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The Role of Nrf2 in the Differential Susceptibility of Male and Female Mice to Acetaminophen-Induced Hepatotoxicity

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**The Role of Nrf2 in the Differential Susceptibility of Male and Female Mice to
Acetaminophen-Induced Hepatotoxicity**

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B.S. University of Pittsburgh, 2006

A Thesis

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Master's of Science Thesis

**The Role of Nrf2 in the Differential Susceptibility of Male and Female Mice to
Acetaminophen-Induced Hepatotoxicity**

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Chapter One

Literature Review

Liver Function and Structure

The function and structure of the liver are well understood. The following information on the function and structure of the liver is adapted from *Casarett and Doull's Toxicology: The Basic Science of Poisons* (Klaassen, 2008), and *Wheater's Functional Histology: A Text and Colour Atlas* (Young, 2006).

Liver Function: The liver resides within the upper abdominal quadrant between the intestinal tract and the rest of the body. It is responsible for numerous functions including metabolism, synthesis and secretion of bile and cholesterol, synthesis of plasma proteins, and detoxification and excretion into bile of endogenous waste products and xenobiotics. The liver is one of the first organs to come into direct contact with orally ingested nutrients, xenobiotics, and toxicants via blood entering the organ through the portal vein from the intestine, and is therefore, critically important in xenobiotic transformation, converting hydrophobic substances into water-soluble derivatives that can be excreted into bile and urine.

Liver Structure: The liver represents approximately 2.5% of total body weight and contains various cell types including hepatocytes (parenchymal cells), sinusoidal lining cells (Kupffer, Ito, endothelial), hematopoietic cells, nerve cells, lymphatic cells, and blood vessel cells.

Hepatocytes represent 60-80% of the entire cell population within the liver. These cells are large polyhedral cells with round nuclei with peripherally dispersed chromatin. The nuclei can vary greatly in size and binucleated hepatocytes are common in normal liver.

Endothelial lining cells are readily distinguishable from hepatocytes by their flattened condensed nuclei. These cells lack a basement membrane and allow for the exchange of fluids and nutrients between hepatocytes and the sinusoid. Kupffer cells are resident macrophages of the liver that phagocytize particles, secrete mediators of inflammation, detoxify endotoxins, process antigens, and catabolize lipids and glycoproteins. Finally, Ito cells are fat-storing cells that serve as a major storage site for vitamin A.

The hepatic lobule is the structural unit of the liver, defined according to structural and functional differences. The hepatic lobule is hexagonal in shape and is centered on a terminal hepatic venule or centrilobular venule. Portal tracts (triads) are positioned at the angles of the hexagon. The portal triads contain branches for the portal vein, hepatic arteriole, and bile duct. Blood flows away from the branches of the portal vein and hepatic arteriole toward the adjacent central veins.

The hepatic lobule is divided into three zones. Zone 1 is the periportal zone. This zone is closest to the portal tract and because hepatocytes in this zone are closest to afferent vessels, this zone receives the most oxygenated blood. Zone 2 is the midzonal region. Zone 3 is the centrilobular zone and has low levels of oxygenated blood. Gradients of proteins involved in bioactivation, detoxification, and transportation of xenobiotics have been observed along these three zones. For example, levels of glutathione (GSH), fatty acid oxidation, and gluconeogenesis are highest in zone 1, but cytochrome P450, essential for drug metabolism, enzyme levels are highest around the centrilobular venule.

Nrf2 Signaling Pathway

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that positively regulates the expression and activity of cytoprotective genes during periods of oxidative stress. Nrf2

was originally identified during a screening process for proteins that bind to the locus control region (LCR) of the β -globin gene cluster (Moi, 1994). The β -globin gene cluster consists of five genes on chromosome 11 which are responsible for the creation of the beta components of hemoglobin. Within the LCR, there are four DNase I hypersensitive sites that serve as binding sites for the transcription factors AP1 (activation protein 1), GATA-1 (an erythroid specific transcription factor), and NF-E2 (nuclear factor erythroid 2). It was during this screening process that a binding protein, given the name Nrf2, was identified and was shown to exhibit remarkable similarity to NF-E2.

Nrf2 was found to bind the same hypersensitive sites as NF-E2 within the LCR of the β -globin gene cluster. Furthermore, both are RNA polymerase activators and belong to the cap 'n' collar family of transcription factors, members of which share a conserved basic leucine zipper structure (Moi, 1994). However, unlike NF-E2 which is predominately expressed in erythroid cells, Nrf2 was found to be ubiquitously expressed in all cell types, most predominantly in the kidney, muscle, lung, heart, liver, and brain (Andrews, 1993, Chan, 1996, Itoh, 1995, McMahon. 2001, Moi, 1994).

Negative Regulation of Nrf2

Under basal conditions, Kelch-like ECH-associated protein 1 (Keap1) is a negative regulator of Nrf2, responsible for the binding and inactivation of Nrf2 in the cytoplasm (Itoh, 1999, Kang, 2004). The double glycine repeat (DGR) region of Keap1 binds the Nrf2-ECH homology 2 (Neh2) domain in the N-terminus region of Nrf2, thereby sequestering Nrf2 in the cytosol and repressing its activity (Itoh, 1999). The BTB (broad complex, tramtrack, bric-a-brac) domain of Keap1 binds Cullin-3 of the Cullin3/Ring Box 1 (Cul3/Rbx1) E3 ubiquitin ligase complex which is responsible for the recruitment of ubiquitin-conjugating enzymes that in turn, label Nrf2 for proteosomal degradation. Consequently, under normal cellular conditions, the cytosolic Keap1/Cul3-Rbx1

complex is constantly degrading Nrf2. However, in the presence of oxidative stress, Nrf2 dissociates from Keap1 and translocates into the nucleus of the cell leading to the activation of antioxidant response element (ARE)-mediated gene transcription.

A number of plausible mechanisms for the dissociation of Nrf2 from Keap1 have been suggested. Phosphorylation of a serine residue at position 40 on Nrf2 in response to electrophilic or oxidative stress has been shown to result in the release of Nrf2 from Keap1 (Bloom, 2003). There are also three cysteine residues on Keap1 crucial for its activity including Cys151, Cys273, and Cys288. Research has shown that modification of Cys151 leads to a change in the conformation of the BTB domain of Keap1, causing a switch of ubiquitination of Nrf2 to Keap1, which is thought to lead to Nrf2 nuclear accumulation (Zhang, 2003). Mutations of a serine residue at position 104 on Keap1 have also been shown to disrupt Keap1 dimerization, leading to the release of Nrf2 (Zipper, 2002). Furthermore, de-phosphorylation of a tyrosine residue at position 141 on Keap1 causes destabilization and degradation of the cytosolic protein leading to the release of Nrf2 (Jain, 2008). It remains uncertain, however, whether the above mechanisms work in concert with one another or are independent in their ability to cause the dissociation of Nrf2 from Keap1.

Nrf2-Mediated Transcription

Once inside the nucleus, Nrf2 heterodimerizes with transcriptional regulatory proteins such as Maf or Jun proteins (Aleksunes, 2007). These Nrf2 partners are often members of the activator protein-1 (AP-1) family of proteins (Itoh, 1997, Wild, 1999). These heterodimeric complexes are then able to bind ARE sequences in the promoter regions of genes involved in cytoprotection. The functional ARE sequence contains a common core 5'-GTGACnnnGC-3' motif, where 'n' represents any nucleotide (Rushmore, 1991). Jun, for example, has been shown to interact with Nrf2 and

overexpression of both Jun and Nrf2 in hepatoma cells activates ARE-mediated transcription (Venugopal, 1998).

Nrf2-Responsive Genes

ARE-containing genes identified as targets of Nrf2 are involved in a variety of cellular functions including xenobiotic metabolism, reactive oxygen species scavenging, GSH homeostasis, and xenobiotic efflux transport pathways (Nguyen, 2003). These genes include, among others, NAD(P)H quinone oxidoreductase 1 (*Nqo1*), heme oxygenase 1 (*Ho-1*), multidrug resistance-associated protein transporters (*Mrp1-4*), glutamate cysteine ligase catalytic subunit (*Gclc*), and glutamate cysteine ligase modifier subunit (*Gclm*) (Aleksunes, 2007). An impaired expression of these genes can increase the sensitivity of cells to oxidative damage.

NAD(P)H Quinone Oxidoreductase 1: NAD(P)H quinone oxidoreductase 1 (*Nqo1*) is a detoxification enzyme that reduces reactive quinones and quinone-imines to less reactive and less toxic hydroquinones (Siegel, 2004). Early studies identified an ARE sequence in the mouse and rat *Nqo1* promoter region (Rushmore, 1991). Additionally, chemical treatment with the Nrf2 activator butylated hydroxyanisole (BHA) increases hepatic and intestinal Nqo1 expression levels in wild-type, but not Nrf2-null, mice (Ishii, 2002), confirming a regulatory role for Nrf2 in the expression of Nqo1.

Heme Oxygenase-1: Heme oxygenase-1 (*Ho-1*) catabolizes prooxidant heme to carbon monoxide, biliverdin, and free iron. Its name is synonymous with heat shock protein 32. Ho-1 is a hallmark target of Nrf2 (Alam, 1999) and is commonly up-regulated following oxidative stress and cellular injury (Guo, 2001). Unlike Nqo1, traditionally considered a drug metabolizing enzyme, Ho-1 is more often thought of as a stress-inducible gene.

Multidrug Resistance-Associated Protein Transporters: In addition to the detoxification of chemicals as a means for ameliorating cellular injury, plasma membrane efflux transport proteins can eliminate chemicals from within the cell, thereby minimizing cellular stress and injury. Literature suggests that ATP-dependent efflux transporters including multidrug resistance-associated proteins (Mrps) may be involved in the antioxidant response (Klaassen, 2005). Mrps belong to the ATP-binding cassette, subfamily c (Abcc). ARE sequences have been identified in the mouse *Mrp1* (Hayashi, 2003) and *Mrp2* (Vollrath, 2006) promoters. Furthermore, treatment of mice with Nrf2 activating chemicals shows induction of Mrps in wild-type mice (Maher, 2005, Aleksunes, 2008), but not in Nrf2-null mice (Aleksunes, 2008), confirming the role of these transporter proteins in the Nrf2 response pathway.

Glutamate Cysteine Ligase: Glutamate cysteine ligase (Gcl), also known as γ -glutamyl cysteine synthetase, catalyzes the formation of γ -glutamylcysteine, the rate-limiting step in GSH synthesis, from glutamine and cysteine in the presence of ATP. Glutathione (GSH) plays an important role in intracellular redox balance and protecting against cellular oxidative stress through the detoxification of chemicals by directly binding or enzymatic conjugation. Gcl is comprised of a catalytic (Gclc) subunit and modifier (Gclm) subunit, both of which contain ARE sequences in their promoter regions (Seelig, 1984, Mulcahy, 1997). The catalytic subunit composes the heavy chain portion of Gcl while the modifier subunit is known to increase the efficiency of the catalytic subunit. Livers from Nrf2-null mice show diminished gene expression of both *Gclc* and *Gclm* as well as lower GSH stores when compared to wild-type counterparts (Chan, 2000, Chanas, 2002). The regulation of Gclc and Gclm by Nrf2 highlights the critical role this nuclear transcription factor undertakes in maintaining cellular GSH homeostasis.

Acetaminophen (APAP) Hepatotoxicity

Incidence: Acetaminophen (APAP) is a commonly used analgesic and antipyretic. APAP is also one of the leading causes of acute liver failure (ALF) in the United States (Reuben, 2010). Since the early 2000's, APAP has been attributed to approximately 50% of all acute liver failure cases across the United States (Reuben, 2010). To address this concern, in January 2011, the United States Food and Drug Administration recommended to manufacturers of prescription combination drug products containing acetaminophen to limit the amount of acetaminophen to no more than 325 milligrams per tablet or capsule (U.S. Food and Drug Administration, 2011).

Currently, *N*-Acetyl cysteine (NAC), a precursor to GSH, is the only standard therapy used to prevent and decrease APAP-induced hepatotoxicity. However, this treatment is administered only in a hospital setting and must be administered within 8 hours following overdose to be most effective (Algren, 2008). In the case of accidental overdose, however, ALF may not be evident within 8 hours, at which point administration of NAC is no longer beneficial (Larson, 2005). It is clear from these data that APAP hepatotoxicity is a significant human health problem.

Metabolism: At therapeutic doses, the majority of APAP is metabolized in the liver by glucuronidation and sulfation to APAP-GLU and APAP-SUL conjugates, respectively (Heard, 2008). Only a small portion of APAP, approximately 5%, is metabolized in the liver by cytochrome P450 enzymes, Cyp1a2, Cyp3a11, and Cyp2e1, to a toxic reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) (Dahlin, 1984) which is efficiently inactivated by hepatic GSH pools (Chen, 2003). At toxic doses, however, the glucuronidation and sulfation pathways become saturated and an excess amount of NAPQI is formed leading to a rapid depletion of hepatic GSH. NAPQI, in turn, covalently binds to cellular macromolecules resulting in the production of reactive oxygen species and leading to oxidative stress and hepatocellular damage (Jaeschke, 2006).

Nrf2 and APAP Hepatotoxicity: APAP leads to the nuclear accumulation of Nrf2 in mouse liver (Goldring, 2004). Various studies have shown increased expression of downstream Nrf2 target genes in mouse liver following APAP administration including Nqo1, Ho-1, and Gclc (Goldring, 2004, Aleksunes, 2006b). Similar observations have also been observed for Nqo1 in human liver tissue samples obtained during transplantation following APAP overdose (Aleksunes, 2006a).

Nrf2-null mice exhibit enhanced hepatocellular injury compared to their wild-type counterparts following APAP administration (Chan, 2001, Enomoto, 2001). Reduced APAP-GLU conjugate formation was hypothesized to contribute to this increased susceptibility of Nrf2-null mice to APAP-induced hepatotoxicity. This was supported by enhanced immunohistochemical staining of APAP-adducted proteins in Nrf2-null mouse liver sections as well as lower mRNA expression and activity of Ugt1a6, an enzyme involved in the glucuronidation and detoxification of APAP (Enomoto, 2001). Nrf2-null mice also exhibit lower basal expression of downstream Nrf2 target genes including *Nqo1* and *Gclc* (Aleksunes, 2008). Furthermore, hepatic mRNA expression of these genes is significantly induced at 24 hours following APAP administration in wild-type mice, with a significant induction in protein seen at 48 hours, but no induction in mRNA or protein is observed in Nrf2-null mice (Aleksunes, 2008). Nrf2 not only regulates the expression of genes involved in the metabolism of APAP, but additionally influences the expression of cytoprotective genes associated with alleviating APAP hepatotoxicity.

Differential Susceptibility of Males and Females to APAP Hepatotoxicity

Sex is known to play an important role in drug absorption, metabolism, distribution, and excretion (Morris, 2003, Tanaka, 1999). Previous work has shown sex differences in susceptibility to APAP hepatotoxicity in mice, however, the mechanism(s) underlying these sex differences remains unknown. Interestingly, in CD-1 mice, one study reported no sex differences with respect to

APAP-induced hepatotoxicity (Hoivik, 1995), however, a more recent study suggests that female CD-1 mice are more resistant to APAP-induced hepatotoxicity than their male counterparts (Masubuchi, 2011). This latter study observed elevated alanine aminotransferase (ALT) activity in male CD-1 mice but not in female CD-1 mice following APAP administration. Furthermore, histological examination of liver tissue sections at 24 hours following APAP administration revealed hemorrhage and centrilobular necrosis only in the male CD-1 mice. The factors responsible for the variations in the results of these studies is unknown but may be a result of differing dosing regimens. Recent studies have also shown that male C57BL/6 mice are more susceptible to APAP hepatotoxicity than their female counterparts (Dai, 2006, McConnachie, 2007). For these reasons, male mouse models are more often employed in APAP-induced hepatotoxicity studies than female mouse models. This subsequently results in only a small amount of literature exploring the differential susceptibility of male and female mice to APAP-induced hepatotoxicity.

A recent study has also suggested that acetaminophen metabolism does not contribute to sex differences in its hepatotoxic effect in mice (Dai, 2006). It is known that APAP is metabolized by Cyp1a2, Cyp3a11, and Cyp2e1 to the toxic reactive metabolite NAPQI. This 2006 study reported observing significantly lower basal level mRNA expression of *Cyp1a2* in livers of female than male mice (Dai, 2006). However, further analysis showed no significant sex differences in NAPQI covalent binding, suggesting that bioactivation of APAP is similar between sexes.

In humans, the differential susceptibility of males and females to APAP hepatotoxicity is less clear. A higher female predominance of acetaminophen-related acute liver failure (ALF) cases has been observed both in the United States (Larson, 2005) and Sweden (Wei, 2007), suggesting that females with acute liver injury are either more predisposed to develop ALF or utilize prescription and/or over-the-counter medications containing acetaminophen more frequently than men. However, these data may be influenced by other factors as well including concomitant alcohol use,

concurrent medications, or nutritional status. Additionally, recent studies show the prevalence, incidence, and morbidity risk of depressive disorders is higher among females than males (Piccinelli, 2000, Pratt, 2008), and depressive disorders can often lead to self-medication, often times compounding the deleterious effects of the condition and leading to suicidal thoughts or actions (Zwolinski, 2010). Furthermore, nearly half of all acetaminophen-related ALF cases are a result of an intentional overdose (Larson, 2005). Although not every acetaminophen-related ALF case resulting from an intentional overdose may be attributed to a concomitant depressive disorder, acetaminophen is also not likely the sole factor resulting in a greater predominance of ALF cases in female than male humans. On the other hand, mouse models of APAP-induced hepatotoxicity are bred and maintained in a tightly controlled environmental setting, without the confounding influence of these added factors or conditions.

Sex Differences of Nrf2-Responsive Genes

NAD(P)H Quinone Oxidoreductase 1: While there are no reports of sex differences of Nqo1 expression in mice, one recent study has reported sex differences in the rat. This study observed significantly higher basal mRNA, protein, and enzymatic activity levels of Nqo1 in Sprague Dawley female than male rats, and these differences remained significant following APAP administration (Augustine, 2008). However, differences in the mRNA, protein, and enzymatic activity of Nqo1 were not observed in August Copenhagen x Irish (ACI) rat strains. While both rat strains are commonly employed in drug-induced enzyme induction and toxicity studies, sex differences in Nqo1 mRNA and protein expression and enzymatic activity levels are strain-dependent. This finding can, therefore, serve as an important tool when using rat models for toxicity studies.

Multidrug Resistance-Associated Protein Transporters: It is known that Nrf2 plays a predominant role in the hepatic induction of the Mrp family of proteins (Maher, 2005). Furthermore, one study has shown that *Mrp4* mRNA expression levels are more pronounced in female than male CD-1 mice at a low dose of APAP (Masubuchi, 2011). The absence of a significant *Mrp4* mRNA response at a higher dose may be due to impaired gene regulation resulting from pronounced hepatocellular necrosis.

Glutamate Cysteine Ligase: A greater number of studies suggest sex differences in glutamate cysteine ligase. One study observed greater mRNA induction of *Gclc* in female than male CD-1 mice following APAP administration at 300 mg/kg (Masubuchi, 2011). An earlier study also reported greater APAP-induced hepatotoxicity in male than female *Gclm* wild-type C57BL/6 mice (McConnachie, 2007). Finally, a more recent study reported that pretreatment of mice with buthionine sulfoximine (BSO), an inhibitor of glutamate cysteine ligase, potentiates APAP hepatotoxicity in female mice. These findings suggest a role for glutamate cysteine ligase in the differential susceptibility of male and female mice to APAP-induced hepatotoxicity. However, glutamate cysteine ligase is involved in the formation of glutathione (GSH) which plays an important role in the detoxification of NAPQI, the toxic reactive metabolite formed as a result of APAP metabolism, and it remains unclear whether there is a differential expression of GSH between males and females. One study shows no basal differences in hepatic GSH levels in overnight-fasted CD-1 male and female mice (Masubuchi, 2011). However, another study reports significantly lower basal GSH levels in male than female mouse livers (Sheng, 2013). These variations in the literature may be due to differences across strains and/or differences across experimental methodologies (e.g. time points of analysis, overnight-fasting versus continued feeding).

While there is a small amount of literature already available that explores the differential susceptibility of males and females to APAP-induced hepatotoxicity, many questions still remain unanswered about the mechanism(s) involved in this differential susceptibility. Because many of the cytoprotective genes associated with APAP hepatotoxicity are regulated by the nuclear transcription factor Nrf2, it is important to understand the potential influence Nrf2 has on the differential susceptibility of males and females to APAP-induced hepatotoxicity.

Chapter Two

Let's Talk Sex: Is Nrf2 Responsible for Sex Differences in Susceptibility to Acetaminophen-Induced Hepatotoxicity in Mice?

ABSTRACT

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that positively regulates the expression and activity of cytoprotective genes during periods of oxidative stress. It has previously been shown that some of these cytoprotective genes are more highly expressed in livers of female than male mice. This could explain previously reported sex-related differences in susceptibility to acetaminophen (APAP) hepatotoxicity in mice, where females show greater resistance to APAP hepatotoxicity. Here, we examined differences in basal mRNA and protein expression for Nrf2 and Nrf2-dependent genes and measured glutathione (GSH) levels in naïve fed wild-type (WT) male and female mice and overnight-fasted WT and Nrf2-null male and female mice following either vehicle or APAP administration. Alanine aminotransferase (ALT) activity was also measured as an indicator of hepatotoxicity. Hepatic mRNA and protein levels were measured by quantitative PCR and western blotting, respectively. Basal *Nrf2* mRNA expression was significantly lower in livers of naïve female than male mice. In agreement with mRNA expression, basal Nrf2 protein expression was lower in livers of both naïve and overnight-fasted WT female than male mice, with significance observed in livers of naïve mice. Basal mRNA expression of the Nrf2-regulated gene NAD(P)H quinone oxidoreductase 1 (*Nqo1*) was significantly greater in livers of overnight-fasted WT female than male mice, however, no significant differences between sexes for the remaining Nrf2-regulated genes were observed. Interestingly, basal Nqo1 protein expression was significantly higher in livers of naïve and overnight-fasted WT and Nrf2-null female than male

mice. Furthermore, a significantly greater induction of *Nqo1* and multidrug resistance-associated protein 4 (*Mrp4*) mRNA expression was observed in both naïve and overnight-fasted WT and Nrf2-null female than male livers following APAP administration, resulting in significantly greater mRNA expression of these genes in female than male livers. ALT activity was also significantly elevated in both overnight-fasted WT and Nrf2-null male mice following APAP administration, but no increases in ALT were observed in either genotype of female mice. These results indicate that other factors, rather than Nrf2, are responsible for the lower susceptibility of female mice to APAP hepatotoxicity.

INTRODUCTION

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that positively regulates the expression and activity of cytoprotective genes during periods of oxidative stress. In its inactive state, Nrf2 is bound to Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm of the cell (Itoh, 1999, Kang, 2004). However, in the presence of oxidative stress, Nrf2 dissociates from Keap1 and translocates into the nucleus of the cell leading to the activation of antioxidant response element (ARE)-mediated gene transcription. ARE-containing genes identified as targets of Nrf2 are involved in a variety of cellular functions including drug metabolism, reactive oxygen species scavenging, GSH homeostasis, and efflux transport pathways (Nguyen, 2003). These genes include, among others, NAD(P)H quinone oxidoreductase 1 (*Nqo1*), heme oxygenase 1 (*Ho-1*), multidrug resistance-associated protein transporters (*Mrp1-4*), glutamate cysteine ligase catalytic subunit (*Gclc*), and glutamate cysteine ligase modifier subunit (*Gclm*) (Aleksunes, 2007). An impaired expression of these genes can increase the sensitivity of cells to oxidative damage. Previously, it has also been shown that many of these genes are more highly expressed in the livers of female than male mice (Masubuchi, 2011, Dai, 2006).

Acetaminophen (APAP) is a commonly used analgesic and antipyretic. APAP is also one of the leading causes of acute liver failure in the United States (Reuben, 2010). Since the early 2000's, APAP has been attributed to approximately 50% of all acute liver failure cases across the United States (Reuben, 2010). At therapeutic doses, the majority of APAP is metabolized in the liver by glucuronidation and sulfation to APAP-GLU and APAP-SUL conjugates, respectively (Heard, 2008). Only a small portion of APAP, approximately 5%, is metabolized in the liver by cytochrome P450 enzymes, Cyp1a2, Cyp3a11 (Cyp3a4 in humans), and Cyp2e1, to a toxic reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) (Dahlin, 1984) which is efficiently inactivated by hepatic glutathione (GSH) pools (Chen, 2003). At toxic doses, however, the glucuronidation and sulfation pathways become saturated and an excess amount of NAPQI is formed leading to a rapid depletion of hepatic GSH. NAPQI, in turn, covalently binds to cellular macromolecules resulting in the production of reactive oxygen species and leading to oxidative stress and hepatocellular damage (Jaeschke, 2006). Currently, *N*-Acetyl cysteine (NAC), a precursor to GSH, is the only standard therapy used to prevent and decrease APAP-induced hepatotoxicity. However, this treatment is administered only in a hospital setting and must be administered within 8 hrs following overdose to be most effective (Algren, 2008). In the case of accidental overdose, however, ALF may not be evident within 8 hrs, at which point administration of NAC is no longer beneficial (Larson, 2005).

APAP has been shown to lead to the nuclear accumulation of Nrf2 in mouse liver (Goldring, 2004). Various studies have also shown increased expression of downstream Nrf2 target genes in mouse liver following APAP administration including *Nqo1*, *Ho-1*, and *Gclc* (Goldring, 2004, Aleksunes, 2006b). Furthermore, Nrf2-null mice exhibit enhanced hepatocellular injury compared to their wild-type counterparts following APAP administration (Chan, 2001, Enomoto, 2001), suggesting a pivotal hepatoprotective role for Nrf2 in APAP-induced hepatotoxicity.

Sex is known to play an important role in drug absorption, metabolism, distribution, and excretion (Morris, 2003, Tanaka, 1999). Previous work has shown sex differences in susceptibility to APAP hepatotoxicity in mice, however, the mechanism(s) underlying these sex differences remains unknown. Recent studies have shown that male C57BL/6 mice are more susceptible to APAP-induced hepatotoxicity than their female counterparts (Dai, 2006, McConnachie, 2007). However, in CD-1 mice, one study reported no sex differences with respect to APAP-induced hepatotoxicity (Hoivik, 1995), while a more recent study suggests that female CD-1 mice are more resistant to APAP-induced hepatotoxicity than their male counterparts (Masubuchi, 2011). The latter study observed elevated alanine aminotransferase (ALT) activity in male CD-1 mice but not in female CD-1 mice following APAP administration, and histological examination of liver tissue sections at 24 hrs following APAP administration revealed hemorrhage and centrilobular necrosis only in the male mice. For these reasons, male mouse models are more often employed in APAP-induced hepatotoxicity studies than female mouse models. This subsequently results in only a small amount of literature exploring the differential susceptibility of male and female mice to APAP-induced hepatotoxicity. Interestingly, in humans, a higher female predominance of acetaminophen-related acute liver failure (ALF) cases has been observed both in the United States (Larson, 2005) and Sweden (Wei, 2007). These data, however, may be influenced by concomitant alcohol use, concurrent medications, or nutritional status, for example.

The present study aims to explore sex differences in the expression of various cytoprotective genes involved in APAP-induced hepatotoxicity in C57BL/6J mice. Here, we hypothesized that resistance to APAP-induced hepatotoxicity by female mice is due to higher basal and inducible expression of Nrf2 and Nrf2-dependent genes. We examined alanine aminotransferase (ALT) levels in male and female mice, measured mRNA and protein expression for Nrf2 and Nrf2-dependent genes, and measured hepatic GSH levels in male and female mice. This study will aid in the

identification of potential new therapeutic gene targets for the treatment and prevention of drug-induced liver injury.

MATERIALS AND METHODS

Reagents. APAP was purchased from Sigma-Aldrich (St. Louis, MO).

Animals and Treatment. Male and female WT C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Nrf2^{-/-} (Nrf2-null) mice with a C57BL/6J background were kindly provided by Dr. Angela Slitt from the University of Rhode Island. The mice utilized were age matched from 8.5 to 10.5 weeks of age. All mice were housed and bred at the University of Connecticut in a temperature-, light-, and humidity-controlled environment that is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Mice were maintained on standard laboratory chow and had free access to water. Male and female WT mice were either fasted overnight or maintained on the laboratory chow. Male and female Nrf2-null mice were fasted overnight. APAP was dissolved in saline (pH 8.0). Mice received either no treatment or were administered APAP (200 mg/kg, 400 mg/kg or 600 mg/kg, i.p.) or saline as a vehicle treatment. Plasma and liver were collected at various time points. A portion of the liver was fixed in 10% zinc formalin and the remaining liver tissue was snap frozen in liquid nitrogen. Frozen tissues were stored at -80°C until assayed. All animal studies were conducted in accordance with National Institutes of Health standards and the *Guide for the Care and Use of Laboratory Animals*. Studies were approved by the University of Connecticut Institutional Animal Care and Use Committee (IACUC Protocol no. A12-050).

Assessment of Hepatotoxicity. Plasma ALT activity was determined as a biochemical indicator of hepatocellular necrosis. Infinity ALT Liquid Stable Reagent (ThermoTrace, Melbourne, Australia) was used according to the manufacturer's protocol. Hepatotoxicity was confirmed by histological examination of liver tissue sections. Formalin-fixed tissue sections were embedded in paraffin, mounted onto glass slides, stained with hematoxylin-eosin, and examined for histopathological changes by a board-certified veterinary pathologist.

Preparation of Crude Membrane, Microsomal, Cytosolic, and Nuclear Fractions. Livers were homogenized in sucrose-Tris (ST) buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4) containing 50 µg/ml aprotinin and centrifuged at $100,000 \times g$ for 60 min at 4°C. The resulting pellet constituted the crude membrane fraction and was resuspended in ST buffer. For isolation of microsomes, homogenates were first centrifuged at $10,000 \times g$ for 20 min at 4°C. The supernatant was then centrifuged at $100,000 \times g$ for 60 min. ST buffer was used to resuspend the microsomal pellet. Liver cytosolic and nuclear extracts were prepared using the NE-PER Nuclear Extraction Kit according to the manufacturer's directions (Pierce Biotechnology, Rockford, IL). Protein concentrations were determined using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Hercules, CA). Bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) was used as a standard.

Western Blot Analysis. Cytosolic and nuclear proteins were boiled for 10 minutes. Proteins were electrophoretically resolved using polyacrylamide gels (8-12% resolving, 5% stacking) and transblotted onto PVDF-Plus membrane (Micron Separations, Westboro, MA). Immunochemical detection of Nrf2, Nqo1, Ho-1, Gclc, Gclm, Mrp3, and Mrp4 proteins are described in Table 2. Briefly, membranes were blocked with 5% non-fat dry milk (NFDM) in Tris-buffered saline-0.1%Tween-20 (pH 7.4) (TBS-Tw) for 1 h and incubated overnight with the primary antibody

diluted in blocking buffer. A species-appropriate peroxidase-labeled secondary antibody (Sigma, St. Louis, MO) was diluted in blocking buffer and incubated with blots for 1 h. Protein-antibody complexes were detected using a chemiluminescent kit (Thermo Scientific, IL) followed by exposure to X-ray film. Intensity values were normalized to TBP for Nrf2 and β -actin for remaining proteins.

Quantitative RT-PCR. Real time PCR (qRT-PCR) was carried out using an Applied Biosystems 7500 Fast qRT-PCR Machine. Primers for *Nrf2*, *Keap1*, *Nqo1*, *Ho-1*, *Gclc*, *Gclm*, *Mrp3*, and *Mrp4* were obtained from Integrated DNA Technologies and gene levels were normalized to β -Tubulin. Primer sequences are shown in Table 3.

Measurement of GSH Levels. Total GSH was measured as previously described (Rahman, 2006). Briefly, livers were homogenized in 500 μ l of buffer containing 0.2% Triton-X 100 and 0.6% sulfosalicylic acid in 0.1 M potassium phosphate buffer with 5 mM EDTA disodium salt (KPE), pH 7.5. 20 μ l of standards and samples were pipetted into a 96-well microtiter plate. 60 μ l of β -NADPH (Sigma, St. Louis, MO) was added followed by 120 μ l of 1:1 3.33 U/ml glutathione reductase (Sigma, St. Louis, MO) with 0.67 mg/ml 5,5'-Dithiobis (2-nitrobenzoic acid, DTNB). Yellow 5-thio-2-nitrobenzoic acid (TNB) formation was monitored spectrophotometrically at 412 nm. The rate of formation of TNB is proportional to the concentration of GSH in the sample.

Statistical Analysis. Data were given as mean \pm SD. Data were analyzed using the Student t-test or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test to determine significant differences between group means. Significance was set at $p < 0.05$.

RESULTS

Plasma ALT activity and histopathology in WT mice following APAP

APAP-induced hepatocellular injury was assessed by plasma ALT levels and confirmed by histological examination of liver tissue sections. At 400 mg APAP/kg, significant elevations in plasma ALT activity at 4 and 24 hrs were detected in male WT mice (Figure 1A). However, ALT activity at 4 and 24 hrs after APAP did not increase in female WT mice. Histological examination and grading of WT male and female liver tissue sections at 4 and 24 hrs after treatment with APAP was further indicative of hepatocellular damage in male but not female WT mice. Centrilobular necrosis was observed in male livers at 4 hrs after treatment with APAP and this damage was even further pronounced at 24 hrs (Figure 1D-E). No significant damage was observed in female livers at either time point (Figure 1B-C). Histological grading further confirms these results (Table 1). In Table 1, liver sections with grades higher than 2 are considered to have significant injury. Treatment with 400 mg APAP/kg resulted in significant injury in 80% of male WT mice at 24 hrs following APAP treatment, however, no significant injury in female livers was observed.

Hepatic mRNA expression of *Nrf2* and *Nrf2*-dependent genes in naïve WT mouse livers

Hepatic mRNA expression was measured for *Nrf2*, *Keap1*, and *Nrf2*-dependent genes *Nqo1*, *Ho-1*, *Gclc*, *Gclm*, *Mrp3*, and *Mrp4* (Figure 2A-H). *Nrf2* hepatic mRNA levels in naïve WT females were significantly lower compared to male values (Figure 2A). No significant differences in mRNA expression between female and male livers were observed for the remaining genes. These results are consistent with previous literature which shows similar basal mRNA levels of *Gclc* in male and female mouse livers (Masubuchi, 2011). Masubuchi (2011) also showed significantly greater basal mRNA expression of *Mrp4* in female mouse livers compared to male livers. In the current study,

however, while greater basal mRNA expression of *Mrp4* was observed in female livers compared to male livers, this difference was not significant.

Hepatic mRNA expression of *Nrf2* and Nrf2-dependent genes in overnight-fasted WT mouse livers following APAP

Hepatic mRNA expression was measured for *Nrf2*, *Keap1*, and Nrf2-dependent genes *Nqo1*, *Ho-1*, *Gclc*, *Gclm*, *Mrp3*, and *Mrp4* (Figure 3A-H). Hepatic basal *Nrf2* mRNA expression was slightly higher in females compared to males, although this difference was not significant. Consistent with the naïve WT mouse data, no significant differences in basal mRNA expression in livers of overnight-fasted WT female and male mice were observed for the Nrf2-dependent genes examined, with the exception of *Nqo1*. Hepatic basal mRNA expression of *Nqo1* was significantly greater in female livers compared to male values at 4 hrs (Figure 3C). In naïve WT female livers, basal mRNA expression of *Nqo1* was greater than in naïve WT male livers, although this difference was not significant (Figure 2C). The significant difference in hepatic basal mRNA expression of *Nqo1* in the overnight-fasted WT mice may be a consequence of fasting-induced oxidative stress; however, previous literature shows no significant difference in basal mRNA expression of *Nqo1* in liver between sexes for fed versus 24h-fasted mice (Zhang, 2013).

A significant induction in *Gclc* mRNA levels was also observed in livers of overnight-fasted WT females at 4 hrs following APAP administration (Figure 3E). This is consistent with previous work which shows elevated *Gclc* mRNA levels in livers of female mice following APAP administration (Masubuchi, 2011). *Mrp4* mRNA expression was also greatly elevated in livers of overnight-fasted WT females at 24 hrs following APAP administration. Mrp1-4 are plasma membrane efflux transporter proteins that are believed to be involved in the antioxidant response through mediating the efflux of xenobiotics from the liver into the bile or blood (Klaassen, 2005).

Previous literature has shown Mrp4 to be a highly inducible antioxidant response protein in the liver of male and female mice following APAP administration (Aleksunes, 2008, Masubuchi, 2011).

Hepatic protein expression of Nrf2 and Nrf2-dependent genes in naïve WT mouse livers

Hepatic protein expression was measured for Nrf2 and Nrf2-dependent genes Nqo1, Gclc, Gclm, Mrp3, and Mrp4 (Figure 4A-F). Similar to mRNA expression, Nrf2 protein expression was significantly lower in female livers compared to male livers (Figure 4A). Hepatic protein expression for the remaining genes, except Gclc, followed a similar pattern with mRNA expression. That is, if mRNA expression was lower in female livers than male livers, protein expression was also observed to be lower in female livers compared to male livers. Significance in protein expression was observed for Nqo1, Gclc, Gclm, and Mrp3. While female livers showed significantly greater Nqo1 protein expression than male livers (Figure 4B), Gclm and Mrp3 protein expression was significantly lower in female than male livers (Figure 4D-E). Gclc protein expression was significantly higher in female than male livers (Figure 4C), though *Gclc* mRNA expression, while not statistically significant, appeared lower in female livers compared to male livers (Figure 2E).

Hepatic protein expression of Nrf2 and Nrf2-dependent genes in overnight-fasted WT mouse livers following APAP

Hepatic protein expression was measured for Nrf2 and Nrf2-dependent genes Nqo1, Ho-1, Gclc, Gclm, Mrp3, and Mrp4 (Figure 5A-N). Similar to protein expression in naïve WT mouse livers, Nrf2 protein expression was less pronounced in livers of overnight-fasted WT female than male mice following either vehicle or APAP administration. Of the Nrf2-dependent genes examined, females did not exhibit a higher global expression of these genes. Consistent with mRNA expression, basal protein expression of Nqo1 was significantly greater in female livers compared to

male livers at 4 hrs following vehicle treatment (Figure 5C). Female livers also showed significantly greater Mrp4 basal protein expression than male livers at both 4 hrs and 24 hrs following vehicle treatment (Figure 5M-N). No significant differences in basal protein expression between sexes were observed for the remaining genes. A significant induction in Ho-1 protein expression was observed in both sexes at 24 hrs following APAP administration (Figure 5D). This is consistent with previous work in our lab which shows Ho-1 is a highly inducible stress protein (Aleksunes, 2008).

Consistent with protein expression in naïve WT livers, basal protein expression of Gclc was higher in livers of overnight-fasted WT females compared to males, although this difference was not significant. Gclc protein expression was significantly decreased in female livers at 4 hrs following APAP administration (Figure 5G), but returned to basal levels at 24 hrs (Figure 5H). No significant differences in Gclc protein expression in male livers were observed following APAP administration. These results are consistent with previous studies showing Gclc protein expression is significantly reduced in female mouse livers following APAP administration while no significant differences in male livers were observed (Sheng, 2013). Furthermore, although not significant, Gclm protein expression was higher in female livers compared to male livers at 4 hrs and 24 hrs following either vehicle or APAP administration (Figure 5I-J). Gclc and Gclm are enzymes involved in the production of GSH, an important detoxification pathway in APAP hepatotoxicity. These data suggest a greater propensity for females to produce more GSH than males following APAP treatment.

Hepatic glutathione measurement in WT mice following APAP

To investigate potential sex differences in hepatic GSH levels, total GSH was measured, as described in the materials and methods, in livers of naïve WT male and female mice as well as overnight-fasted WT male and female mice following either vehicle or APAP administration. No

significant differences in basal GSH levels were observed between livers of naïve or overnight-fasted WT male and female mice (Figures 6-7). However, a significantly greater reduction in GSH at 4 hrs following APAP administration was observed in overnight-fasted WT male livers compared to female livers (Figure 7). This is consistent with observations of lower toxicity and liver damage in female livers compared to male livers. Glutathione levels rebound in both sexes at 24 hrs following APAP administration (Figure 7), consistent with an increase in *Gclc* mRNA expression in both sexes at 4 hrs following APAP administration (Figure 3E).

Plasma ALT activity and histopathology in Nrf2-null mice following APAP

To better understand whether Nrf2 is responsible for the sex differences in susceptibility of male and female mice to APAP-induced hepatotoxicity, ALT activity was measured in Nrf2-null male and female mice following APAP treatment. At 200 mg APAP/kg, significant elevation in plasma ALT activity at 24 hrs was detected in male Nrf2-null mice (Figure 8A). However, no significant change in ALT activity was observed in females of the same genotype (Figure 6A). Histological examination and grading of liver tissue sections from male and female Nrf2-null mice at 24 hrs following APAP administration further confirms these results (Figures 8C-D, Table 1). Treatment with 200 mg APAP/kg resulted in significant injury in 60% of male Nrf2-null mice compared to 0% of female Nrf2-null mice (Table 1). Furthermore, at 400 mg APAP/kg, significantly elevated ALT activity was still not observed in female Nrf2-null mice (Figure 8B) and histological grading did not detect significant injury in female Nrf2-null mice following APAP treatment at 400 mg/kg (Table 1). However, at 600 mg APAP/kg, significantly elevated ALT activity was observed in female Nrf2-null mice (Figure 8B). Histological examination and grading further confirmed the presence of hepatocellular damage in liver tissue sections of female Nrf2-null

mice following APAP treatment at 600 mg APAP/kg (Figure 8F, Table 1). Significant injury in 75% of female Nrf2-null mice resulted following treatment with 600 mg APAP/kg (Table 1).

Hepatic mRNA expression of Nrf2-dependent genes in Nrf2-null mouse livers following APAP

Hepatic mRNA expression was measured for *Keap1* and Nrf2-dependent genes *Nqo1*, *Ho-1*, *Gclc*, *Gclm*, *Mrp3*, and *Mrp4* (Figure 9A-G). Significant induction of *Ho-1* was observed in male livers at 24 hrs following APAP treatment (Figure 9B). Ho-1 catabolizes prooxidant heme to carbon monoxide, biliverdin, and free iron, thereby preventing free heme from facilitating the formation of reactive oxygen species (Aleksunes, 2007). These results are consistent with previous literature that show *Ho-1* is upregulated following oxidative stress and cellular injury (Guo, 2001, Aleksunes, 2008).

Similar to the overnight-fasted WT mouse livers, Nrf2-null female mouse livers showed significant induction of *Nqo1* and *Mrp4* mRNA expression at 24 hrs following APAP treatment (Figure 9C & G). Furthermore, at 24 hrs following APAP treatment, female livers showed significantly greater mRNA expression of *Nqo1*, *Mrp3*, and *Mrp4* than male livers (Figure 9C, F & G). These data suggest that other mechanisms in addition to Nrf2 or in compensation for the absence of Nrf2 may regulate the transcription of *Nqo1* and *Mrp4* in females.

Hepatic protein expression of Nrf2-dependent genes in Nrf2-null mouse livers following APAP

Hepatic protein expression was measured for Nrf2-dependent genes *Nqo1*, *Ho-1*, *Gclc*, *Gclm*, *Mrp3*, and *Mrp4* (Figure 10A-F). Nrf2-null female livers showed significantly greater *Nqo1* basal protein expression than their male counterparts at 24 hrs following vehicle treatment (Figure 10B). No significant differences between sexes were observed for the remaining genes. A

significant reduction in Gclc protein expression was observed in female livers at 24 hrs following APAP treatment (Figure 10C). A similar reduction was observed at 4 hrs following APAP treatment in female overnight-fasted WT mouse livers (Figure 5G).

Hepatic glutathione measurement in Nrf2-null mice following APAP

Total GSH was measured in livers of Nrf2-null male and female mice following either vehicle or APAP administration. A significantly greater reduction in GSH was observed in male than female livers at 24 hrs following APAP treatment (Figure 11). This is consistent with observations in livers of overnight-fasted WT male and female mice (Figure 7) and observations of lower toxicity and liver damage in Nrf2-null female than male livers.

Figure 1. Sex differences in APAP hepatotoxicity in mice. Male and female mice were sacrificed at 4 and 24 hrs following APAP administration. (A) Serum ALT activity in overnight-fasted WT mice following APAP administration (400mg/kg, i.p.). Data are presented as mean \pm SD (n = 6 animals). Data were analyzed using ANOVA followed by Tukey's multiple comparison test. Asterisks (*) represent statistical differences ($p < 0.05$) between groups. (B-E) Histopathology of liver sections. Liver sections from male and female overnight-fasted WT mice at 4 and 24 hrs following vehicle or APAP treatment were subjected to hematoxylin-eosin staining (10x final magnification). Samples are as follows: B, female/WT/400mg/kg APAP/4h; C, female/WT/400mg/kg APAP/24h; D, male/WT/400mg/kg APAP/4h; E, male/WT/400mg/kg APAP/24h. Vehicle not shown.

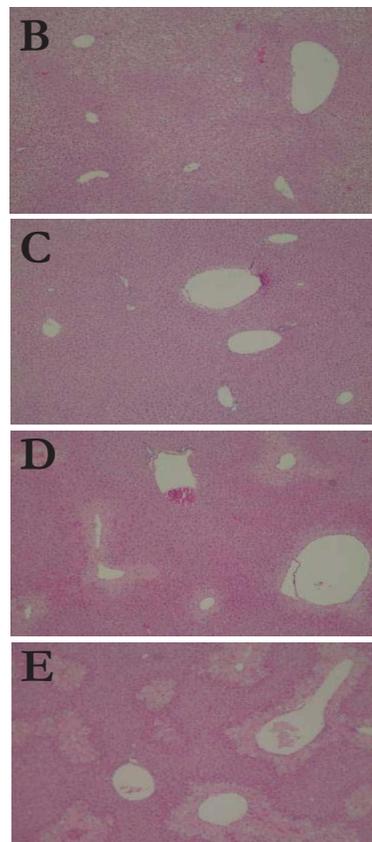
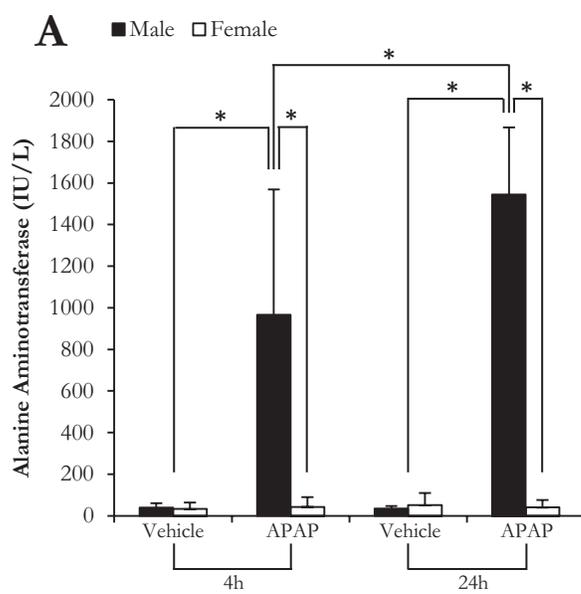


Table 1. Histopathological analysis of livers from APAP-treated WT and Nrf2-null mice. Livers were removed from WT and Nrf2-null mice 4 and 24 hrs following vehicle or APAP (200 mg/kg, 400 mg/kg, or 600 mg/kg) administration and fixed in 10% formalin prior to paraffin embedding and staining with hematoxylin and eosin. Liver sections were evaluated for the severity of degenerative and necrotic changes in the centrilobular regions as previously described (Manautou *et al.*, 1994, Aleksunes *et al.*, 2005). Liver samples with grades greater than 2 are considered to have significant injury. Data were rank ordered prior to statistical analysis. Asterisks (*) represent a statistical difference ($p < 0.05$) from control mice of the same genotype and sex. Daggers (†) represent a statistical difference ($p < 0.05$) from WT mice of the same treatment and sex.

| | Histological Grade | | | | | | | | |
|------------------|--------------------|----------|----------|----------|----------|----------|-----------------|--------------|--|
| WT | 0 | 1 | 2 | 3 | 4 | 5 | % > 2 | Total | |
| 4 hrs | | | | | | | | | |
| Male | | | | | | | | | |
| Vehicle | 3 | | | | | | 0 | 3 | |
| 400 mg APAP/kg | | | 5 | | | | 0* | 5 | |
| Female | | | | | | | | | |
| Vehicle | 3 | | | | | | 0 | 3 | |
| 400 mg APAP/kg | 4 | | | | | | 0 | 4 | |
| 24 hrs | | | | | | | | | |
| Male | | | | | | | | | |
| Vehicle | 3 | | | | | | 0 | 3 | |
| 400 mg APAP/kg | | | 1 | 4 | | | 80* | 5 | |
| Female | | | | | | | | | |
| Vehicle | 3 | | | | | | 0 | 3 | |
| 400 mg APAP/kg | | 5 | | | | | 0 | 5 | |
| Nrf2-null | | | | | | | | | |
| 24 hrs | | | | | | | | | |
| Male | | | | | | | | | |
| Vehicle | 4 | | | | | | 0 | 4 | |
| 200 mg APAP/kg | | 1 | 1 | 1 | | 2 | 60* | 5 | |
| Female | | | | | | | | | |
| Vehicle | 6 | | | | | | 0 | 6 | |
| 200 mg APAP/kg | | 3 | 1 | | | | 0 | 4 | |
| 400 mg APAP/kg | 1 | 2 | 1 | 1 | | | 20 | 5 | |
| 600 mg APAP/kg | | | 1 | | 2 | 1 | 75* | 4 | |

Figure 2. Messenger RNA expression of *Nrf2*, *Keap1*, and Nrf2-regulated genes in male and female naïve WT mouse livers. Data are presented as mean \pm SD. Data were analyzed using the Student t-test. Asterisks (*) represent statistical differences ($p < 0.05$) between sexes.

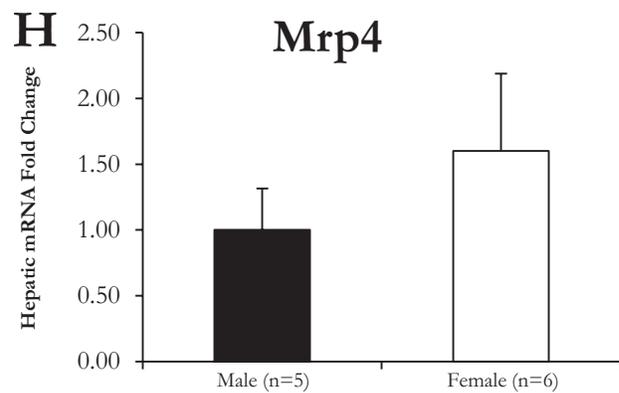
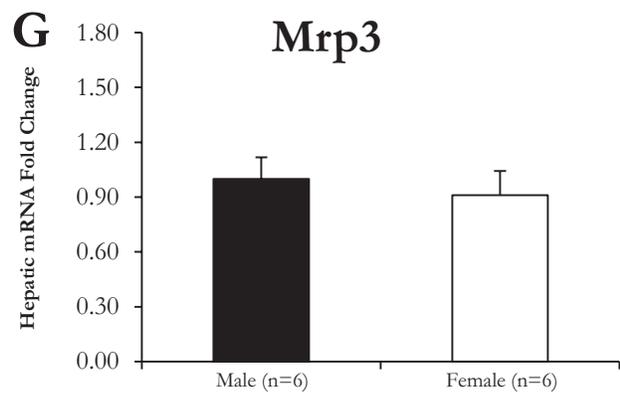
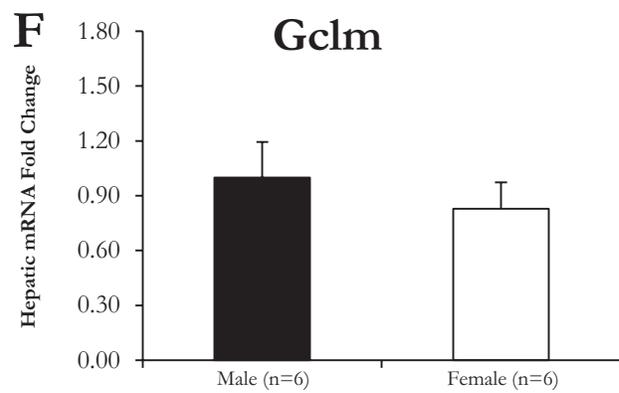
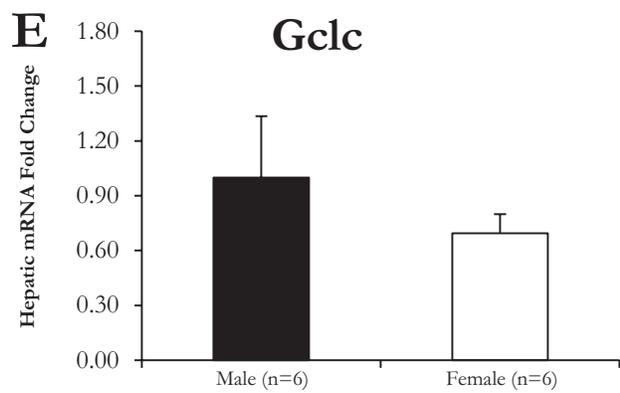
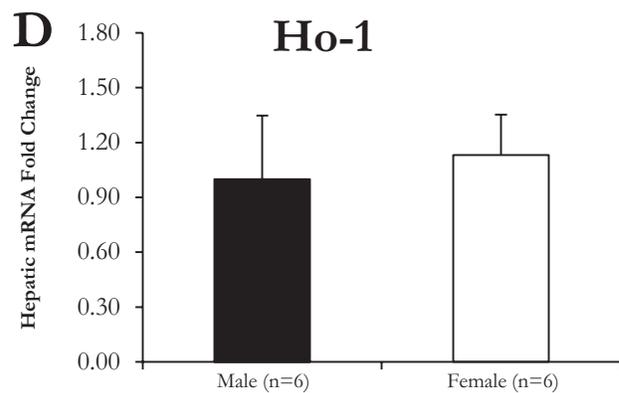
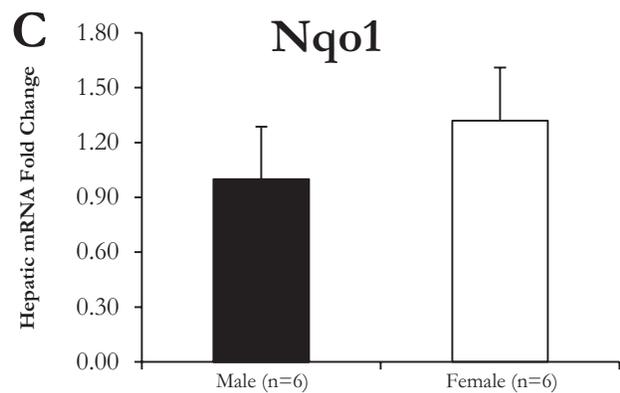
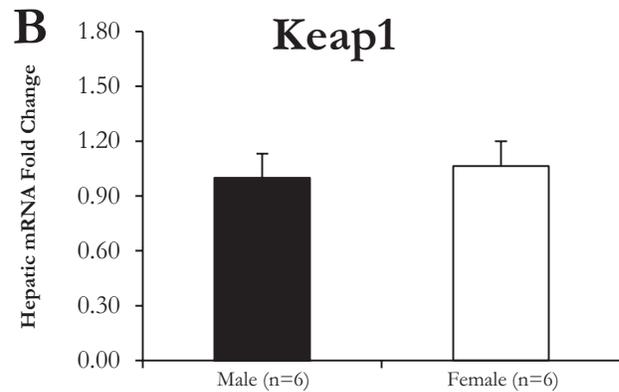
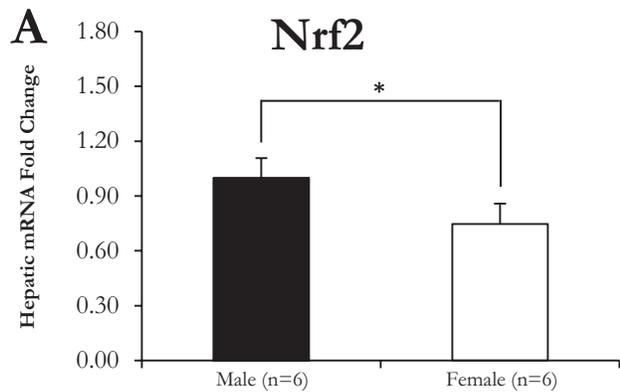


Figure 3. Hepatic messenger RNA expression of *Nrf2*, *Keap1*, and Nrf2-regulated genes in vehicle- and APAP-treated (400 mg/kg, i.p.) overnight-fasted WT male and female mice. Data are presented as mean \pm SD (n = 6 animals). Data were analyzed using ANOVA followed by Tukey's multiple comparison test. Asterisks (*) represent statistical differences ($p < 0.05$) between groups.

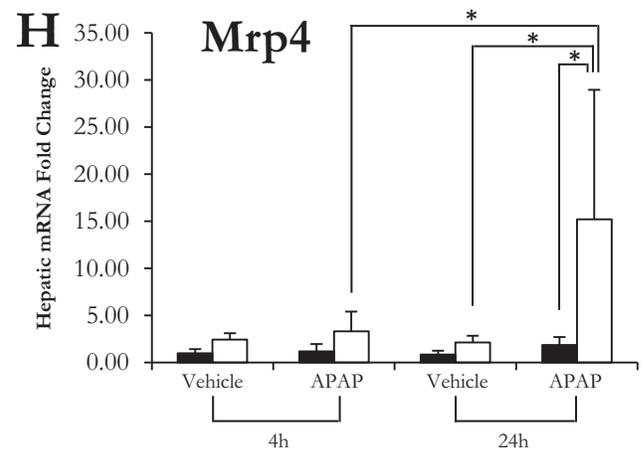
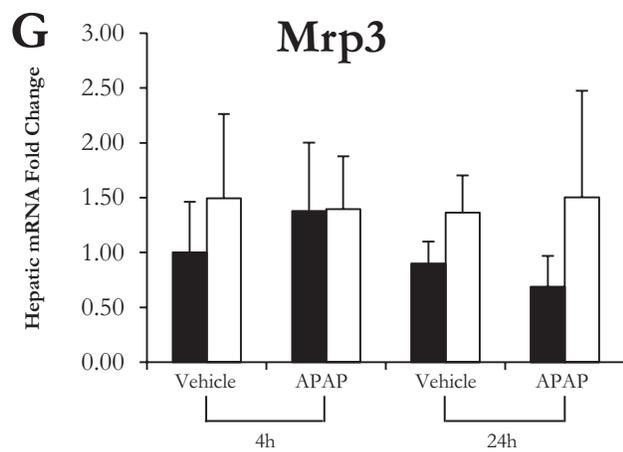
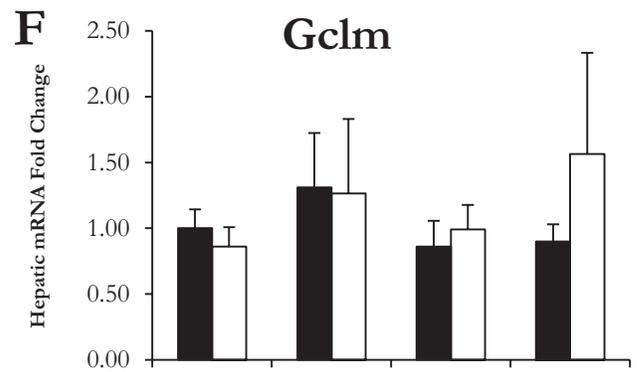
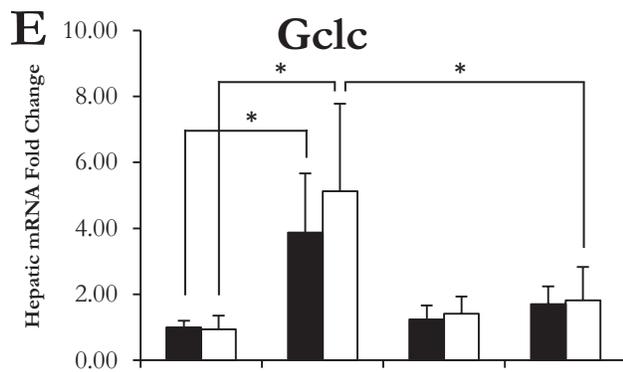
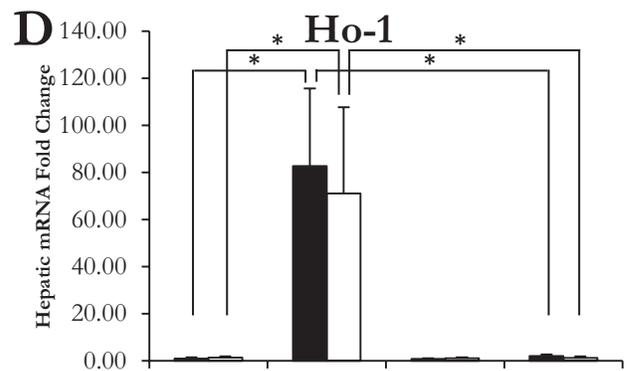
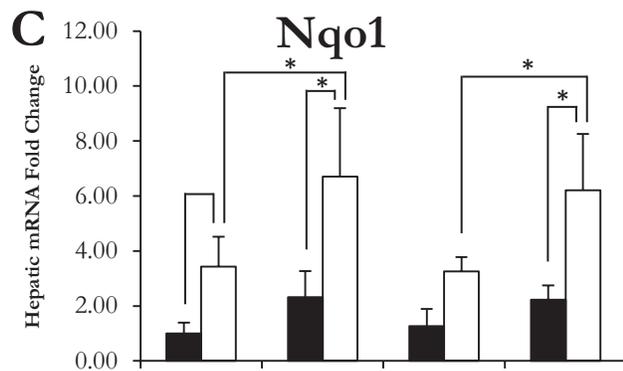
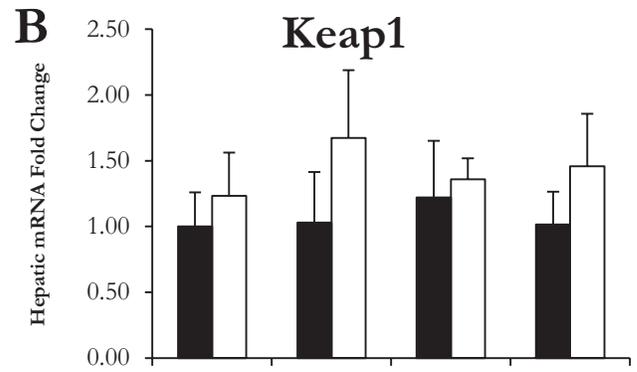
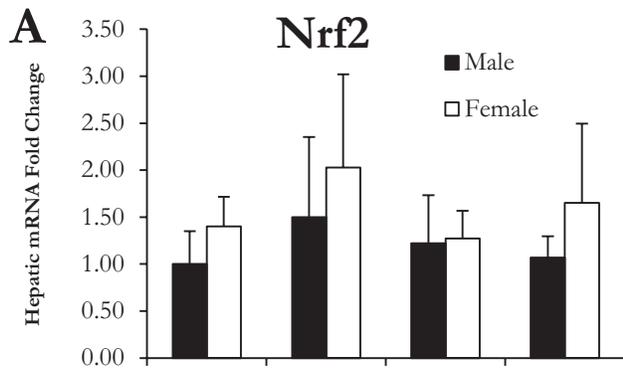


Figure 4. Western blots for Nrf2 and Nrf2-regulated genes were performed using liver membrane (Mrp3 and Mrp4), cytosolic (Nqo1, Gclc, and Gclm), and nuclear (Nrf2) preparations from naïve WT male and female mice. Human hepatocyte-derived HCO4 cells treated with 100 μ M tBHP for 2 hrs were used as a positive control for Nrf2 detection. Nrf2-null mouse liver tissue was used as a negative control for Nrf2 detection. Equal protein loading was confirmed by detection of TBP for Nrf2 and β -actin. Data are presented as blots (upper panel) and as mean \pm SD (n = 6 animals) (lower panel). Data were analyzed using the Student t-test. Asterisks (*) represent statistical differences ($p < 0.05$) between sexes.

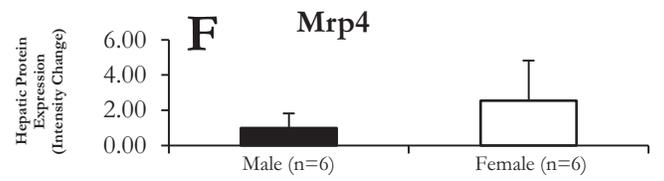
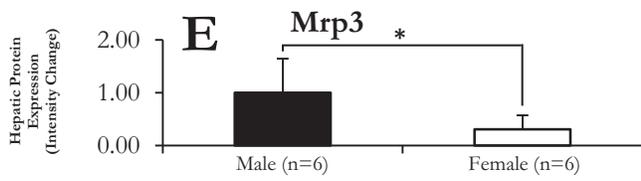
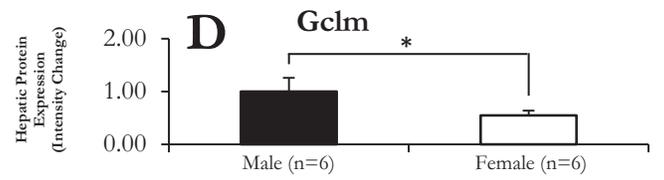
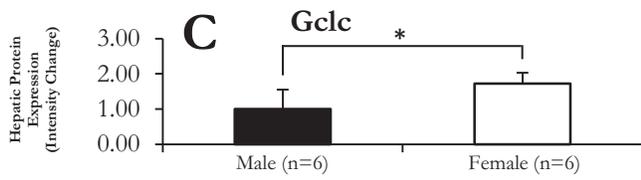
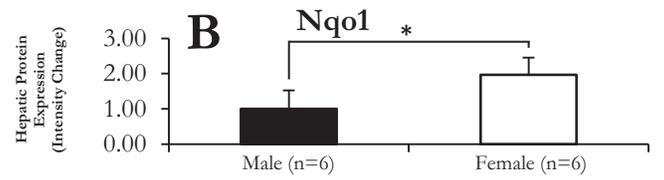
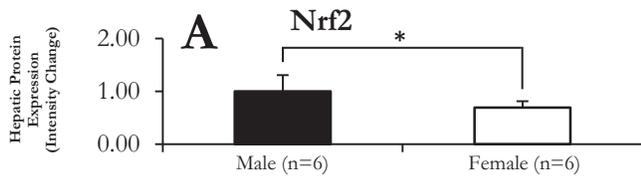
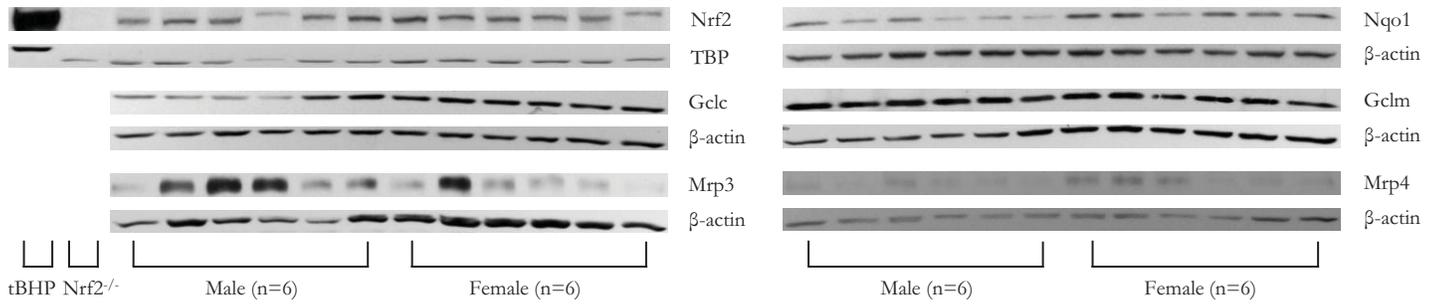


Figure 5. Western blots for Nrf2 and Nrf2-regulated genes were performed using liver membrane (Mrp3 and Mrp4), cytosolic (Nqo1, Ho-1, Gclc, and Gclm), and nuclear (Nrf2) preparations from vehicle- and APAP-treated (400 mg/kg, i.p.) overnight-fasted WT male and female mice. Human hepatocyte-derived HCO4 cells treated with 100 μ M tBHP for 2 hrs were used as a positive control for Nrf2 detection. Equal protein loading was confirmed by detection of TBP for Nrf2 and β -actin. Data are presented as blots (upper panel) and as mean \pm SD (n = 3 animals) (lower panel). Data were analyzed using ANOVA followed by Tukey's multiple comparison test. Asterisks (*) represent statistical differences ($p < 0.05$) between groups.

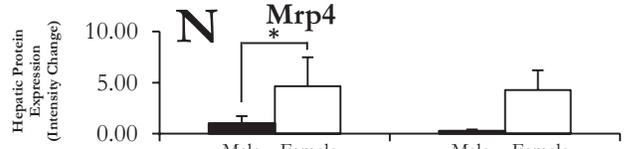
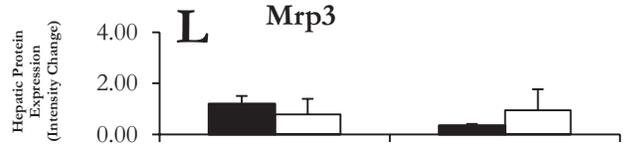
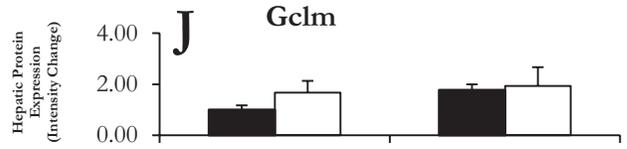
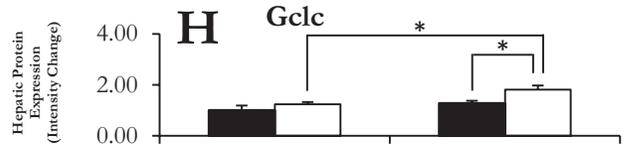
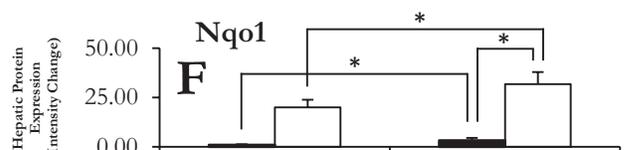
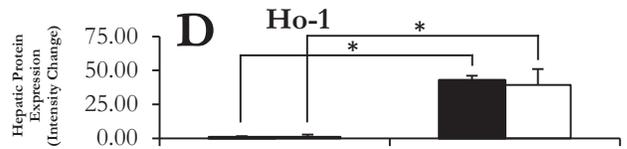
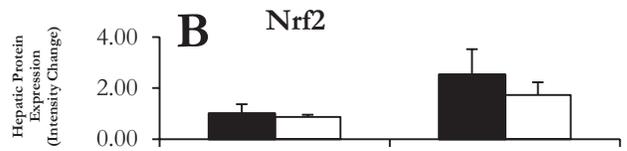
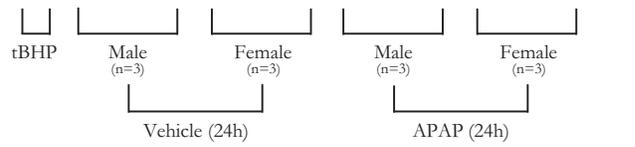
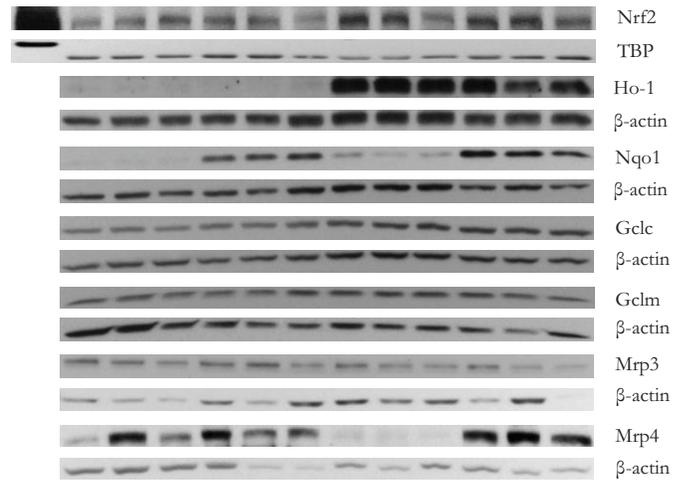
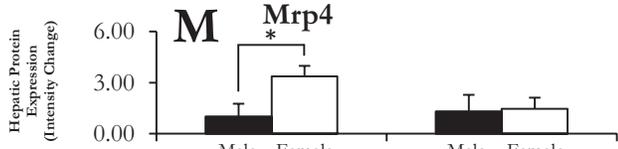
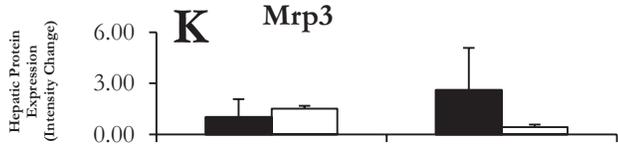
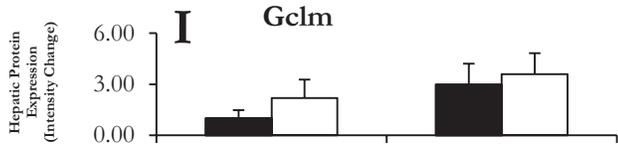
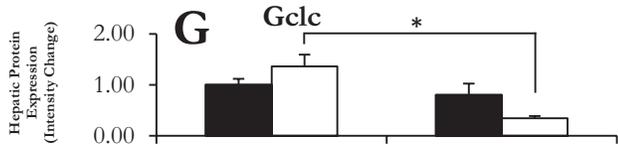
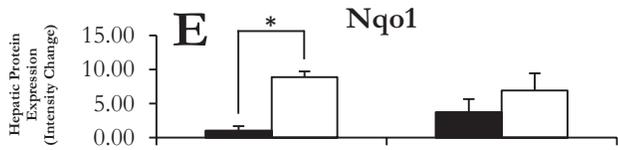
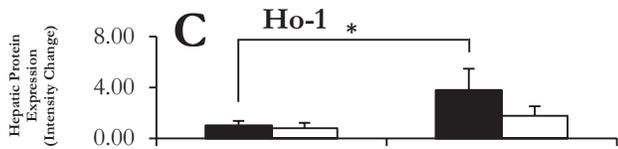
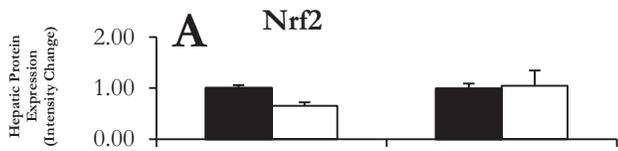
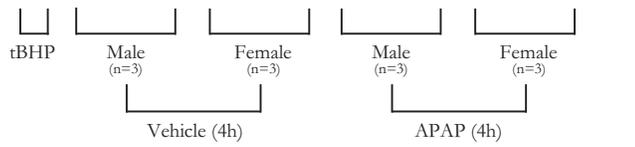
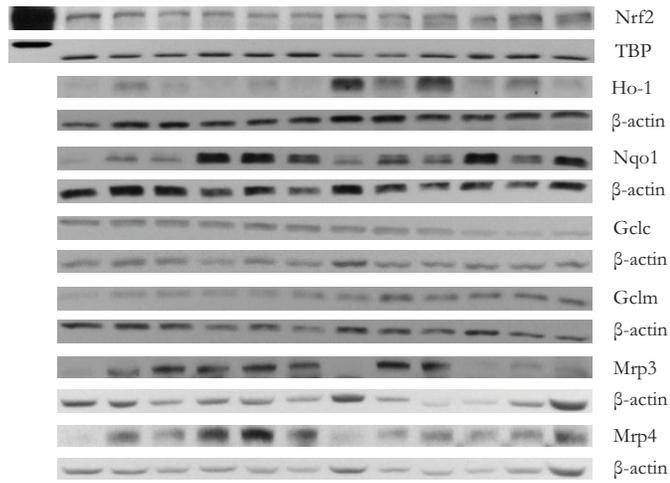


Figure 6. GSH content in naïve wild-type mouse livers. Hepatic GSH levels were measured in male and female naïve WT mice. Data were analyzed using the Student t-test. Data are presented as mean \pm SD (n=6 animals). Data were analyzed using the Student t-test. Asterisks (*) represent a statistical difference ($p<0.05$) between sexes.

Total GSH

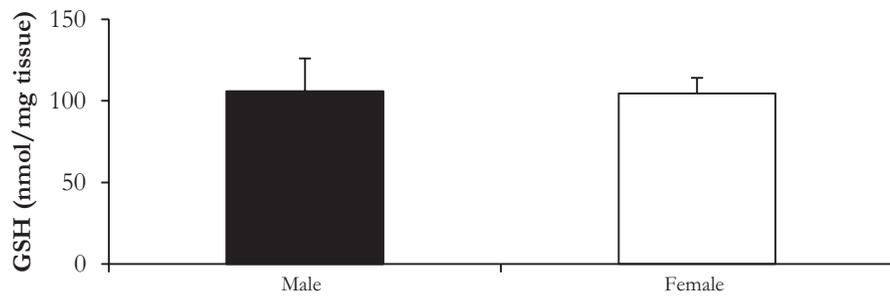


Figure 7. Hepatic GSH levels were measured in vehicle- and APAP-treated (400 mg/kg, i.p.) overnight-fasted WT male and female mice. Data are presented as mean \pm SD (n=6 animals). Data were analyzed using ANOVA followed by Tukey's multiple comparison test. Asterisks (*) represent statistical differences ($p < 0.05$) between groups.

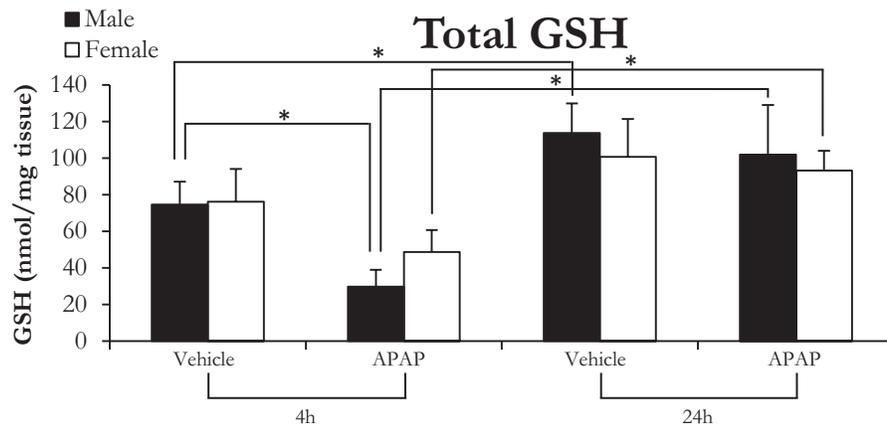


Figure 8. Sex differences in APAP hepatotoxicity in mice. Male and female mice were sacrificed at 24 hrs following APAP administration. (A) Serum ALT activity in Nrf2-null mice at 24 hrs following APAP administration (200mg/kg, i.p., n = 4 to 5 animals). (B) Serum ALT activity in Nrf2-null female mice at 24 hrs following APAP administration (400mg/kg and 600mg/kg, i.p., n = 4 to 5 animals). Data are presented as mean \pm SD. Data were analyzed using ANOVA followed by Tukey's multiple comparison test. Asterisks (*) represent statistical differences ($p < 0.05$) between groups. (C-F) Histopathology of liver sections. Liver sections from male and female Nrf2-null mice at 24 hrs following vehicle or APAP administration were subjected to hematoxylin-eosin staining (10x final magnification). Samples are as follows: C, female/Nrf2-null/200mg/kg APAP/24h; D, male/Nrf2-null/200mg/kg APAP/24h; E, female/Nrf2-null/400mg/kg APAP/24h; F, female/Nrf2-null/600mg/kg APAP/24h. Vehicle not shown.

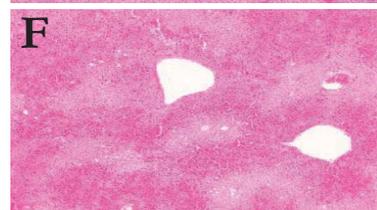
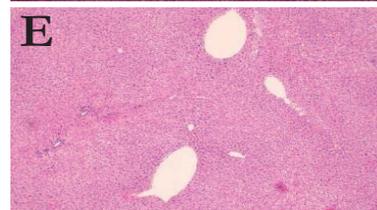
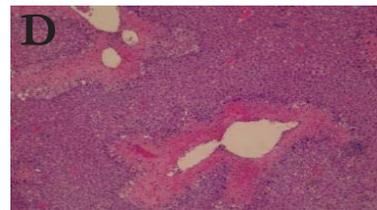
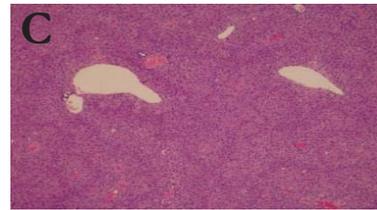
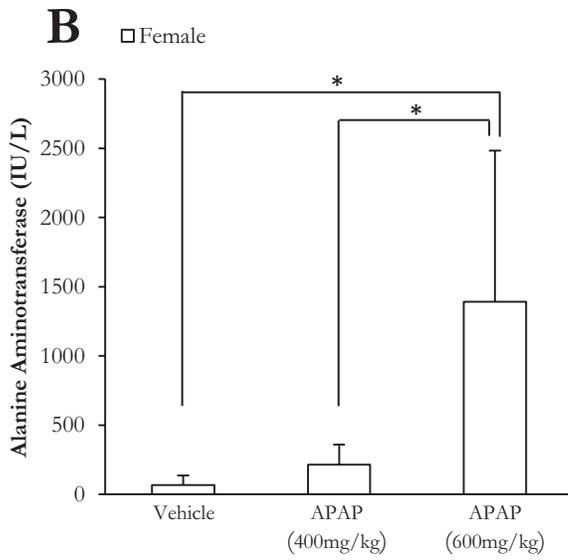
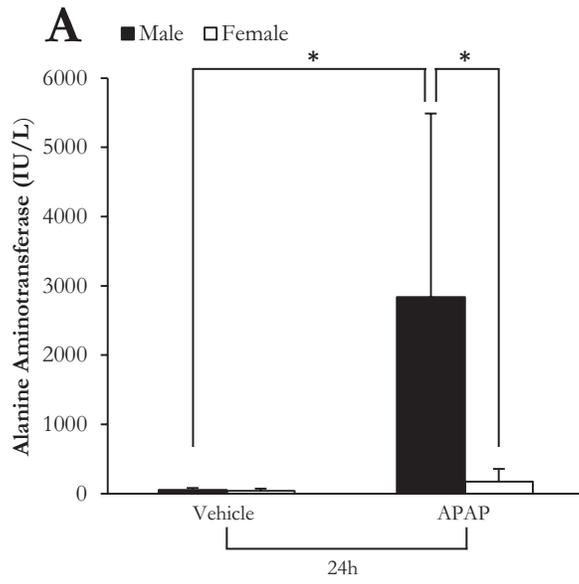


Figure 9. Hepatic messenger RNA expression of *Keap1*, and Nrf2-regulated genes in vehicle- and APAP-treated (200 mg/kg, i.p.) male and female Nrf2-null mice. Data are presented as mean \pm SD (n = 4 to 5 animals). Data were analyzed using ANOVA followed by Tukey's multiple comparison test. Asterisks (*) represent statistical differences ($p < 0.05$) between groups.

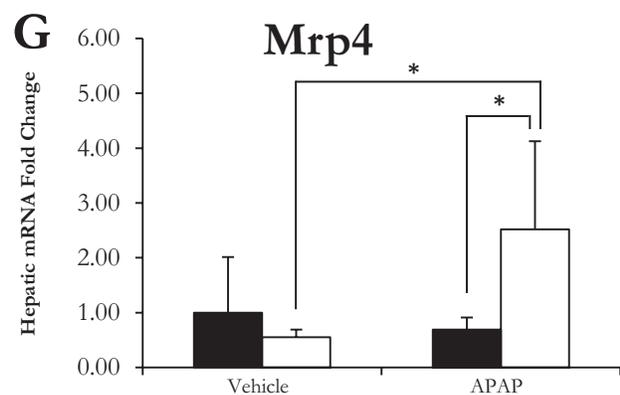
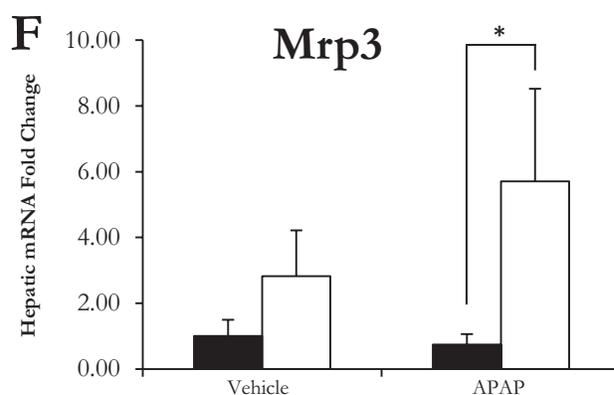
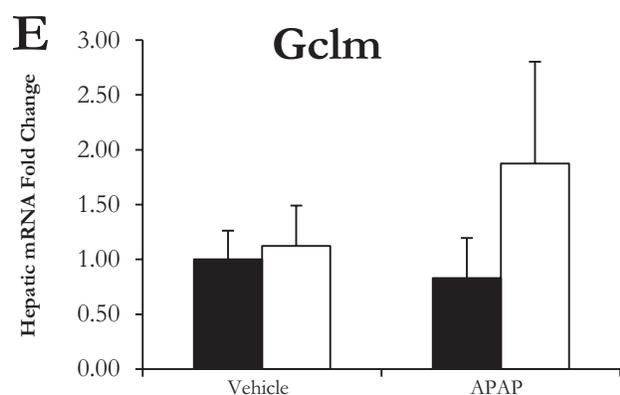
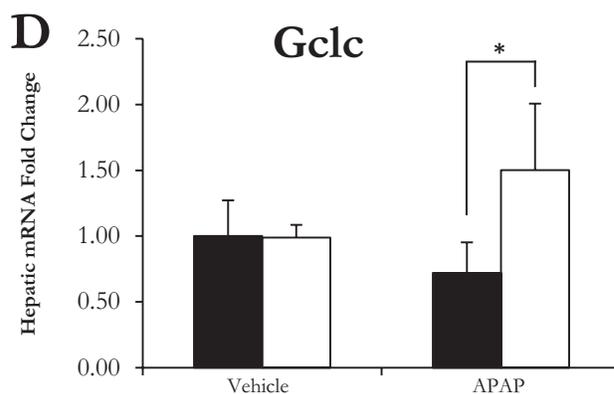
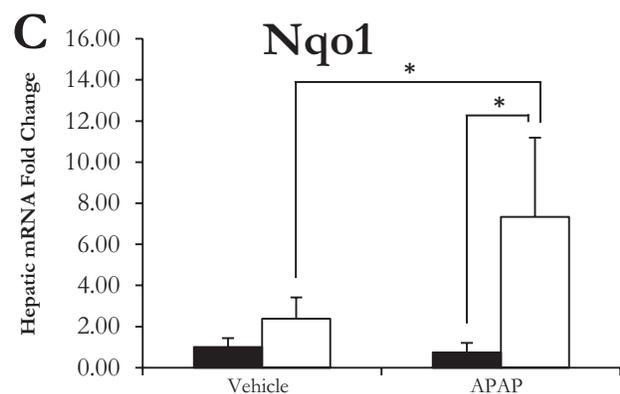
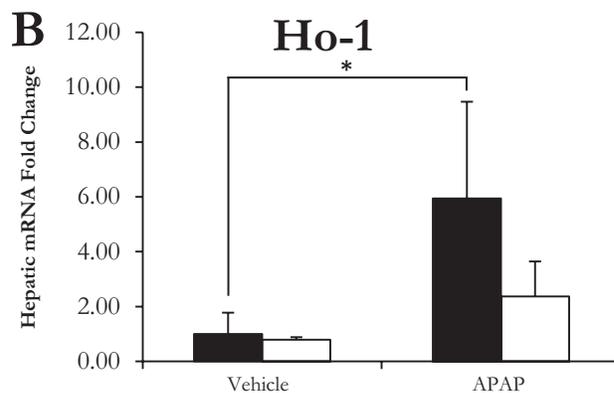
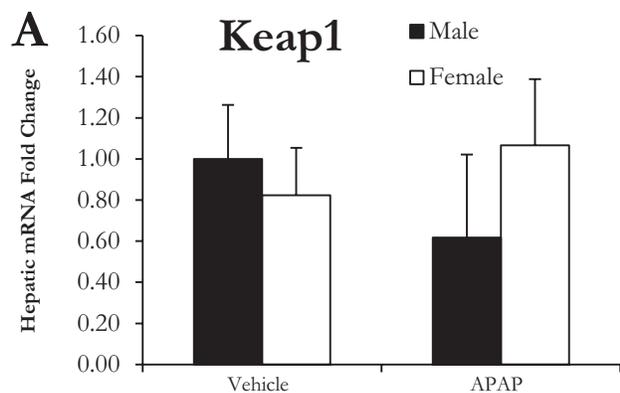


Figure 10. Western blots for Nrf2-regulated genes were performed using liver membrane (Mrp3 and Mrp4) and cytosolic (Nqo1, Gclc, and Gclm) preparations from Nrf2-null male and female mice. Equal protein loading was confirmed by detection of β -actin. Data are presented as blots (upper panel) and as mean \pm SD (n = 3 animals) (lower panel). Data were analyzed using ANOVA followed by Tukey's multiple comparison test. Asterisks (*) represent statistical differences ($p < 0.05$) between groups.

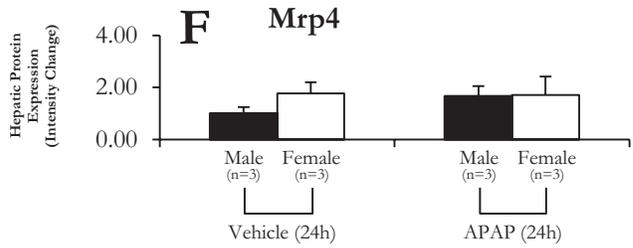
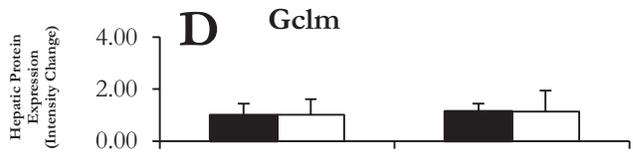
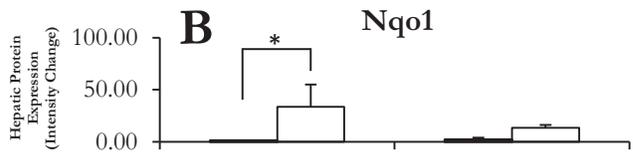
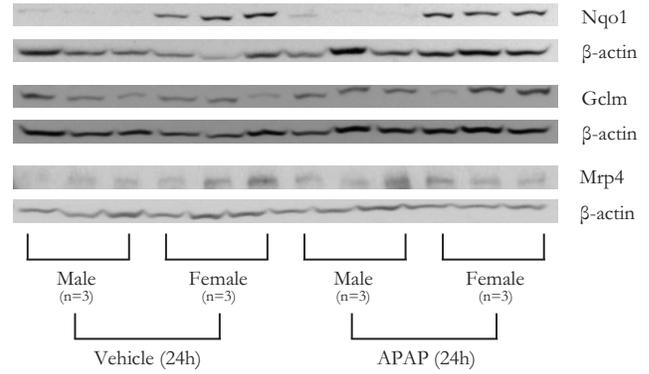
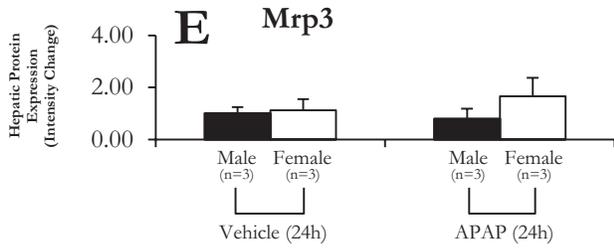
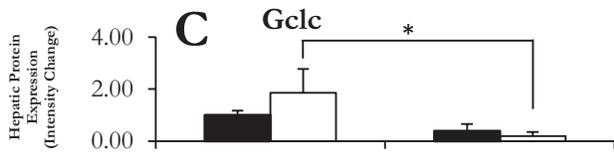
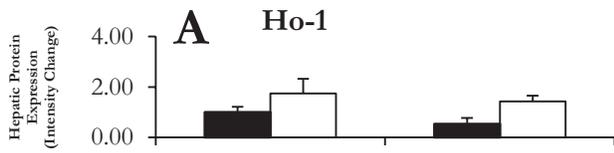
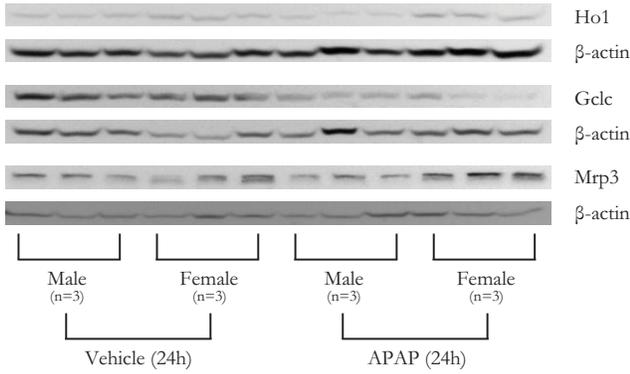


Figure 11. Hepatic GSH levels were measured in vehicle- and APAP-treated (200 mg/kg, i.p.) male and female Nrf2-null mice at 24h. Data are presented as mean \pm SD (n=4 to 5 animals). Data were analyzed using ANOVA followed by Tukey's multiple comparison test. Asterisks (*) represent statistical differences ($p < 0.05$) between groups.

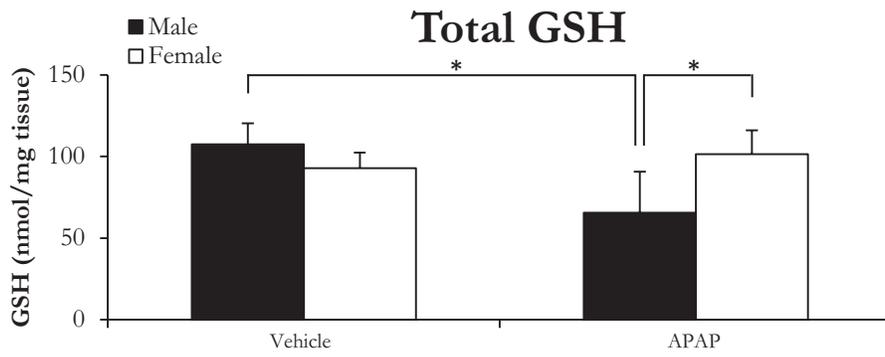


Table 2 - Methods For Immunoblot Analysis

| Protein | Primary Antibody | Primary Antibody Source | Cell Fraction | Primary Antibody Concentration | Primary Antibody Blocking Buffer | Secondary Antibody | Secondary Antibody Source | Secondary Antibody Concentration | Secondary Antibody Blocking Buffer | Protein Molecular Weight (kDa) |
|---------|------------------|------------------------------------|---------------|--------------------------------|----------------------------------|--------------------|----------------------------|----------------------------------|------------------------------------|--------------------------------|
| Nrf2 | 8882S | Cell Signaling ^a | Nuclear | 1:1000 | 5% BSA | anti-rabbit | Sigma ^f (A4914) | 1:1000 | 2% NFDM | ~110 |
| Nqo1 | ab2346 | Abcam ^b | Cytosolic | 1:1000 | 2% NFDM | anti-goat | Sigma ^f (A8919) | 1:10,000 | 2% NFDM | ~32 |
| Ho-1 | SPA-895 | Stressgen Bioreagents ^c | Cytosolic | 1:2000 | 1% NFDM | anti-rabbit | Sigma ^f (A4914) | 1:2000 | 2% NFDM | ~33 |
| Gclc | -- | Washington ^d | Cytosolic | 1:20,000 | 2% NFDM | anti-rabbit | Sigma ^f (A4914) | 1:20,000 | 2% NFDM | ~75 |
| Gclm | -- | Washington ^d | Cytosolic | 1:20,000 | 2% NFDM | anti-rabbit | Sigma ^f (A4914) | 1:20,000 | 2% NFDM | ~28 |
| Mrp3 | SC-5775 | Santa Cruz ^e | Membrane | 1:2000 | 2% NFDM | anti-rat | Sigma ^f (A9037) | 1:2000 | 2% NFDM | ~180 |
| Mrp4 | ab15602 | Abcam ^b | Membrane | 1:2000 | 2% NFDM | anti-rat | Sigma ^f (A9037) | 1:2000 | 2% NFDM | ~160 |
| TBP | ab51841 | Abcam ^b | Nuclear | 1:1000 | 2% NFDM | anti-mouse | Sigma ^f (A4416) | 1:3000 | 2% NFDM | ~38 |
| β-actin | ab8227 | Abcam ^b | Cytosolic | 1:5500 | 2% NFDM | anti-rabbit | Sigma ^f (A4914) | 1:2000 | 2% NFDM | ~42 |

^a Cell Signaling Technology (Danvers, MA)

^b Abcam (Cambridge, MA)

^c Stressgen Bioreagents (Ann Arbor, MI)

^d University of Washington, Seattle, WA, Terry Kavanagh

^e Santa Cruz Biotechnologies (Santa Cruz, CA)

^f Sigma (St. Louis, MO)

Table 3 - Primer Sequences for Quantitative RT-PCR

| Gene | Primer Sequences |
|--------------|---|
| <i>Nrf2</i> | Forward: 5' – TCTATGTCTTGCCTCCAAAGG – 3' Reverse: 5' – CTCAGCATGATGGACTTGGGA – 3' |
| <i>Keap1</i> | Forward: 5' – GGCAGTGTGACAGGTTGAAG – 3' Reverse: 5' – GATCGGCTGCACTGAACTG – 3' |
| <i>Nqo1</i> | Forward: 5' – TTTAGGGTCGTCTTGGCAAC – 3' Reverse: 5' – GTCTTCTCTGAATGGGCCAG – 3' |
| <i>Ho-1</i> | Forward: 5' – GAGCCTGAATCGAGCAGAAC – 3' Reverse: 5' – CCTTCAAGGCCTCAGACAAA – 3' |
| <i>Gcl</i> | Forward: 5' – CTGCACATCTACCACGCAGT – 3' Reverse: 5' – TTCATGATCGAAGGACACCA – 3' |
| <i>Gclm</i> | Forward: 5' – CGGGAACCTGCTCAACTG – 3' Reverse: 5' – TTGGGAACTCCATTCA – 3' |
| <i>Mrp3</i> | Forward: 5' – AGTGGCTGTGATAGTCTTGCTGATA – 3' Reverse: 5' – CCGTTCAGGATCTCGCTCAT – 3' |
| <i>Mrp4</i> | Forward: 5' – ACCTCTGCTCGCGGTGTTCT – 3' Reverse: 5' – CCAGTACCGTTGAAGCTCCTCTCC – 3' |

DISCUSSION

Nrf2 regulates a plethora of genes shown to protect against drug-induced liver toxicity following toxic doses of APAP (Aleksunes, 2007). Furthermore, sex differences in susceptibility to APAP-induced hepatotoxicity have also been reported, where female mice show greater resistance to APAP-induced hepatotoxicity than their male counterparts. In the present study, we have demonstrated that Nrf2 is not responsible for this sex difference. Lower Nrf2 mRNA and protein expression was observed in livers of naïve WT female mice as well as overnight-fasted WT female mice following vehicle- or APAP-treatment than in livers of male mice. Most notably, while ALT activity was significantly elevated in both WT and Nrf2-null male mice following APAP administration, no increases in ALT were observed in either genotype of female mice.

Interestingly, hepatic basal and APAP-inducible mRNA levels of *Nqo1* and *Mrp4* were consistently higher in naïve and overnight-fasted WT and Nrf2-null females than males. This finding suggests that other factors differentially expressed in male and female mice, with greater expression in females than males, may regulate the transcription of these two genes, thereby resulting in greater resistance of female than male mice to APAP-induced hepatotoxicity. Future studies are needed to better understand the sex-dependent susceptibility of mice to APAP hepatotoxicity.

In addition to Nrf2, other nuclear transcription factors including peroxisome proliferator-activated receptor alpha (PPAR α), aryl hydrocarbon receptor (AhR), and c-Jun N-terminal kinase (JNK) also regulate the expression and activity of many cytoprotective genes associated with APAP hepatotoxicity. For example, JNK facilitates the transcription of genes involved in cytoprotection containing activator protein-1 (AP-1) binding sites within their promoter regions including Glutathione *S*-Transferases (*Gst*) (Elsby, 2003). APAP is known to induce AP-1 binding activity, suggesting that JNK may play a protective role against APAP-induced hepatotoxicity. Interestingly,

recent literature has shown 10-fold greater basal expression of mRNA for the *Gst* isoform *Gst π* in livers of male mice compared to females (Dai, 2006) and there is evidence that *Gst π* may be a direct inhibitor of JNK (Elsby, 2003). Recent studies have also shown that *Gst π* -null mice show increased resistance to APAP-induced hepatotoxicity (Henderson, 2000) and the absence of *Gst π* elevates the expression of Ho-1 in liver (Elsby, 2003). *Gst π* is the most abundant *Gst* isoform in mouse liver (Kitteringham, 2003). Further research is necessary to understand whether sex differences in *Gst π* expression may influence JNK expression and sex-dependent susceptibility to APAP hepatotoxicity.

Recent literature has also demonstrated the protective effect of 17 β -Estradiol against APAP-induced hepatotoxicity (Chandrasekaran, 2011). Estrogens are known to elicit antioxidant effects, protecting numerous tissues including the liver (Huh, 1994) from oxidative stress-induced damage. Estradiol has been shown to significantly reduce malondialdehyde (MDA) levels in rats and inhibit iron-mediated lipid peroxidation in rat liver microsomes (Huh, 1994). MDA is a thiobarbituric acid reactive substance (TBAR) formed as a byproduct of lipid peroxidation. Iron is well known to be involved in the initiation and/or progression of lipid peroxidation in the liver, particularly through the Fenton reaction resulting in the production of the free radical and powerful oxidant, the hydroxyl radical. A study by Huh (1994) showed a pronounced sex difference in lipid peroxide levels in rats, with male rats exhibiting a greater level of lipid peroxides than female rats. Literature has also suggested a synergistic interaction between 17 β -Estradiol and Interleukin-6 (Il-6), an inflammatory cytokine that aids in stimulating an immune response. Il-6 promotes sex bias in experimental drug-induced liver injury (DILI) by reducing regulatory T cells (Tregs) (Cho, 2013). Additional studies need to be performed to better understand how 17 β -Estradiol may contribute to sex-related differences in susceptibility to APAP-induced hepatotoxicity.

The present study demonstrates that Nrf2 is not responsible for the resistance of female mice to APAP-induced hepatotoxicity and indicates that other mechanisms must contribute to this

sex difference. Future studies will allow for a better understanding of the different mechanisms that may contribute to the differential susceptibility of male and female mice to APAP-induced hepatotoxicity, and the identification of new gene targets involved in APAP hepatotoxicity will create opportunities for the development of new therapeutic interventions to aid in the prevention and treatment of drug-induced liver injury.

Chapter Three

Summary

Sex is known to play an important role in drug absorption, metabolism, distribution, and excretion (Morris, 2003, Tanaka, 1999). It is, therefore, imperative when studying drug toxicity to understand the differences in susceptibility of drugs between males and females. In turn, clinicians can better adjust dosages of pharmaceuticals to compensate for any differences between sexes. The work presented in this thesis explores the role of Nrf2, a nuclear transcription factor that positively regulates the expression and activity of cytoprotective genes during periods of oxidative stress, in the differential susceptibility of males and females to APAP-induced hepatotoxicity. This work will aid in the progression of identifying new potential gene targets involved in APAP hepatotoxicity, allowing for future opportunities to develop new therapeutic interventions aiding in the prevention and treatment of drug-induced liver injury.

Previous work has shown differences in susceptibility of male and female mice to APAP-induced hepatotoxicity, where female mice show greater resistance to APAP-induced hepatotoxicity than their male counterparts, however, the mechanism(s) underlying these sex differences remains unknown. Current literature reports sex differences in various genes involved in cytoprotection (Dai, 2006, McConnachie, 2007, Masubuchi, 2011, Sheng, 2013). Many of these genes are more highly expressed in females than males and are transcriptionally regulated by Nrf2. Therefore, the experiments in Chapter 2 examine mRNA and protein expression of Nrf2 and Nrf2-dependent genes between male and female mice. The mouse closely resembles the type of response seen in humans intoxicated with APAP in terms of susceptibility to hepatotoxicity and the sequence and timing of events in hepatocyte injury and recovery. Additionally, the availability of genetically-

modified mice to explore mechanisms involved in APAP-induced hepatotoxicity, specifically with respect to Nrf2, is further justification for using a mouse model for these studies.

It was demonstrated in Chapter 2 that Nrf2 is not responsible for the differential susceptibility of male and female mice to APAP-induced hepatotoxicity. Lower Nrf2 mRNA and protein expression was observed in livers of naïve WT female mice as well as overnight-fasted WT female mice following vehicle- or APAP-treatment than in livers of male mice. Most notably, while ALT activity was significantly elevated in both WT and Nrf2-null male mice following APAP administration, no increases in ALT were observed in either genotype of female mice. Interestingly, hepatic mRNA levels of *Nqo1* and *Mrp4* were consistently higher in naïve wild-type, overnight-fasted wild-type, and Nrf2-null females than males. This finding suggests that other factors differentially expressed in male and female mice with greater expression in females than males may regulate the transcription of these two genes, thereby resulting in greater resistance of female than male mice to APAP-induced hepatotoxicity. Future studies, however, are needed to better understand the sex-dependent susceptibility of mice to APAP hepatotoxicity.

The experiments in Chapter 2 were designed to explore the sex differences in the role of various gene targets involved in APAP-induced hepatotoxicity in mice. The results show that other factors, rather than Nrf2 are responsible for the difference in susceptibility of male and female mice to APAP-induced hepatotoxicity. The question remains then, if Nrf2 is not responsible for this sex difference, what is? As mentioned previously, the basal and APAP-inducible mRNA expression levels of *Nqo1* and *Mrp4* were consistently higher in female than male livers. While the transcriptional activity of these genes is known to be regulated by Nrf2, other transcriptional regulators of these genes are known (Aleksunes, 2012). Future *in silico* studies examining the promoter regions of these genes may aid in the identification of new regulatory sequences associated with transcription factors differentially expressed between males and females. Further

understanding of the mechanism(s) contributing to the differential susceptibility of males and females to APAP hepatotoxicity will allow for the identification of new drug targets, thereby improving the development of new therapeutics targeting the treatment and prevention of drug-induced liver injury.

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