated ALDH2 activity played a protective

role in the liver during exposure to VC metabolites. That provided a strong rationale to assume that ALDH2 activation attenuated liver injury caused by HFD and VC.

### **3. ALDH2 activation reduced liver injury of VC and HFD through protecting mitochondrial function**

Previously we have found that VC exacerbated liver damage in the HFD mice involving multiple mechanisms including metabolic dysfunction, oxidative stress, and to a lesser extent, inflammation (34). Thus, these hallmarks were measured as described in Chapter IV for evaluating the role of ALDH2 in the combination of VC and HFD. Indices of liver injury, transaminases activity (AST, ALT) were decreased by ALDH2 activation in the VC and HFD group. The effect of VC inhalation and dietary fat on the hepatic general morphology was also improved by ALDH2 activity, particularly neutrophil infiltration was decreased. Increased lipid accumulation (e.g. TG, FFA) mediated by HFD and VC was alleviated by ALDH2, while the reversed effect of ALDH2 on depleted glycogen was only observed in HFD group. Oxidative stress-mediated liver damage has been studied previously, as aldehydes derived from lipid peroxidation are known substrates of ALDH2 (58). Steatosis caused by HFD+VC was associated with elevated toxic aldehyde production such as MDA. ALDH2 catalyzed MDA degradation and ameliorated oxidative stress in liver of HFD and VC. Furthermore, we measured the expression of CYP2E1 in response to ALDH2 activation. CYP2E1 is not only known as the critical enzyme for drug metabolism but

also serves as an inducer of ROS. ALDH2 decreased CYP2E1 levels and partly ameliorated oxidative stress.

Cells often evoke an adaptive process in response to such stress-mediated injury. Autophagy is one of the key pathways allowing cells to eliminate damaged components for quality control, homeostasis of organelles, proteins and portions of cytoplasm. Atg7, LC3 and p62 are the factors typically regulating autophagy (71, 72). ALDH2 did not alter Atg7 and LC3 expression in the HFD and VC but decreased p62 expression, which suggested an increase in autophagy by ALDH2. Selective autophagy termed mitophagy is initiated by PINK1/PARKIN signal pathway and these markers in the HFD and VC were suppressed by ALDH2. Previous work in our group has demonstrated mitochondrial dysfunction was induced by aldehydes such as 4-HNE in the in vitro experiments (45). We hypothesized that ALDH2 decreases mitophagy due to its function in reducing oxidative stress in mitochondria. Moreover, decreased OCRs in hepatocytes after CAA addition was blunted by pre-treatment of Alda-1, indicating that impaired mitochondrial respiration was prevented by ALDH2 activation. However, we found that this protection did not persist. Moreover, ALDH2 showed no effect on cytotoxicity indices (e.g. mitochondrial membrane potential) in the HFD fed mice. It is possible for ALDH2 to play a role in protecting liver via different patterns according to different time point exposure of risk factors.

**Summarized table 1: The effect of ALDH2 in indices change in VC metabolites exposure**

<b>Indices</b>	<b>CE/CAA</b>	<b>Alda-1+CE/CAA</b>
Lipid and glycogen phenotype	TG↑ FFA-, cholesterol -	TG↓ FFA-, cholesterol -
Lipid and carbohydrate gene	No change	<i>Cpt1</i> ↑
Oxidative stress	4-HNE↑	4-HNE↓
Pro-inflammatory cytokine	TNF-α -, Pai-1 -	TNF-α -, Pai-1 -
ALDH2 activity	↓	↑
OCRs	↓	↑

**Summarized table 2: The effect of ALDH2 in indices change VC and HFD exposure**

<b>Indices</b>	<b>HFD+VC</b>	<b>Alda-1+HFD+VC</b>
ALDH2 function	↓	/
AST, ALT	↑	↓
Metabolism phenotype	TG↑, FFA↑, body weight ↑ Glycogen store↓, glucose tolerance ↑	TG↓, FFA↓, body weight ↓ Glycogen store↑, glucose tolerance ↓
Oxidative stress Inflammation	MDA↑ Neutrophils infiltration↑	MDA↓ Neutrophils infiltration↓
Autophagy	Atg7↑, LC3II↑, p62 - PINK1-, PARKIN↑	Atg7-, LC3II -, p62↓ PINK1↓, PARKIN↓
Cytotoxicity Mitochondrial function	TOTO-3 -, Fluo-4 - TMRM -, miSOX-	TOTO-3 -, Fluo-4 - TMRM -, miSOX-

### **C. Significance**

In the United States, NAFLD is becoming the most common cause of liver disease that leads to a non-negligible health problem. Obesity and environmental toxicants are two independent risk factors causing liver injury. VC, as the representative toxicant has been demonstrated to exacerbate liver damage at a low-dose level in the presence of a HFD (34). This model allowed us a better understanding of the underlying mechanisms in the interaction of VC and obesity, which provided the rationale for determining a target to protect the liver from injury. ALDH2 is a mitochondrial enzyme known for its role in ethanol metabolism and its participation in protecting organs from not only alcohol but also other stress (78). The effect of ALDH2 in the interaction of VC and HFD induced liver injury remains elusive. In this project we found that ALDH2 impairment is, at least in part, involved in the deleterious effects of VC and HFD. Indeed, ALDH2 activation by Alda-1 protected the liver from the detrimental effects of not only acute VC metabolite exposure but also of chronic exposure to HFD and VC.

Our model of low-dose VC inhalation and HFD feeding mimics the situation of subjects living/working near manufacturing and/or superfund sites who are also likely to ingest a Western style diet, rich in fat and carbohydrates. Based on our findings, we raised several concerns for this kind of population: 1) subjects with ALDH2 dysfunction or deficiency (*ALDH2*\*2 allele) might be more susceptible to liver damage caused by VC exposure and dietary fat. Therefore, we suggest that screening for these

ALDH2 activity defects might be beneficial for these subjects. 2) For those subjects who have ALDH2 impairment, ALDH2 activation by Alda-1 will serve as a potential therapeutic target. These proposed concerns are based on the experimental animal data. The effect of ALDH2 in humans needs further investigation.

#### **D. Strength**

Liver disease caused by high-level VC exposure has been studied extensively, however, the effect of low-level VC exposure on liver function is largely understudied. Our group focuses on the impact of chronic administration of sub-OSHA concentrations of VC exposure (<1 ppm) on liver. Indeed, in our previous work, we observed the importance of VC exposure on hepatic metabolism, oxidative stress activation, and mitochondrial dysfunction. This effect was exacerbated when combined with a HFD. These findings suggested that even low-dose VC exposure may not be safe for people in combination with other factors, such as diets rich in fat. Moreover, there are no potential targets for therapy known to halt or reverse the disease. Therefore, seeking such a target was a major focus of this study.

In this work, we focused on the effect of ALDH2 in liver injury. Although ALDH2 has been shown to reduce liver injury caused by alcohol, the role of ALDH2 in our animal model remains unclear. We employed an animal model to closely mimic human exposure of VC and co-exposure of HFD. The major goal was to investigate whether activation of ALDH2 by Alda-1 changed the



disease phenotype. ALDH2 activation was found to improve the dysregulated energy metabolism and to decrease oxidative stress in this model. We also used the state-of-the-art techniques such as Cellomics Assay Scan VTI HCS reader and XF96 Extracellular Flux Analyzer to gain insight into the role of ALDH2 in mitochondrial function. Indeed, ALDH2 activation protected mitochondrial respiration from the toxic effects of CAA.

This dissertation revealed that ALDH2 plays a role in the mechanisms involved in liver injury caused by VC and HFD, and that its activation may protect the liver in this model. Other related organochlorine contaminants such as TCE and PCE, that have also been demonstrated to cause liver injury, could also be following a similar pathway (e.g. oxidative stress) (70). This work provides rational evidence that ALDH2 is a potential target in preventing liver injury from chlorinated solvent mixtures.

#### **E. Weakness**

In this dissertation, the role of ALDH2 in liver protection against VC and HFD was shown in an animal model. Animal models may not completely mimic human liver disease progression. Therefore, the effect of ALDH2 in regulating liver function may not be applicable to a human population. Moreover, multiple factors participate in the liver damage, it is therefore difficult to identify an exact pathway. We here showed the role of ALDH2 in altering disease phenotype in mice, however, we did not perform a direct regulation of ALDH2 at molecular level in liver protection in the animal model.

Additionally, only male mice were used in this study. It is important to note the effects observed may vary with sex. Indeed, recent data by Wahlang et al., (unpublished observation) showed that female mice are less susceptible to liver injury caused by VC and HFD.

## **F. Future direction**

### **1. Investigate how CAA modulates ALDH2 activity**

ALDH2 contains a catalytic domain and a NAD<sup>+</sup> binding domain. Cys302 is known as the main aldehyde binding site in the catalytic domain of ALDH2 for aldehydes clearance (38). It has been also proposed that Cys302 is the target for ALDH2 inactivation by 4-HNE. Additionally, it has been observed that the Lys residue in the NAD<sup>+</sup> binding site of ALDH2 is susceptible to be targeted by toxic aldehydes such as acrolein (58). In our work, we have shown that chronic VC exposure decreased ALDH2 activity in an animal model, and that CAA pre-incubation binds to the active site and therefore inhibiting NADH formation. These findings suggested that VC or its metabolites react with residues within the ALDH2 enzyme and therefore decrease its catalytic activity. However, how these active sites were regulated remains subject to further studies. Although ALDH2 shows a high affinity of toxic aldehydes, this enzyme also was sensitive to inactivation reversibly or irreversibly by these toxicants. It would be beneficial to have a more in-depth understanding of the interaction between enzyme and aldehydes.

### **2. Investigate whether Alda-1 protects liver injury from VC and HFD in defect ALDH2 mice**

The most prevalent ALDH2 variant is *ALDH2* 2\*2 allele, which is found in the 35-45% Asian population (38). The *ALDH2* 2\*2 has a lower catalytic activity than the wild-type allele. For those subjects with an ALDH2 mutation, it would be beneficial to know whether Alda-1 still protects liver function. *ALDH2* 2\*2 knock-in mice may serve as an ideal experimental model for the research of human diseases associated with ALDH2 deficiency. In our study, we demonstrated that activation of ALDH2 by Alda-1 reduced liver injury caused by VC and HFD in the wild-type mice. Moreover, Alda-1 is shown to activate both human and mouse *ALDH2* 2\*2 enzyme with a similar potency profile and activation kinetics (38). It is likely that Alda-1 treatment also protects ALDH2 deficient livers against environmental toxicants and dietary fat.

### **3. Investigate whether ALDH2 activation prevents liver against chronic VC and HFD**

In Chapter IV, we demonstrated that ALDH2 intervention reduced liver injury caused by chronic exposure of VC and HFD. In mice exposed to long-term noxious compounds, ALDH2 impairment may occur in an early stage of liver damage (ALDH2 dysfunction was measured at the 6 week time-point of the animal model; data not shown). Aldehydes including 4-HNE, MDA and CAA caused ALDH2 inactivation may not be completely reversed by Alda-1 treatment. As the toxicant exposure continues, the effect of Alda-1 on ALDH2 activation may not be strong enough to maintain the protective effect in liver damage. In Chapter III, we observed that pre-treatment of Alda-1 protected

livers from acute CE exposure. It is promising that pre-treatment of Alda-1 prevent liver damage from chronic VC and HFD.

### **G. Summary and conclusion**

Taken together, this dissertation described ALDH2, an enzyme as a potential therapeutic target in the novel interaction between of chronic VC exposure and dietary fat. The experiments described in Chapter III revealed that ALDH2 played a role in the preventing liver injury caused by acute CE exposure. This effect was due to the decreased oxidative stress and reversed metabolic dysfunction. Inhibition of ALDH2 activity by CAA was prevented by Alda-1 pre-incubation. These findings showed that ALDH2 function in this phenotype might be mediated via a preservation of enzymatic activity by Alda-1. Chapter IV demonstrated that ALDH2 dysfunction was a part of the mechanisms mediating liver damage in the interaction of chronic VC exposure and HFD. Indeed, activation of ALDH2 reduced indices of liver injury in this combination. Additionally, metabolic stress, oxidative damage and impaired autophagy were observed to be affected by ALDH2. This could result from an intact mitochondrial function. Activation of ALDH2 by Alda-1 increased toxic substrate elimination, which attenuated oxidative stress and protected mitochondrial respiration. Therefore, energy metabolism and autophagy (mitophagy) were also improved. Overall, this project demonstrated the role of ALDH2 in liver protection using an experimental

animal model. In our future studies, we will further explore the mechanisms in this model.

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## ABBREVIATIONS

4-HNE	4-Hydroxynoneal
ACLY	ATP citrate lyase
AFLD	Alcoholic fatty liver diseases
ALDH 2	Aldehyde dehydrogenases 2
ALT	Alanine aminotransferase
ANOVA	Analysis variation
ASL	Angiosarcoma of the liver
AST	Aspartate aminotransferase
ATF4	Activating transcription factor 4
ATSDR	Agency for Toxic Substances and Disease Registry
BMI	Body mass index
CAA	Chloroacetaldehyde
CAE	Chloroacetate esterase
CE	Chloroethanol
CPT1	Carnitine palmitoyltransferase 1

CREB	CAMP responsive element binding protein 1
CYP	Cytochrome P450
DCE	Dichloroethylene
EPA	Environment Protection Agency
ER	Endoplasmic reticulum
FAS	Fatty acid synthase
FFA	Free fatty acid
GCK	Glucokinase
GLUT4	Glucose transporter 4
GSK3b	Glycogen synthase kinase 3b
H&E	Hematoxylin and eosin
HCC	Hepatocellular carcinoma
HFD	High-fat diet
IHC	Immunohistochemistry
KEAP1	Kelch-like ECH-associated protein 1
LC3	Microtubule-associated protein light chain 3
LFD	Low-fat control
LIX	Lipopolysaccharide-induced CXC chemokine

MDA	Malondialdehyde
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NEFA	Non-essential fatty acid
Nrf2	Nuclear factor-like 2
OCRs	Oxygen consumption rates
OGTT	Glucose tolerance test
ORO	Oil Red-O
OSHA	Occupational Safety and Health Association
p62	Sequestosome 1
PAI-1	Plasminogen activator inhibitor 1
PAS	Periodic Acid-Schiff
PCE	Tetrachloroethene
PCK	Phosphoenolpyruvate carboxykinase
PINK1	PTEN-induced kinase 1
PPAR $\gamma$	Peroxisome proliferated-activated receptor $\gamma$
Ppm	Parts per million
PVC	Polyvinyl chloride
ROS	Reactive oxygen species

SREBP-1c	Sterol regulatory element-binding protein-1c
TASH	Toxicant-associated steatohepatitis
TBARS	Thiobarbituric acid reactive substances
TCE	Trichloroethene
TG	Triglyceride
TNF- $\alpha$	Tumor necrosis factor- $\alpha$
VC	Vinyl chloride
VDAC	Voltage-dependent anion channel

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### Publication

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

1. Poster, 07/17. "Vinyl Chloride exacerbates liver injury induced by high-fat diet via causing ALDH2 dysfunction in mice." *OVSOT Student Summer Meeting*, Cincinnati, OH. OVSOT Summer Student Meeting, Louisville, KY.
2. Poster, 09/17. Vinyl Chloride exacerbates liver injury induced by high-fat diet via causing ALDH2 dysfunction in mice. Research!Louisville, Louisville, KY.
3. Poster, 10/17. Vinyl Chloride exacerbates liver injury induced by high-fat diet via causing ALDH2 dysfunction in mice. AASLD annual meeting, Washington, DC.
4. Poster, 11/18. Environmental toxicant Vinyl Chloride causes hepatic mitochondrial dysfunction in mice. AASLD annual meeting, San Francisco, CA.

### **Abstracts**

#### **National/International**

1. Lang AL, **Chen L**, and Beier JI (2017) Vinyl Chloride Inhalation Exacerbates Experimental Fatty Liver Disease in Mice. *The Toxicologist. Supplement to Toxicological Sciences* 156(1):359.
2. Lang AL, **Chen L**, Poff GD, and Beier JI (2017) Vinyl chloride inhalation causes mitochondrial dysfunction and exacerbates experimental fatty liver disease in mice. *Hepatology*, 66:415A.
3. **Chen L**, Lang AL, and Beier JI (2017) Vinyl Chloride exacerbates liver injury induced by high-fat diet via causing ALDH2 dysfunction in mice. *Hepatology*, 66:413A.
4. Lang AL, **Chen L**, and Beier JI (2018) Vinyl Chloride Inhalation Causes Hepatic Metabolism Dysfunction in Mice. *The Toxicologist. Supplement to Toxicological Sciences* in press.

5. Lang AL, **Chen L**, Cave MC, and Beier JI (2018) Environmental toxicant vinyl chloride causes hepatic mitochondrial dysfunction in mice. *Hepatology*, 68:429A.

#### **Local/Regional**

1. Lang AL, **Chen L**, Poff GD, and Beier JI (2017) Vinyl Chloride Inhalation Exacerbates Experimental Fatty Liver Disease in Mice. *OVSOT Student Summer Meeting*, Louisville, KY. (Selected for Podium Presentation).
2. **Chen L**, Lang AL, and Beier JI (2017) Vinyl Chloride exacerbates liver injury induced by high-fat diet via causing ALDH2 dysfunction in mice. *OVSOT Student Summer Meeting*, Louisville, KY.
3. Lang AL, **Chen L**, Poff GD, and Beier JI (2017) Vinyl Chloride Inhalation Exacerbates Experimental Fatty Liver Disease in Mice. Research!Louisville, Louisville, KY.
4. **Chen L**, Lang AL, and Beier JI (2017) Vinyl Chloride exacerbates liver injury induced by high-fat diet via causing ALDH2 dysfunction in mice. Research!Louisville, Louisville, KY.
5. Lang AL, **Chen L**, Poff GD, and Beier JI (2017) Vinyl Chloride Inhalation Exacerbates Experimental Fatty Liver Disease in Mice. *OVSOT Student Summer Meeting*, Louisville, KY. (Selected for Podium Presentation).