Is Nuclear Factor Erythroid 2–Related Factor 2 Responsible for Sex Differences in Susceptibility to Acetaminophen-Induced Hepatotoxicity in Mice?

Philip R. Rohrer, Swetha Rudraiah, Michael J. Goedken, and José E. Manautou

Department of Pharmaceutical Sciences, University of Connecticut, Storrs, Connecticut (P.R.R., S.R., J.E.M.); and Office of Translational Science, Rutgers University, Piscataway, New Jersey (M.J.G.)

Received May 10, 2014; accepted August 4, 2014

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 Nrf2 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, for the first time, differences in mRNA and protein expression for Nrf2 and a battery of Nrf2-dependent genes in naïve wild-type (WT) and overnight-fasted WT and Nrf2-null male and female mice following APAP treatment. Alanine aminotransferase (ALT) activity was measured as an indicator of hepatotoxicity. Hepatic mRNA and

protein levels were measured by quantitative polymerase chain reaction and western blotting, respectively. Contrary to expectations, basal Nrf2 mRNA and protein expression were significantly lower in livers of naïve female than male mice. Although mRNA and/or protein expression of quinone oxidoreductase 1 and multidrug resistance-associated protein 4 was more pronounced in livers of female than male mice under some of the conditions examined, no higher global expression of Nrf2-dependent genes was detected in female mice. Furthermore, ALT activity was significantly elevated in overnight-fasted WT and Nrf2-null male mice following APAP treatment, but no increases in ALT were observed in either genotype of female mice. These results indicate that factors other than Nrf2 are responsible for the lower susceptibility of female mice to APAP hepatotoxicity.

This work was supported by the National Institutes of Health [Grant DK069557].

Part of this work was presented as follows: Rohrer P and Manautou JE (2014) Let's talk about sex: is Nrf2 responsible for sex differences in susceptibility to acetaminophen-induced hepatotoxicity in mice? Poster session presented at: 53rd Society of Toxicology Annual Meeting and ToxExpo; 2014 Mar 23–27; Phoenix, AZ.

Rohrer P and Manautou JE (2013) Let's talk about sex: is Nrf2 responsible for sex differences in susceptibility to acetaminophen-induced hepatotoxicity in mice? Poster session presented at: Northeast Society of Toxicology 2013 Fall Annual Meeting, Toxicological Aspects of the Immune System; 2013 Sep 27; Cambridge, MA.

Rohrer P and Manautou JE (2013) Gender differences in mRNA and protein expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and Nrf2-dependent genes. Poster session presented at: Gordon Research Conference, 2nd Biannual Conference on Cellular & Molecular Mechanisms of Toxicity; 2013 Aug 11–16; Andover, NH.

Rohrer P and Manautou JE (2013) Gender differences in mRNA and protein expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and Nrf2-dependent genes. Poster session presented at: Gordon-Kenan Research Seminar, 1st Biannual Seminar on Cellular & Molecular Mechanisms of Toxicity; 2013 Aug 10–11; Andover, NH.

Rohrer P and Manautou JE (2013) Gender differences in mRNA and protein expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and Nrf2-dependent genes. Poster session presented at: 52nd Society of Toxicology Annual Meeting and ToxExpo; 2013 Mar 10–14; San Antonio, TX.

dx.doi.org/10.1124/dmd.114.059006.

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 et al., 1999; Kang et al., 2004). However, during oxidative stress, Nrf2 dissociates from Keap1, translocates into the nucleus, and activates antioxidant response element–mediated gene transcription. Antioxidant response element–containing genes identified as targets of Nrf2 are involved in many cellular functions, including drug metabolism, reactive oxygen species scavenging, glutathione homeostasis, and efflux transport pathways (Nguyen et al., 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 and Manautou, 2007). Previously, it has been shown that many of these genes are more highly expressed in the livers of female than male mice (Dai et al., 2006; Masubuchi et al., 2011).

Acetaminophen (APAP), a commonly used analgesic and antipyretic, has been attributed to approximately 50% of all acute liver failure cases in the United States (Reuben et al., 2010). At therapeutic doses, the majority of APAP is metabolized in the liver by glucuronidation and sulfation (Heard, 2008). Only a minority of APAP is metabolized in the

ABBREVIATIONS: ALT, alanine aminotransferase; ANOVA, analysis of variance; APAP, acetaminophen; Gclc, glutamate cysteine ligase catalytic subunit; Gclm, glutamate cysteine ligase modifier subunit; GSH, glutathione; Ho-1, heme oxygenase 1; JNK, c-Jun N-terminal kinase; Keap1, Kelch-like ECH-associated protein 1; Mrp, multidrug resistance–associated protein; NAPQI, *N*-acetyl-*p*-benzoquinone imine; Nqo1, NAD(P)H quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2–related factor 2; ST, sucrose-Tris; TBP, TATA-binding protein; WT, wild type.

liver by cytochrome P450 enzymes—Cyp1a2, Cyp3a11 (Cyp3a4 in humans), and Cyp2e1—to a toxic reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) (Dahlin et al., 1984), which is efficiently inactivated by hepatic glutathione (GSH) (Chen et al., 2003). At toxic doses, however, the glucuronidation and sulfation pathways become saturated and excess NAPQI is formed, leading to a rapid depletion of hepatic GSH. Un-neutralized 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 and Bajt, 2006).

Toxic APAP treatment has been shown to trigger nuclear accumulation of Nrf2 in mouse liver (Goldring et al., 2004) and increase expression of downstream Nrf2 target genes, including *Nqo1*, *Ho-1*, and *Gclc* (Goldring et al., 2004; Aleksunes et al., 2006). Furthermore, Nrf2-null mice exhibit enhanced hepatocellular injury compared with their wild-type counterparts following APAP treatment (Chan et al., 2001; Enomoto et al., 2001), thus illustrating that Nrf2 has a pivotal hepatoprotective role in APAP-induced hepatotoxicity.

Sex is known to play an important role in drug absorption, metabolism, distribution, and excretion (Tanaka and Hisawa, 1999; Morris et al., 2003). Previous work has shown sex differences in susceptibility to APAP hepatotoxicity in mice; however, the underlying mechanism(s) remains unknown. Recent studies have shown that male C57BL/6 mice are more susceptible than females to APAP-induced hepatotoxicity (Dai et al., 2006; McConnachie et al., 2007). However, in CD-1 mice, one study reported no sex differences with respect to APAP-induced hepatotoxicity (Hoivik et al., 1995), whereas a more recent study suggests that female CD-1 mice are indeed more resistant to APAP-induced hepatotoxicity than males (Masubuchi et al., 2011). The latter study observed elevated alanine aminotransferase (ALT) activity in male CD-1 mice, but not in female mice, following APAP treatment, and histologic examination further confirmed the higher susceptibility of male CD-1 to APAP toxicity compared to females, as also seen in C57BL/6 mice. Consequently, only a small amount of literature is available 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 cases has been observed in the United States (Larson et al., 2005) and Sweden (Wei et al., 2007). These data, however, may be influenced by concomitant alcohol use, concurrent medications, nutritional status, or differential rates of suicide ideation between genders. Despite this

difference in gender prevalence of APAP hepatotoxicity among humans and mice, the mouse is a useful experimental model to study APAP-induced hepatotoxicity, with males more often used in APAP hepatotoxicity studies. The mechanisms of APAP toxicity in the mouse closely resemble those in humans (McGill et al., 2012). Additionally, the availability of genetically modified mice to explore mechanisms involved in APAP-induced hepatotoxicity is a further justification for using this species as a preferred animal model.

The present study aims to explore sex differences in the expression of cytoprotective genes involved in APAP-induced hepatotoxicity in 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. For this purpose, we measured mRNA and protein expression for Nrf2 and various Nrf2-dependent genes, and hepatic GSH levels in untreated and APAP-treated male and female mice. To further study the role of Nrf2 in the resistance of female mice to APAP hepatotoxicity, we examined the susceptibility of Nrf2-null female mice to APAP.

Materials and Methods

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

Animals and Treatment. Male and female wild-type (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 used were age matched from 8 to 10 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. APAP was dissolved in saline (pH 8.0). Naïve (fed) WT male and female mice were maintained on the laboratory chow and received no treatment. Overnight-fasted WT as well as Nrf2-null male and female mice were fasted overnight prior to treatment with APAP (200, 400, or 600 mg/kg, i.p.) or saline. Plasma and liver were collected at 4 and 12 hours after treatment. 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 (Protocol A12-050).

Assessment of Hepatotoxicity. Plasma ALT activity was determined as a biochemical indicator of hepatocellular necrosis. Infinity ALT Liquid Stable

TABLE 1
Methods for immunoblot analysis

Protein	Primary Antibody	Primary Antibody Source	Cell Fraction	Primary Antibody Concentration	Primary Antibody Blocking Buffer Dilution	Secondary Antibody	Secondary Antibody Source	Secondary Antibody Concentration	Secondary Antibody Blocking Buffer Dilution	Protein Molecular Mass
Nrf2	8882S	Cell Signaling ^a	Nuclear	1:1000	5% BSA	Antirabbit	Sigma ^b	1:1000	2% NFDm	kDa ~110
Nqo1	ab2346	Abcam ^c	Cytosolic	1:1000	2% NFDm	Antigoat	Sigma ^b	1:10,000	2% NFDm	~32
Ho-1	SPA-895	Stressgen Bioreagents ^d	Cytosolic	1:2000	1% NFDm	Antirabbit	Sigma ^b	1:2000	2% NFDm	~33
Gclc	—	Washington ^e	Cytosolic	1:20,000	2% NFDm	Antirabbit	Sigma ^b	1:20,000	2% NFDm	~75
Gclm	—	Washington ^e	Cytosolic	1:20,000	2% NFDm	Antirabbit	Sigma ^b	1:20,000	2% NFDm	~28
Mrp3	SC-5775	Santa Cruz ^f	Membrane	1:2000	2% NFDm	Antirat	Sigma ^b	1:2000	2% NFDm	~180
Mrp4	ab15602	Abcam ^c	Membrane	1:2000	2% NFDm	Antirat	Sigma ^b	1:2000	2% NFDm	~160
TBP	ab51841	Abcam ^c	Nuclear	1:1000	2% NFDm	Antimouse	Sigma ^b	1:3000	2% NFDm	~38
β-actin	ab8227	Abcam ^c	Cytosolic	1:3500	2% NFDm	Antirabbit	Sigma ^b	1:2000	2% NFDm	~42

BSA, bovine serum albumin; NFDm, nonfat dry milk.

^aCell Signaling Technology (Danvers, MA).

^bSigma-Aldrich (St. Louis, MO).

^cAbcam (Cambridge, MA).

^dStressgen Bioreagents (Ann Arbor, MI).

^eUniversity of Washington (Seattle, WA), Terry Kavanagh.

^fSanta Cruz Biotechnologies (Santa Cruz, CA).

TABLE 2

Primer sequences for quantitative real-time polymerase chain reaction

Gene	Primer Sequences
<i>Nrf2</i>	Forward: 5' - TCTATGTCTTGCCTCCAAAGG - 3' Reverse: 5' - CTCAGCATGATGGACTTGGG - 3'
<i>Keap1</i>	Forward: 5' - GGCAGTGTGACAGGTGAAG - 3' Reverse: 5' - GATCGGCTGCACTGAACG - 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>Gclc</i>	Forward: 5' - CTCGACATCTACCACGAGT - 3' Reverse: 5' - TTCATGATCGAAGGACACCA - 3'
<i>Gclm</i>	Forward: 5' - CGGGAACCTGCTCAACTG - 3' Reverse: 5' - TTGGGAATCCATTTCATCA - 3'
<i>Mrp3</i>	Forward: 5' - AGTGGCTGTGATAGTCTTGCTGATA - 3' Reverse: 5' - CCGTTCAGGATCTCGTCTCAT - 3'
<i>Mrp4</i>	Forward: 5' - ACCTCTGCTCGCGGTGTTCT - 3' Reverse: 5' - CCAGTACCGTTGAAGCTCCTCTCC - 3'

Reagent (Thermo Fisher Scientific Inc., Waltham, MA) was used according to the manufacturer's protocol. Hepatotoxicity was confirmed by histologic 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 (Dr. Michael Goedken).

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 minutes 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 10,000g for 20 minutes at 4°C. The supernatant was then centrifuged at 100,000g for 60 minutes. 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 (Sigma-Aldrich) 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 polyvinylidene fluoride-Plus membrane (Micron Separations, Westboro, MA). Immunochemical detection of Nrf2, Nqo1, Ho-1, Gclc, Gclm, Mrp3, and Mrp4 proteins is described in Table 1. In brief, membranes were blocked with 5% nonfat dry milk in Tris-buffered saline-0.1% Tween-20 (pH 7.4) for 1 hour and incubated overnight with the primary antibody diluted in blocking buffer. A species-appropriate peroxidase-labeled secondary antibody (Sigma-Aldrich) was diluted in blocking buffer and incubated with blots for 1 hour. Protein-antibody complexes were detected using a chemiluminescent kit (Thermo Fisher Scientific) followed by exposure to X-ray film. Intensity values were normalized to TATA-binding protein (TBP) for Nrf2 and β -actin for remaining proteins.

Quantitative Real-Time Polymerase Chain Reaction. Quantitative real-time PCR was carried out using an Applied Biosystems 7500 Fast qRT-PCR Machine (Applied Biosystems, Foster City, CA). Primers for *Nrf2*, *Keap1*, *Nqo1*, *Ho-1*, *Gclc*, *Gclm*, *Mrp3*, and *Mrp4* were obtained from Integrated DNA Technologies (Coralville, IA), and expression levels were normalized to β -tubulin. Primer sequences are shown in Table 2. Amplification was carried out in a 20-µl reaction volume containing 8 µl of diluted cDNA (~100 ng), Fast SYBR Green Master Mix (Applied Biosystems), and 1 µM of each primer. The reaction was held at 95°C for 20 seconds followed by a denaturing stage at 95°C for 3 seconds and an annealing and extension stage at 60°C for 30 seconds. The denaturing, annealing, and extension stages were performed for 40 cycles.

Measurement of GSH Levels. Total GSH was measured as previously described (Rahman et al., 2006). In brief, livers were homogenized in 500 µl 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, pH 7.5. Twenty microliters of standards and samples was pipetted into a 96-well microtiter plate. Sixty microliters of β -NADPH (Sigma-Aldrich) was added followed by 120 µl of 1:1 3.33 U/ml glutathione reductase (Sigma-Aldrich) with 0.67 mg/ml 5,5'-dithiobis (2-nitrobenzoic acid). Yellow 5-thio-2-nitrobenzoic acid formation was monitored spectrophotometrically at 412 nm. The rate of formation of 5-thio-2-nitrobenzoic acid is proportional to the concentration of GSH in the sample.

Statistical Analysis. Data are expressed as the mean $\pm$ S.D. Data were analyzed using Student's *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 $P < 0.05$.

Fig. 1. Sex differences in APAP hepatotoxicity in mice. Male and female mice were sacrificed at 4 and 24 hours following APAP treatment. (A) Serum ALT activity in overnight-fasted WT mice following APAP treatment (400 mg/kg, i.p.). Data are presented as the mean $\pm$ S.D. ($n = 6$ animals). Data were analyzed using ANOVA followed by Tukey's multiple comparison test. *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 hours following vehicle or APAP treatment were subjected to hematoxylin-eosin staining (10 $\times$ final magnification). Samples are as follows: female/WT/400 mg/kg APAP/4 hours (B); female/WT/400 mg/kg APAP/24 hours (C); male/WT/400 mg/kg APAP/4 hours (D); male/WT/400 mg/kg APAP/24 hours (E). Vehicle not shown.

Results

Plasma ALT Activity and Histopathology in WT Mice following APAP. APAP-induced hepatocellular injury was assessed by plasma ALT levels and confirmed by histologic examination of liver tissue sections. At 400 mg of APAP/kg, significant elevations in plasma ALT activity at 4 and 24 hours were detected in male WT mice (Fig. 1A). However, ALT activity at 4 and 24 hours after APAP did not increase in female WT mice. Histologic examination and grading of WT male and female liver tissue sections at 4 and 24 hours 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 hours after treatment with APAP, and this damage was even further pronounced at 24 hours (Fig. 1, D, and E). No significant damage was observed in female livers at either time point (Fig. 1, B and C). Histologic grading further confirms these results. In Table 3, liver sections with grades higher than 2 are considered to have significant injury. Treatment with 400 mg of APAP/kg resulted in significant injury in 80% of male WT mice at 24 hours 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* (Fig. 2, A–H). *Nrf2* hepatic mRNA levels in naïve WT females were significantly lower compared with male values (Fig. 2A). No significant differences in mRNA expression between genders were observed for the remaining genes. These results are consistent with the literature, which shows similar basal mRNA levels of *Gclc* in male and female mouse livers (Masubuchi et al., 2011). Masubuchi et al. (2011) also showed significantly greater basal

mRNA expression of *Mrp4* in female mouse livers compared with males. In the current study, however, no statistical sex difference in basal mRNA expression of *Mrp4* was observed. This discrepancy between the prior study and our current study may be a result of differences in gene expression across different mouse strains.

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* (Fig. 3, A–H). No statistical sex difference in hepatic basal *Nrf2* mRNA expression was observed (Fig. 3A). Consistent with the naïve WT mouse data in Fig. 2, no significant differences in mRNA expression in livers of overnight-fasted WT female and male mice treated with vehicle were observed for the Nrf2-dependent genes examined, with the exception of *Nqo1*. Hepatic mRNA expression of *Nqo1* was significantly greater in vehicle-treated female livers compared with male values at 4 hours (Fig. 3C). In the naïve WT livers, no statistical sex difference in basal mRNA expression of *Nqo1* was observed (Fig. 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 between sexes in basal mRNA expression of *Nqo1* in the liver for fed versus 24-hour-fasted mice (Zhang et al., 2013).

A significant induction of *Gclc* mRNA levels was also observed in livers of overnight-fasted WT females at 4 hours following APAP treatment (Fig. 3E). This is consistent with previous work which shows elevated *Gclc* mRNA levels in livers of female mice following APAP treatment (Masubuchi et al., 2011). *Mrp4* mRNA expression was also greatly elevated in livers of overnight-fasted WT females at 24 hours following APAP treatment. Mrp1–4 are plasma membrane efflux transporter proteins that are believed to be involved in the antioxidant response by mediating the efflux of xenobiotics from the liver into the bile or blood (Klaassen and Slitt, 2005). Previous literature has shown *Mrp4* to be a highly inducible antioxidant response protein in the liver of male and female mice following APAP treatment (Aleksunes et al., 2008; Masubuchi et al., 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* (Fig. 4, A–F). Similar to mRNA expression, Nrf2 protein expression was significantly lower in female compared with male livers (Fig. 4A). Hepatic protein expression for the remaining genes, except *Gclc*, followed a pattern similar to 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 with male livers. Significance in protein expression was observed for *Nqo1*, *Gclc*, *Gclm*, and *Mrp3*. Although female livers showed significantly greater *Nqo1* and *Gclc* protein expression than male livers (Fig. 4B), *Gclm* and *Mrp3* protein expression was significantly lower in female than male livers (Fig. 4, D and E).

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* (Fig. 5, A–N). Similar to protein expression in naïve WT mouse livers (Fig. 4), Nrf2 protein expression was less pronounced in livers of overnight-fasted WT female than male mice following either vehicle or APAP treatment. Of the Nrf2-dependent genes examined, females did not exhibit a higher global expression of these genes. Consistent with mRNA expression, protein expression of *Nqo1* was significantly greater in female compared with male livers at 4 hours following vehicle treatment (Fig. 5E). Female livers also showed significantly greater *Mrp4* protein

TABLE 3

Histopathological analysis of livers from APAP-treated WT and Nrf2-null mice

Livers were removed from WT and Nrf2-null mice 4 and 24 hours following vehicle or APAP (200, 400, or 600 mg/kg) treatment 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.

	Histologic Grade						% > 2	Total
	0	1	2	3	4	5		
WT								
4 hours								
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 hours								
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 hours								
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

*Statistical difference ($P < 0.05$) from control mice of the same genotype and sex.

Fig. 2. Messenger RNA expression of *Nrf2* (A), *Keap1* (B), and Nrf2-regulated genes *Nqo1* (C), *Ho-1* (D), *Gclc* (E), *Gclm* (F), *Mrp3* (G), and *Mrp4* (H) in naïve WT male and female mouse livers. Data are presented as the mean $\pm$ S.D. Data were analyzed using Student's *t* test. *Statistical differences ($P < 0.05$) between sexes.

expression than male livers at both 4 and 24 hours following vehicle treatment (Fig. 5, M and N). No statistical differences in protein expression between sexes following vehicle treatment were observed for the remaining genes. A significant induction of Ho-1 protein expression was observed in both sexes at 24 hours following APAP treatment, although no sex difference was observed (Fig. 5D).

Gclc protein expression was significantly decreased in female livers at 4 hours following APAP treatment (Fig. 5G), but returned to basal levels at 24 hours (Fig. 5H). No significant differences in

Gclc protein expression in male livers were observed following APAP treatment. These results are consistent with previous studies showing Gclc protein expression is significantly reduced in female mouse livers following APAP treatment, whereas no significant differences in male livers were observed (Sheng et al., 2013). No statistical differences in Gclm protein expression were observed between female and male livers at either time point tested following either vehicle or APAP treatment (Fig. 5, I and J). Gclc and Gclm are enzymes involved in the production of GSH, an important detoxification pathway in APAP

Fig. 3. Hepatic messenger RNA expression of *Nrf2* (A), *Keap1* (B), and Nrf2-regulated genes *Nqo1* (C), *Ho-1* (D), *Gclc* (E), *Gclm* (F), *Mrp3* (G), and *Mrp4* (H) in vehicle- and APAP-treated (400 mg/kg, i.p.) overnight-fasted WT male and female mice. Data are presented as the mean $\pm$ S.D. ($n = 6$ animals). Data were analyzed using ANOVA followed by Tukey's multiple comparison test. *Statistical differences ($P < 0.05$) between groups.

hepatotoxicity. These data suggest a greater propensity for females to produce more hepatic 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 *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 treatment. No significant differences in total GSH levels were observed between livers of

naïve or overnight-fasted WT male and female mice (Fig. 6, A and B). However, a significantly greater reduction in GSH at 4 hours following APAP treatment was observed in overnight-fasted WT male livers compared with female livers (Fig. 6B). This is consistent with observations of lower toxicity and liver damage in female livers compared with male livers. Glutathione levels rebounded in both sexes at 24 hours following APAP treatment (Fig. 6B), consistent with an increase in *Gclc* mRNA expression at 4 hours following APAP treatment (Fig. 3E).

Fig. 4. Western blots for Nrf2 (A) and Nrf2-regulated genes were performed using liver membrane [Mrp3 (E) and Mrp4 (F)], cytosolic [Nqo1 (B), Gclc (C), and Gclm (D)], and nuclear (Nrf2) preparations from naïve WT male and female mice. Human hepatocyte-derived HCO4 cells treated with 100 μ M *tert*-butyl hydroperoxide (tBHP) for 2 hours 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 the mean $\pm$ S.D. ($n = 6$ animals) (lower panel). Data were analyzed using Student's *t* test. *Statistical differences ($P < 0.05$) between sexes.

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 of APAP/kg, significant elevation in plasma ALT activity at 24 hours was detected in male Nrf2-null mice (Fig. 7A). However, no significant changes in ALT activity were observed in females of the same genotype (Fig. 7A). Histologic examination and grading of liver tissue sections from male and female Nrf2-null mice at 24 hours following APAP treatment further confirms these results (Fig. 7, C and D; Table 3). Treatment with 200 mg of APAP/kg resulted in significant injury in 60% of male Nrf2-null mice compared with 0% of their female counterparts (Table 3). Furthermore, at 400 mg of APAP/kg, significantly elevated ALT activity was still not observed in female Nrf2-null mice (Fig. 7B), and histologic grading did not detect significant injury at this higher dose in female Nrf2-null mice (Table 3). However, at 600 mg of APAP/kg, significantly elevated ALT activity was observed in female Nrf2-null mice (Fig. 7B). Histologic 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 of APAP/kg, with significant injury resulting in 75% of female Nrf2-null mice at this dose (Fig. 7F; Table 3).

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* (Fig. 8, A–G). Significant induction of *Ho-1* was observed in male livers at 24 hours following APAP treatment

(Fig. 8B). *Ho-1* catabolizes pro-oxidant heme to carbon monoxide, biliverdin, and free iron, thereby preventing free heme from facilitating the formation of reactive oxygen species (Aleksunes and Manautou, 2007). The role of oxidative stress in the induction of *Ho-1* during cellular injury is well documented (Guo et al., 2001; Aleksunes et al., 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 hours following APAP treatment (Fig. 8, C and G). Furthermore, at 24 hours following APAP treatment, female livers showed significantly greater mRNA expression of *Nqo1*, *Mrp3*, and *Mrp4* than male livers (Fig. 8, C, F, and 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 (Fig. 9, A–F). Nrf2-null female livers showed significantly greater Nqo1 basal protein expression than their male counterparts at 24 hours following vehicle treatment (Fig. 9B). 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 hours following APAP treatment (Fig. 9C). A similar reduction was observed at 4 hours following APAP treatment in female overnight-fasted WT mouse livers (Fig. 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 treatment. A significantly greater reduction in GSH was observed in male than female livers at 24 hours

Fig. 5. Western blots for Nrf2 (A and B) and Nrf2-regulated genes were performed using liver membrane [Mrp3 (K and L) and Mrp4 (M and N)], cystolic [Nqo1 (E and F), Ho-1 (C and D), Gclc (G and H), and Gclm (I and J)], 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 *tert*-butyl hydroperoxide (tBHP) for 2 hours 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 the mean $\pm$ S.D. ($n = 3$ animals) (lower panel). Data were analyzed using ANOVA followed by Tukey's multiple comparison test. *Statistical differences ($P < 0.05$) between groups.

following APAP treatment (Fig. 6C). This is consistent with observations in livers of overnight-fasted WT male and female mice (Fig. 6B) and observations of lower toxicity and liver damage in Nrf2-null female compared with male livers.

Discussion

Nrf2 regulates a plethora of genes shown to protect against drug-induced liver toxicity following toxic doses of APAP (Aleksunes and Manautou, 2007). Furthermore, sex differences in susceptibility to

Fig. 6. Hepatic GSH levels were measured in untreated naïve WT male and female mice (A), vehicle- and APAP-treated (400 mg/kg, i.p.) overnight-fasted WT male and female mice (B), and vehicle- and APAP-treated (200 mg/kg, i.p.) overnight-fasted Nrf2-null male and female mice (C). Data are expressed as the mean $\pm$ S.D. ($n = 6$ animals). Data were analyzed using Student's t test or ANOVA followed by Tukey's multiple comparison test when more than two groups were present. *Statistical difference ($P < 0.05$) between groups.

APAP-induced hepatotoxicity have also been reported, where female mice show greater resistance to APAP-induced hepatotoxicity than their male counterparts (Dai et al., 2006; Masubuchi et al., 2011). 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 compared with livers of male mice. Most notably, although ALT activity was significantly elevated in both WT and Nrf2-null male mice following APAP treatment, 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 α , aryl hydrocarbon receptor, and c-Jun N-terminal kinase (JNK), also regulate the expression and

Fig. 7. Sex differences in APAP hepatotoxicity in mice. Male and female mice were sacrificed at 24 hours following APAP treatment. (A) Serum ALT activity in Nrf2-null mice at 24 hours following APAP treatment (200 mg/kg, i.p., $n = 4-5$ animals). (B) Serum ALT activity in Nrf2-null female mice at 24 hours following APAP treatment (400 and 600 mg/kg, i.p., $n = 4-5$ animals). Data are presented as the mean $\pm$ S.D. Data were analyzed using ANOVA followed by Tukey's multiple comparison test. *Statistical differences ($P < 0.05$) between groups. (C-F) Histopathology of liver sections. Liver sections from male and female Nrf2-null mice at 24 hours following vehicle or APAP treatment were subjected to hematoxylin-eosin staining (10 $\times$ final magnification). Samples are as follows: female/Nrf2-null/200 mg/kg APAP/24 hours (C); male/Nrf2-null/200 mg/kg APAP/24 hours (D); female/Nrf2-null/400 mg/kg APAP/24 hours (E); and female/Nrf2-null/600 mg/kg APAP/24 hours (F). Vehicle not shown.

activity of many cytoprotective genes associated with APAP hepatotoxicity. For example, JNK facilitates the transcription of genes involved in cytoprotection containing activator protein-1 binding sites within their promoter regions including glutathione *S*-transferases (*Gsts*) (Elsby et al., 2003). APAP is known to induce activator protein-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 with females (Dai et al., 2006), and there is evidence that *Gst π* may be a direct inhibitor of JNK (Elsby et al., 2003). Recent studies have also shown that *Gst π* -null mice show increased resistance to APAP-induced hepatotoxicity (Henderson et al., 2000), and the absence of *Gst π* elevates the expression of Ho-1 in the liver (Elsby et al., 2003). *Gst π* is the most abundant *Gst* isoform in mouse liver (Kitteringham et al., 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 et al., 2011). Estrogens are known to elicit antioxidant effects, protecting numerous tissues, including the liver (Huh et al., 1994), from oxidative stress-induced damage. Estradiol has been shown to

Fig. 8. Hepatic messenger RNA expression of *Keap1* (A) and Nrf2-regulated genes *Ho-1* (B), *Nqo1* (C), *Gclc* (D), *Gclm* (E), *Mrp3* (F), and *Mrp4* (G) in vehicle- and APAP-treated (200 mg/kg, i.p.) male and female Nrf2-null mice. Data are presented as the mean $\pm$ S.D. ($n = 4-5$ animals). Data were analyzed using ANOVA followed by Tukey's multiple comparison test. *Statistical differences ($P < 0.05$) between groups.

significantly reduce malondialdehyde levels in rats and inhibit iron-mediated lipid peroxidation in rat liver microsomes (Huh et al., 1994). Malondialdehyde is a thiobarbituric acid reactive substance 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 et al. (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. The literature has also suggested a synergistic interaction between 17β -estradiol and interleukin-6, an inflammatory cytokine that aids in stimulating an immune response. Interleukin-6 promotes sex bias in experimental drug-induced liver injury by reducing regulatory T cells (Cho et al., 2013). Additional studies need to be performed to better understand how 17β -estradiol may contribute to sex-related differences in susceptibility to APAP-induced hepatotoxicity.

Fig. 9. Western blots for Nrf2-regulated genes were performed using liver membrane [Mrp3 (E) and Mrp4 (F)] and cytosolic [Nqo1 (B), Ho-1 (A), Gclc (C), and Gclm (D)] 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 the mean $\pm$ S.D. ($n = 3$ animals) (lower panel). Data were analyzed using ANOVA followed by Tukey's multiple comparison test. *Statistical differences ($P < 0.05$) between groups.

These findings are the first evidence that female mice do not have higher basal hepatic Nrf2 expression or greater global expression of Nrf2-dependent genes. Our data also demonstrate that, in response to APAP, female mice do not exhibit a more robust Nrf2 response than male mice. Furthermore, our studies with the Nrf2-null mice demonstrate conclusively that this transcription factor is not responsible for the resistance of female mice to APAP-induced hepatotoxicity, and indicate that other mechanisms must contribute to this sex difference. The "ruling out" of a potential involvement of Nrf2 in the resistance of female mice to acetaminophen hepatotoxicity is a seminal scientific contribution due to the well documented role of Nrf2 in establishing thresholds for tolerance to chemicals that produce oxidative stress. 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. The identification of new gene targets involved in APAP hepatotoxicity will lead to new opportunities for the development of new therapeutic interventions to aid in the prevention and treatment of drug-induced liver injury.

Authorship Contributions

Participated in research design: Rohrer, Rudraiah, Manautou.

Conducted experiments: Rohrer.

Performed data analysis: Rohrer, Goedken.

Wrote or contributed to the writing of the manuscript: Rohrer, Manautou.

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Address correspondence to: José E. Manautou, University of Connecticut, Department of Pharmaceutical Sciences, 69 North Eagleville Road, Storrs, CT 06269-3092. E-mail: jose.manautou@uconn.edu