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The Role of CYP2A5 in Cadmium-Induced Liver Injury

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The Role of CYP2A5 in Cadmium-Induced Liver Injury

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ABSTRACT

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Cadmium is present in food and groundwater. Tobacco smoking and occupational exposure are also major sources for cadmium. Cadmium is primarily accumulated in liver, a major organ metabolizing exogenous chemicals. Chemical metabolism may cause detoxification, but it can also cause bio-activation resulting in liver damage. Cytochrome P450s (CYP) are major liver metabolism enzymes, and cadmium chloride ($CdCl_2$) can induce CYP2A5 in mice. We examined the effect of CYP2A5 on $CdCl_2$-induced liver injury using CYP2A5-knockout (*cyp2a5*$^{-/-}$) mice. The *cyp2a5*$^{-/-}$ mice and their control WT mice were injected $CdCl_2$ intraperitoneally at 5 mg/kg body weight, respectively, to induce liver injury. The control group of *cyp2a5*$^{-/-}$ mice and WT mice were injected saline at the same volume. Twenty-four hours later, all the mice were sacrificed. As indicated by biochemical assays and pathological evaluation, $CdCl_2$-treated WT mice exhibited more severe liver injury than $CdCl_2$-treated *cyp2a5*$^{-/-}$ mice, suggesting that CYP2A5 contributes to Cd-induced liver injury.

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CHAPTER 1

INTRODUCTION

Cadmium is an extremely toxic heavy metal classified as a Group 1 human carcinogen by the International Agency for Research on Cancer (WHO 1997). Cadmium is present in groundwater and soil at low levels, but through bioaccumulation, cadmium levels in tobacco plants, crops and rice may increase dramatically. An average person ingests about 10-40 μg cadmium a day from just food and water, and 1-3 μg remains in the body. A single cigarette contains 1-2 μg of cadmium, and a smoker has double the amount of cadmium retained in the body compared to non-smokers (Cupertino et al. 2013). In addition, Cd can also be obtained from occupational exposure, such as in batteries, paint, and plastic (Wang et al. 2014). Cadmium has a half-life of about thirty years and is not easily eliminated from the body, due to slow excretion (Dumkova et al. 2016). The liver is the primary organ that accumulates cadmium, followed by the kidney. While chronic cadmium exposure affects kidney the most, acute exposure to this heavy metal has the most effect on the liver (Baba et al. 2014). This heavy metal is also known to target other organs such as the testes, bones, and the cardiovascular system (Messner et al. 2016).

Soil heavy metals are positively associated with obesity, diabetes, and fatty liver disease. One mouse study showed that cadmium exposure in water altered liver genes associated with lipid metabolism, cell death and survival regulatory pathways, and mitochondrial oxidative phosphorylation (Go et al. 2015). A cross sectional study in the Taiwanese population showed that soil heavy metal exposure (such as arsenic, cadmium, mercury, and lead) is associated with fatty liver disease (Lin et al. 2017). In a more recent study, the presence of cadmium in maternal blood during pregnancy is associated with increased risk of juvenile obesity in the offspring, and

adiposity is increased in the zebra fish developed from those larvae that were exposed to cadmium (Green et al. 2018). Exposure to cadmium leads to lipid accumulation in the liver, which can eventually lead to the development of non-alcoholic fatty liver disease (Tinkov et al. 2017).

Liver is the main organ involved in metabolizing exogenous chemicals and detoxification. The cytochrome P450 enzymes (CYPs) serve as terminal oxidases in the mixed-function oxidase system for metabolizing various endogenous substrates and xenobiotics, including drugs, toxins, and carcinogens (Lamsa et al. 2010; Pan et al. 2016). CYPs can be found in all biological organisms and are the most essential group involved in Phase I metabolism (Li et al. 2008). All CYP450 enzymes have a heme-binding core (Hasemann et al. 1995). Members of the human CYP enzymes play a vital role in oxidation of a myriad of compounds originating both internally and externally. The enzymes are designated CYP for the superfamily cytochrome P450, followed by a number designating the family of the gene (greater than 40% identity on amino acid sequence level), a capital letter representing the subfamily (greater than 55% identity), and lastly a numeral denoting the individual gene in the subfamily (Lewis et al. 1998). Many different compounds of diverse structures can be metabolized by CYPs. A major function of CYP-catalyzed reactions is to convert a compound into a more polar metabolite through various processes such as oxidation, hydrolysis, and hydroxylation so that they can be easily excreted. However, for some compounds such as carbon tetrachloride or acetaminophen, metabolism by CYP2E1 can give rise to toxic metabolites which damage cells (Lu and Cederbaum 2008).

CYP2A6 enzymes are part of the CYPs that metabolize specific potentially toxic compounds including some drugs, nicotine, coumarin, and procarcinogens e.g. aflatoxin B1 and

nitrosamines (Pelkonen et al. 1997). CYP2A6 can bio-activate tobacco specific carcinogen called Nicotine-derived Nitrosamine Ketone (NNK) and this contributes to tobacco smoke-induced carcinogenesis (Xue et al. 2014). Aflatoxin B1 is metabolized by CYP2A6 to a genotoxic metabolite and a role in hepato-carcinogenesis has been suggested. CYP2A6 also activates N-nitrosamines to genotoxic intermediates (Lin et al. 2016). Additionally, CYP2A6 expression is increased in patients with alcoholic or non-alcoholic fatty liver. CYP2A5 is the mouse orthologue of human CYP2A6 (Abu-Bakar et al. 2012). Mouse CYPA5 and human CYP2A6 have an 86% amino acid sequence similarity (Honkakoski and Negishi 1997). Several studies have suggested that CYP2A5 is a useful indicator for carcinogenesis in the liver (Lamsa et al. 2010). It was reported that an increased expression of this enzyme was observed in bacterial, viral, and parasitic hepatitis (Kirby et al. 1994) and a more recent study also suggested that CYP2A5/A6 acts as catalysts in oxidizing the compound bilirubin, therefore suggesting that these enzymes have a role in heme homeostasis in cells (Abu-Bakar et al. 2011). In relation to this, Heme Oxygenase-1 (HO-1), a protein that can also be induced by oxidative stress, metabolizes heme to biliverdin (Emerson and Levine 2008). HO-1 breaks down heme into products such as biliverdin and ferrous iron. Biliverdin would then be converted to bilirubin, a powerful antioxidant. However, excess production of heme, and therefore excess formation of bilirubin in the body can potentially be cytotoxic (Stocker et al. 1987).

Alcohol feeding induced CYP2A5 in mice, and alcohol induced fatty-liver disease was enhanced in CYP2A5 knockout (*cyp2a5*$^{-/-}$) mice suggesting that CYP2A5 protects against alcoholic fatty liver disease (Lu et al. 2012). CYP2A5 is also suggested to work in regulation of lipid metabolism and that CYP2A5 protects against high fat diet-induced metabolic syndrome since obesity and hepatic steatosis was more severe in *cyp2a5*$^{-/-}$ mice than their wildtype

counterpart (Wang et al. 2018). A study by Hong et al. 2015, also showed that CYP2A5 prevented liver injury and fibrosis when mice were injected with thioacetamide, an organosulfur hepatotoxicant.

In both the mice and humans, CYP2A5/A6 enzymes are distinctive among the other members of the CYP enzymes as both are stimulated by varied, unrelated substrates (Abu-Bakar et al. 2012). Among the conditions that induce CYP2A5 expression, the most common factor present is oxidative stress i.e. generating reactive oxygen species (ROS), one of the fundamental processes for chemical toxicity, chronic inflammation and carcinogenesis (Abu-Bakar et al. 2012). Oxidative stress pertains to the disproportion resulting from excess ROS or oxidants produced over the cell's capacity to initiate an antioxidant response to counteract the ROS. ROS can originate exogenously or endogenously. Externally, it can be acquired from environmental pollutants, drugs, and radiation. However, the majority of ROS in living organisms originates intracellularly during mitochondrial oxidative metabolism from the electron transport chain, as well as in cellular reactions to foreign substances, cytokines, bacterial invasion, as well as in photosynthetic processes (Nemmiche et al. 2007; Ray et al. 2012). ROS unselectively oxidizes all molecules in living organisms and can induce cell and tissue damage through several ways such as through lipid peroxidation, triggering DNA strand breaks, and inducing cell death through necrotic and apoptotic mechanisms (Mccord 2000; Li et al. 2015).

Oxidative stress is one of the main mechanisms responsible for cadmium-induced toxicity to tissues and organs (Nemmiche 2017). It has been associated with cadmium-related development of immunotoxicity, nephrotoxicity, and carcinogenesis (Waisberg et al. 2003). Interestingly, the element cadmium itself is not capable of generating ROS directly, as it is not capable of direct production of free radicals (Nemmiche 2017). Free radicals are molecules that

consist of extremely reactive and unstable unpaired electrons (Finkel and Holbrook 2000). Elemental cadmium only has one oxidation state; thus, it is incapable of generating free radicals. However, it can indirectly promote the generation of ROS such as superoxide radicals, hydroxyl anions and hydrogen peroxide (Nemmiche 2017).

Cadmium causes liver damage (Harstad and Klaassen 2002; Fouad et al. 2009) and some studies showed that cadmium can induce CYP2A5 (Abu-Bakar et al. 2004; Lu et al. 2011). This study aims to investigate if CYP2A5 has an effect on cadmium-induced liver injury by using *cyp2a5*$^{-/-}$ and their WT mice. CYP2A6 in humans and CYP2A5 in mice are mainly expressed in liver, making them a good animal model for studying biological function of CYP2A6.

CHAPTER 2

METHODS

Animals and Treatment

The C57BL/6 background CYP2A5 knock-out (*cyp2a5*$^{-/-}$) mouse colony was created by mating female C57BL/6 wild type (WT) mice (Charles River Laboratory) with male C57BL/6 *cyp2a5*$^{-/-}$ mice (generously provided by Dr. Xinxin Ding). All the mice were created at Icahn School of Medicine at Mount Sinai (New York, NY) and were transferred to East Tennessee State University and housed in the Animal Facility in Brown Hall. The animal rooms are temperature-controlled with 12-hour light/dark cycles. The mice received humane care and all experiments performed were according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals as well as the protocol approved by Mount Sinai and ETSU's Division of Laboratory Animal Resources.

Male *cyp2a5*$^{-/-}$ mice and WT mice (8-10 weeks) were administered cadmium chloride ($CdCl_2$) at 5 mg/kg body weight intraperitoneally (i.p.). The control mice were injected i.p. with the same volume of saline. All mice had access to their water and food *ad libitum*. For the time-course study, wherein cadmium activity was observed at specific time points, the mice were divided into four groups of three mice each: 1 hour after injection, 3 mice were sacrificed. Another 3 were sacrificed 4 hours after injection, and 3 were sacrificed 8 hours after injection. For the role of CYP2A5 in Cd-induced liver injury study, 16 mice were divided into 4 groups of 4 mice each: WT control, *cyp2a5*$^{-/-}$ control, *cyp2a5*$^{-/-}$ $CdCl_2$, and WT $CdCl_2$. 24 hours after injection, the mice were sacrificed by decapitation.

Mice were weighed before sacrificing. Blood was collected, and serum was separated to be used for biochemical assays. Whole liver was collected from each of the mice and were rapidly excised into fragments and washed with precooled saline. Liver sections collected from the same lobe from each mouse and were fixed in 10% formalin solution and sent to ETSU's Pathology Department for paraffin embedding as follows:

1. The tissue was rinsed in running water for 30-60 minutes.
2. Put in 70% Alcohol for 20 minutes
3. 85% Alcohol for 20 minutes
4. 95% Alcohol for 20 minutes
5. First absolute Alcohol for 15 minutes
6. Second absolute Alcohol for 15 minutes
7. Xylene-I as a clearing agent for 15 minutes
8. Xylene-II for 15 minutes
9. 60°C Paraffin-I for 15 minutes
10. Paraffin-II for 15 min
11. Paraffin-III for 15 min
12. Embedding in cassettes

Other liver tissue aliquots were stored at -80°C for further analyses.

Homogenate

Liver homogenates were prepared in ice-cold 0.15M potassium chloride (KCl).

Liver histology and immunohistochemistry

H&E staining and Immunohistochemical (IHC) staining

Paraffin liver blocks were cut into five-micrometer sections for haematoxylin and eosin (H&E) staining or IHC staining.

IHC staining for 3-Nitrotyrosine was performed by using mouse monoclonal anti-3-NT antibody (Santa Cruz cat. # sc-101358, with a dilution of 1:200) followed by IHC Select Chemicon Histostain-Plus Kit (EMD Millipore cat. # DAB150). Single antibody staining procedure was done.

Biochemical assays

Serum alanine aminotransferase (ALT) and Serum aspartate transaminase (AST) were assayed using a MaxDiscovery ALT/AST Color Endpoint Assay Kit (Purchased from Bioo Scientific Corp., cat. # ALT: 3460-08, AST: 5605-01) following manufacturer's instructions. The serum was also used to test for hepatic thiobarbituric acid reactive substances (TBARS) as a marker for lipid peroxidation. Hepatic homogenates were incubated with 0.2 ml of TBARS reactive solution containing 15% (wt/vol) trichloroacetic acid (TCA), 0.375% (wt/vol) thiobarbituric acid (TBA) in 0.25 N HCl for 10 minutes in a boiling water bath. Followed by centrifugation at 200 rcf for 5 minutes. The resulting supernatant was used to determine the formation of TBARS by evaluating the absorbance at 535 nm. Tetramethoxypropane served as a standard.

Western Blot Analysis

Hepatic protein extracts from liver homogenates were subjected to protein assay to determine protein concentrations using a Pierce BCA Protein Assay Kit (cat. # 23225). After protein quantification, 20 μL per sample were separated by 10% SDS- polyacrylamide gel electrophoresis with PageRuler ™ Prestained Protein Ladder used as protein ladder (Thermo Fisher

Scientific, cat. # 26616). The gel was then transferred to a 0.45μM nitrocellulose membrane (Bio-Rad, cat. # 1620115). Membranes were blocked for 1 hour with 2% fat-free milk then incubated overnight with chicken monoclonal anti-CYP2A5 (dilution of 1:5000, gift from Dr. Risto Juvonen, Department of Pharmacology and Toxicology, University of Kuopio, Kupio, Finland) and rabbit polyclonal anti-HO-1 (dilution of 1:1000, Santa Cruz Biotechnology) primary antibodies. For testing acute effects of Cd at specific time points, Calnexin or β-actin were used as a protein loading control to look at the role of CYP2A5 in Cd-induced liver injury. Afterwards, membranes were incubated with respective peroxidase secondary antibodies (Millipore) for 1 hour. Chemiluminescent signals were detected by adding ECL Prime Detection chemiluminescent reagent (GE Healthcare, cat. # RPN2232). The bands of proteins were quantified with the Automated Digitizing System (ImageJ gel programs, version 1.34S; National Institutes of Health, Bethesda, MD).

Statistical Analysis

Results are expressed as mean ± standard deviation. Statistical evaluation was carried out by using one-way analysis of variance (ANOVA) followed by Student-Neuman-Keuls post hoc test. $P < 0.05$ was considered as statistically significant.

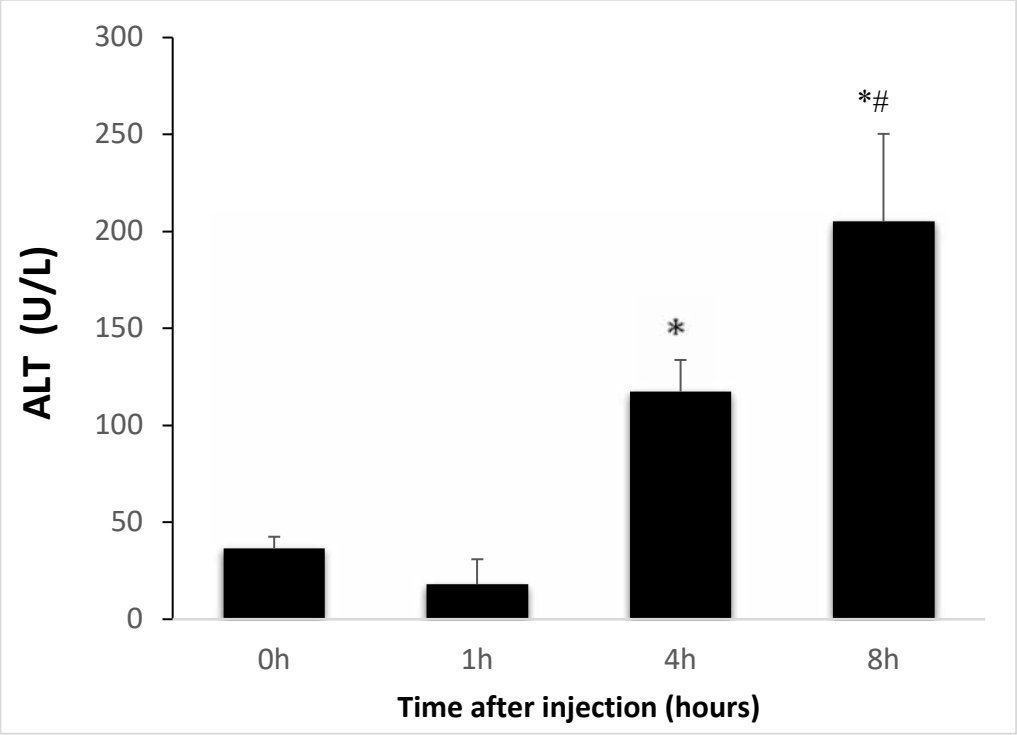
CHAPTER 3

RESULTS

Time-specific Increase in Liver Injury Markers Upon $CdCl_2$ Administration

Alanine aminotransferase (ALT) and aspartate amino transferase (AST) are known markers for liver injury. When the liver is damaged, these enzymes, which are usually housed in liver cells, spill into the blood stream (Huang et al. 2006). Serum ALT and AST were detected from blood of mice at time 0, 1, 4, and 8 hours after $CdCl_2$ administration. The serum levels of ALT significantly increased after 4 hours of $CdCl_2$ injection and further increased after 8 hours (Fig. 1A). The initial decrease after 1 hour of injection cannot be explained. However, the serum levels of AST significantly increased after 4 and 8 hours of $CdCl_2$ injection (Fig. 1B). These results suggest that liver injury occurs after 4 hours of CdCl2 administration.

A)



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B)

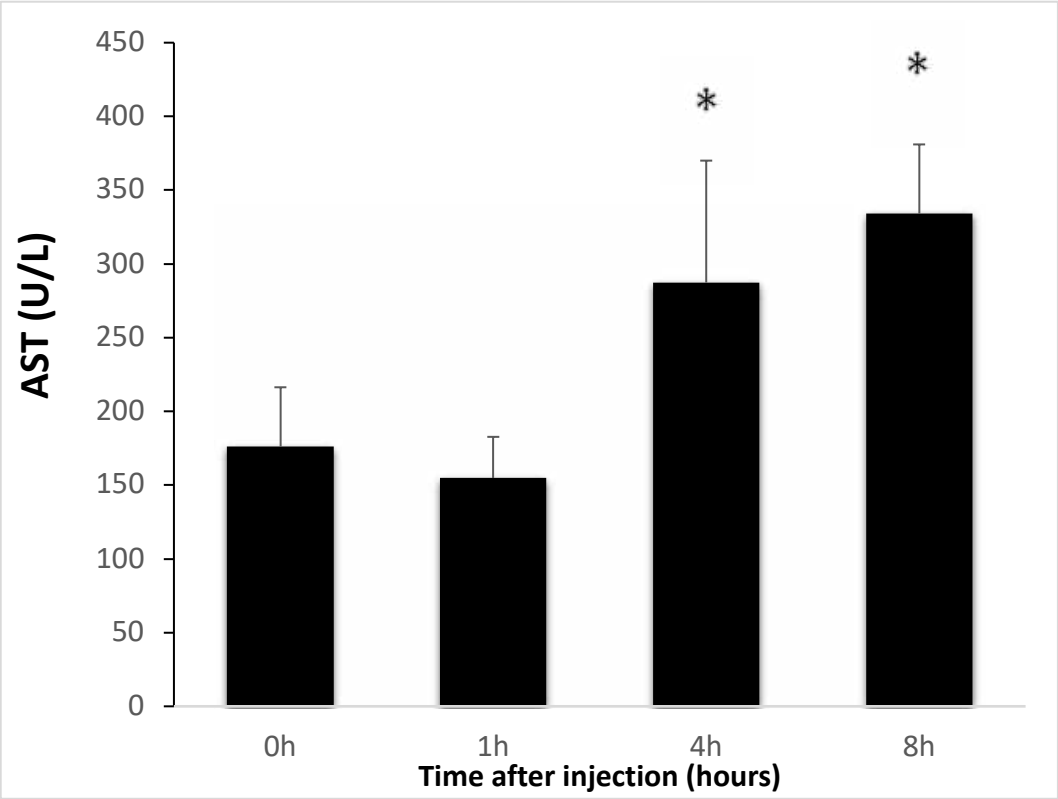
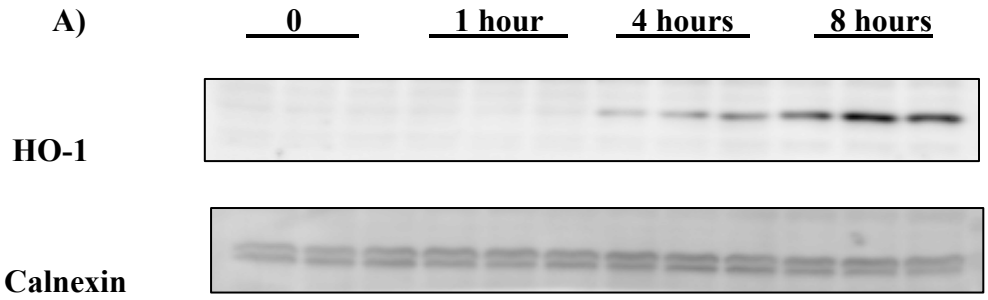


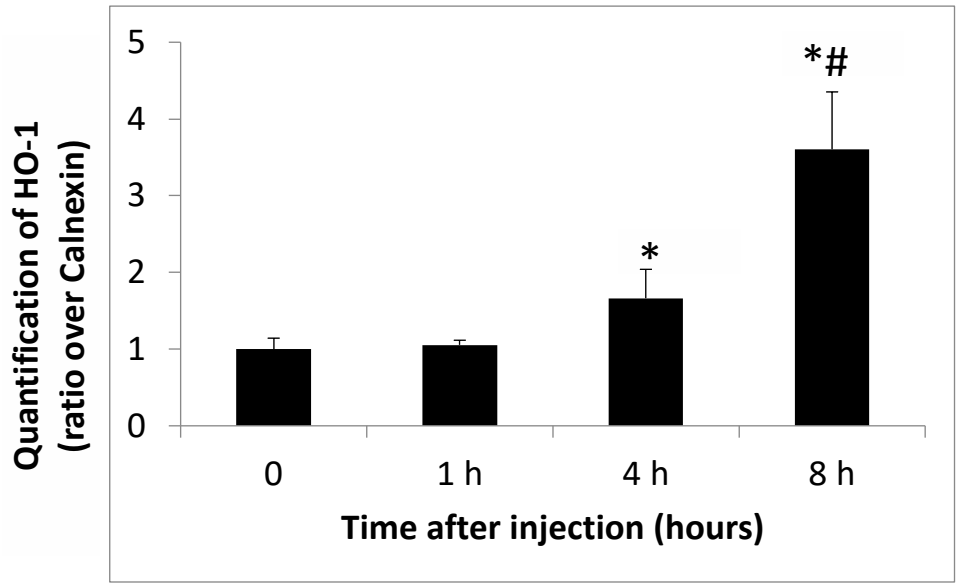
Figure 1. — *Liver injury increases in a time specific manner after $CdCl_2$ administration.* (A)Serum Alanine transaminase; (B) Serum Aspartate Transaminase. Data is represented by mean ± S.D. *P < 0.05 compared to 0h time point; # P<0.05, compared with 4h.

Expression of antioxidant enzyme HO-1 upon $CdCl_2$ administration

Western blot analysis was used to determine liver HO-1 and CYP2A5 protein levels in mice injected with $CdCl_2$ and sacrificed 1, 4, or 8 hours after administration. Calnexin was used as loading control.

HO-1 was undetected at time 0, and 1 hour but significantly increased after 4 hours and further increased after 8 hours (Fig. 2A). CYP2A5 levels did not have significant changes from time 0-8 hours (Fig. 2B).





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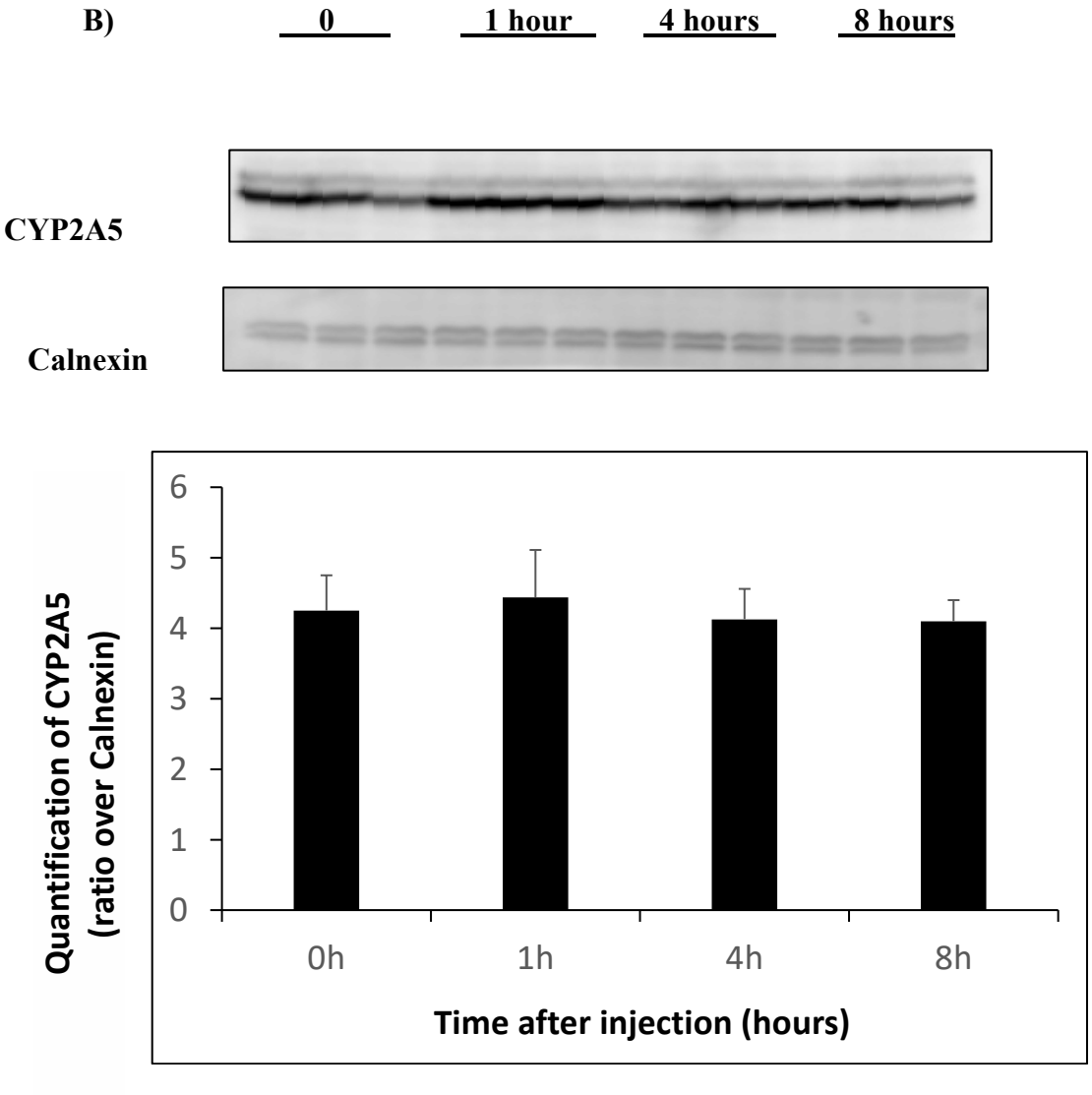


Figure 2. — *Liver HO-1 and CYP2A5 content*. Western Blot analysis and quantification of liver HO-1 (A) and CYP2A5 (B). *P < 0.05 compared to previous time point. # P<0.05, compared with 4h. Data is represented by mean ± S.D, N=3.

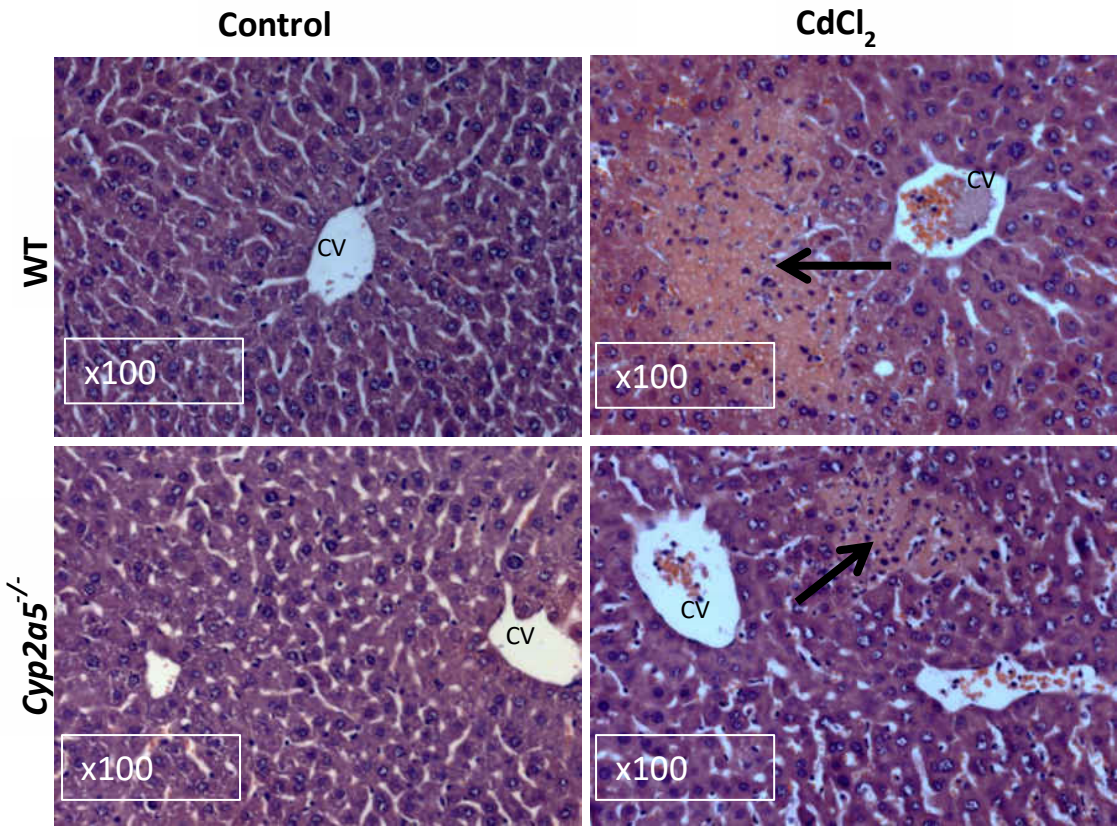
Liver Injury Induced by $CdCl_2$ in WT Mice Compared to *cyp2a5*$^{-/-}$

Exposure of Cd may cause the simultaneous activation of different cell death signals. These signals, such as apoptosis and autophagy, all culminate in necrosis (Messner et al. 2016).

After 24 hours of $CdCl_2$ exposure, hepatic necrosis was observed in the liver sections of both WT

and *cyp2a5*$^{-/-}$ exposed to $CdCl_2$. Moreover, the WT group exhibited more severe necrotic injury compared to the *cyp2a5*$^{-/-}$ mice (Fig. 3A). The serum levels of ALT and AST, indicators of liver injury, were significantly elevated in the WT mice exposed to $CdCl_2$ compared to the *cyp2a5*$^{-/-}$ counterpart (Fig. 3B).

A)



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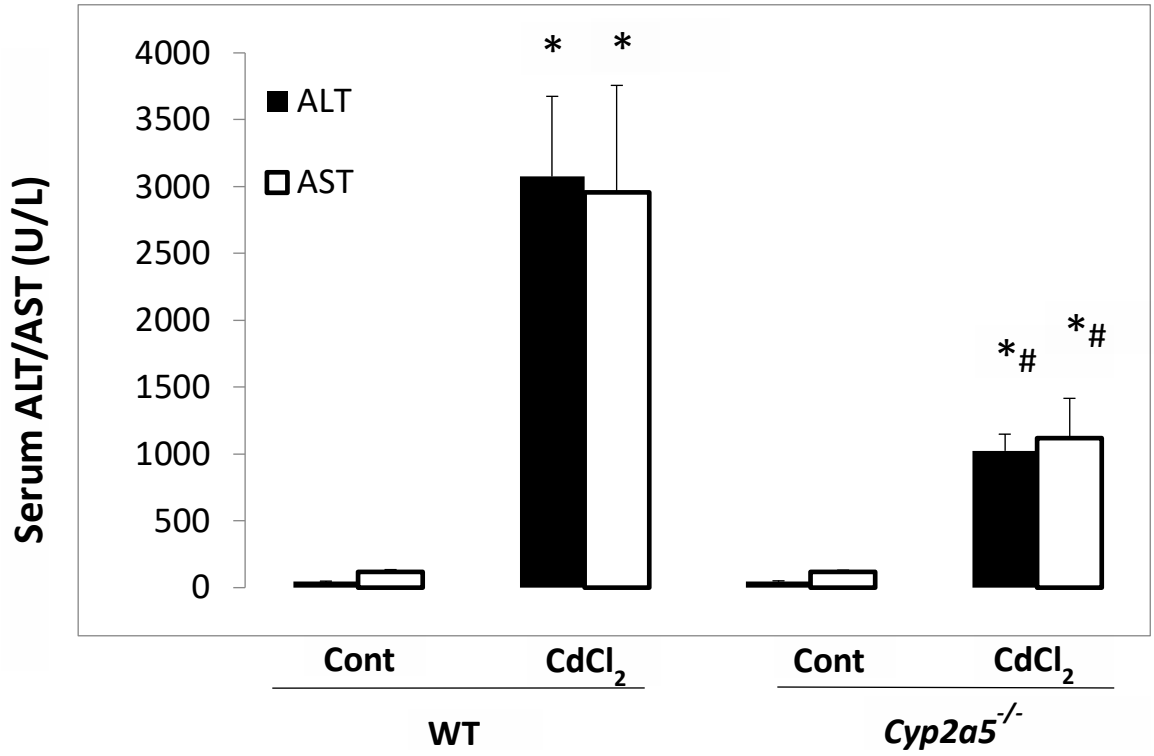


Figure 3. — *Liver injury is minor in cyp2a5-/- compared to WT*. H&E staining of liver sections at 100x. Arrows point to necrotic area (A). The serum levels of ALT and AST (B) in $CdCl_2$-exposed WT were much higher than *cyp2a5*$^{-/-}$ exposed to $CdCl_2$. *P < 0.05 compared to Control group and #P<0.05 compared to WT $CdCl_2$ group.

Expression of antioxidant enzyme HO-1 upon $CdCl_2$ administration in *CYP2A5-/-* vs.WT

Western Blot analysis was used to determine HO-1 and CYP2A5 protein levels in mice sacrificed 24 hours after injection of $CdCl_2$. β-actin was used as loading control (Fig. 4A). HO-1 was significantly increased in both groups exposed to $CdCl_2$ and was upregulated in the WT group exposed to $CdCl_2$, although not significantly different. There was no significant difference in the CYP2A5 levels between WT exposed to $CdCl_2$ and control (Fig. 4B).

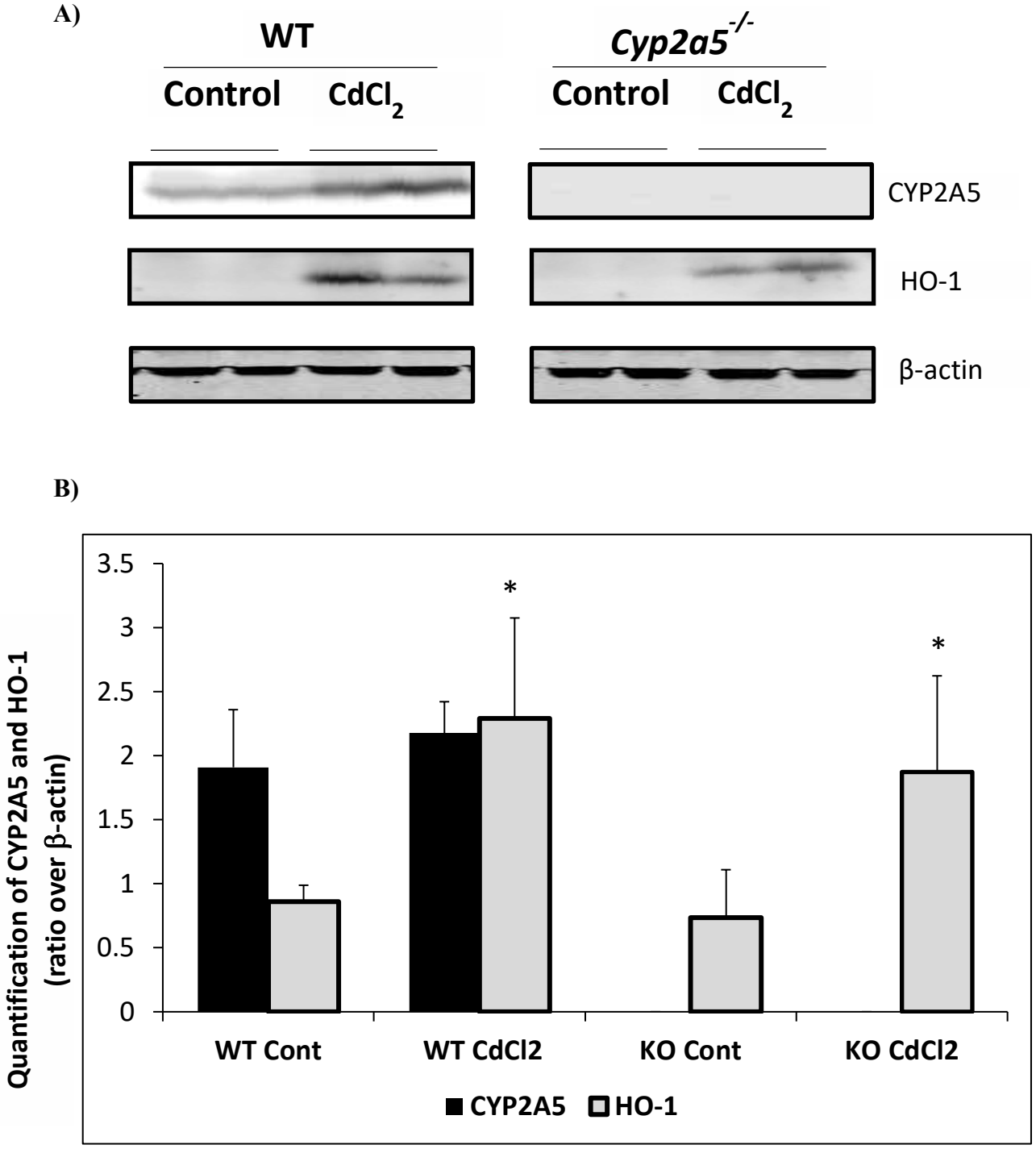
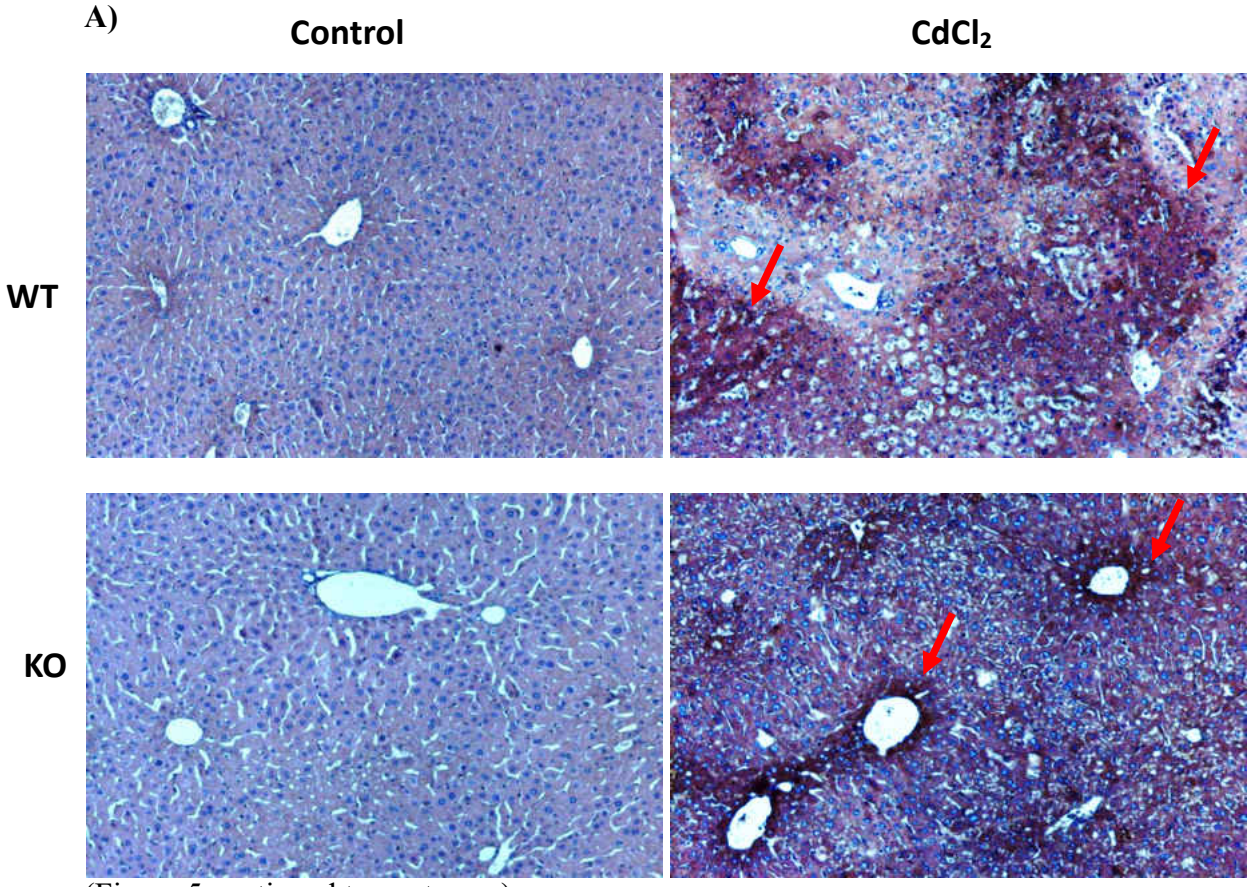


Figure 4. — *Western Blot analysis of liver HO-1 and CYP2A5 in WT and cyp2a5$^{-/-}$ mice*. (A) Representative bands of HO-1 and CYP2A5. (B) Quantification of HO-1 and CYP2A5. * P<0.05, compared with Cont.

Markers of ROS

Immunohistochemical staining was used to detect 3-Nitrotyrosine (3-NT), a marker of oxidative stress, in liver sections of both WT and *cyp2a5*$^{-/-}$ exposed to $CdCl_2$. Mouse monoclonal anti-3-NT antibody was used to detect the presence of 3-Nitrotyrosine. Although both WT and *cyp2a5*$^{-/-}$ groups exposed to $CdCl_2$ showed an increase in 3-NT as exhibited by the darker coloration after staining, the level was higher in the *cyp2a5*$^{-/-}$ compared to the WT (Fig. 5A). Furthermore, the assay on Thiobarbicturic Acid Reactive Substances (TBARS), a byproduct of lipid peroxidation, was also evaluated in WT and *cyp2a5*$^{-/-}$ mice exposed to $CdCl_2$. The TBARS levels in *cyp2a5*$^{-/-}$, were significantly higher in both control and $CdCl_2$ group (Fig. 5B). These results suggest that the presence of CYP2A5 can inhibit oxidative stress.



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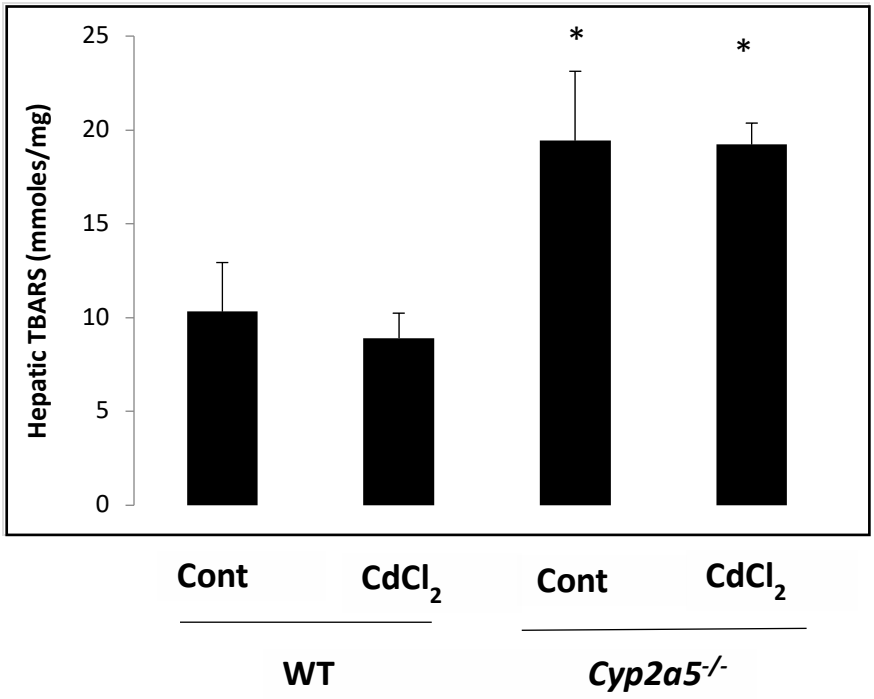

Figure 5. — *Markers of Oxidative Stress between WT and cyp2a5$^{-/-}$.* Liver oxidative stress is more severe in *cyp2a5$^{-/-}$* mice than WT mice as indicated by the red arrows in IHC staining of 3-NT in liver sections (A) and hepatic formation of TBARS (B). *P< 0.05 compared with WT.

CHAPTER 4

DISCUSSION

As mentioned earlier, CYP2A5 is unique in that this enzyme is induced by various chemicals. It is also distinctive compared to other members of the CYPs because it is both protective when it comes to ethanol-induced liver injury (Hong et al. 2015), thioacetamide-induced liver damage (Hong et al. 2016), and high fat diet-induced metabolic syndrome (Wang et al. 2018), and detrimental because this enzyme can also activate certain substances to be carcinogenic, such as aflatoxin B1 and nitrosamines (Lin et al. 2016).

It has long been known that cadmium is an extremely toxic environmental and industrial chemical (Wang et al. 2014). It was also proven to produce oxidative stress and immunotoxicity in cells under diverse experimental conditions (Liu et al. 2015). As mentioned earlier, Cd is capable of indirectly producing ROS and although the body is capable of getting rid of ROS derived from this heavy metal up to a certain degree, this still interrupts signaling processes in the body resulting in signaling dysfunction (Mitler 2002; Matovic et al. 2015).

In the present study, we assessed how cadmium administration would affect specific markers of liver injury. We also examined whether CYP2A5 will have a role in the liver injury induced by this heavy metal. We hypothesized that as time increases, damage resulting from cadmium exposure worsens, even resulting in lipid accumulation in the liver. However, the presence of the enzyme CYP2A5 will protect against liver injury induced by this heavy metal. Recently it was found that acute exposure to a low dose of cadmium increases lipid content in hepatocytes isolated from cholesterol-fed mice by affecting the autophagy process (Rosales-Cruz et al. 2018). The *in vivo* study showed that mice fed with 10mg/L of cadmium in their drinking water for 20 weeks developed fatty liver disease. The altered genes and liver metabolites related

to lipid metabolism resulted in high cholesterol and triglycerides therefore caused these mice to accumulate fat in their liver (Go et al. 2015).

We found that upon $CdCl_2$ injection of 5 mg/kg body weight intraperitoneally, liver damage starts as soon as 4 hours after exposure as shown by hallmarks of liver damage such as ALT and AST. Both enzymes had a significant increase in serum 4 hours after injection and 8 hours after injection. There was no increase in the CYP2A5 enzyme level in between time points as indicated by the western blot, however, the enzyme Heme Oxygenase-1 (HO-1), was significantly upregulated 4 and 8 hours after $CdCl_2$ administration, suggesting that $CdCl_2$ can induce this enzyme. As mentioned in the introduction, HO-1 is the enzyme in-charge of the degradation of heme, therefore making it unavailable for CYP450 protein to incorporate heme in their structure. This would then lead to the decrease of the CYP450 enzymes affecting CYP 450-catalyzed activity (Chung et al. 2009; Karuzina and Archakov 1994). Activation of the HO-1 gene is regulated by a transcription factor called Nuclear erythroid-related factor 2 (Nrf2) and Cadmium can impede the disintegration of this transcription factor, therefore, leading to upregulation of HO-1 (Abu-Bakar 2004).

With the WT showing more severe liver injury than the knockouts, it seems that CYP2A5 metabolizes $CdCl_2$ to a more toxic metabolite, therefore promoting $CdCl_2$-induced liver injury. Additionally, no abnormal lipid accumulation was witnessed in both groups exposed to $CdCl_2$, instead pathological evaluation showed necrosis. Sub-micromolar quantities of this heavy metal might actually lead to delayed apoptosis, while high concentrations can cause necrosis (Luevano and Damodaran 2014). Unlike apoptosis and autophagy, necrosis is unregulated. But the obstruction of certain proteins involved in regulation of apoptosis or autophagy can change the type of cell death to necrosis (Golstein and Kroemer 2007). Although the wildtype exposed to

$CdCl_2$ showed more liver damage as shown by the transaminases and by H&E staining, lipid peroxidation exhibited by TBARS assay and 3-NT IHC staining, was greater in CYP2A5 knock-out, suggesting that the presence of CYP2A5 is protective against oxidative degradation of lipids.

$CdCl_2$ exposure is able to induce oxidative stress. A study by Wang et al. 2004, suggested that this heavy metal affects the electron transport chain of cellular respiration in the cell's mitochondria. This heavy metal possibly binds between semi-ubiquinone and cytochrome b566 of cytochrome b in Complex III, therefore resulting in buildup of semi-ubiquinone at this site. Semi-ubiquinones are radicals, making them highly reactive (Quinlan et al. 2012). The semi-ubiquinone will then have an affinity to transfer one electron to molecular oxygen, forming a superoxide. This is a possible mechanism to how Cd can produce ROS in the mitochondria. However, the process of how CYP2A5 interacts with this heavy metal, as well as the role of HO-1 have to be further investigated. As this thesis project was an acute study, it will also be interesting to study both enzymes under chronic exposure.

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