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Lower Susceptibility of Female Mice to Acetaminophen Hepatotoxicity: Role of Mitochondrial Glutathione, Oxidant Stress and c-Jun N-Terminal Kinase

Kuo Du, C. David Williams, Mitchell R. McGill, and Hartmut Jaeschke

Department of Pharmacology, Toxicology & Therapeutics, University of Kansas Medical Center, Kansas City, Kansas, USA

Abstract

Acetaminophen (APAP) overdose causes severe hepatotoxicity in animals and humans. However, the mechanisms underlying the gender differences in susceptibility to APAP overdose in mice have not been clarified. In our study, APAP (300 mg/kg) caused severe liver injury in male mice but 69-77% lower injury in females. No gender difference in metabolic activation of APAP was found. Hepatic glutathione (GSH) was rapidly depleted in both genders, while GSH recovery in female mice was 2.6 fold higher in mitochondria at 4h, and 2.5 and 3.3 fold higher in the total liver at 4h and 6h, respectively. This faster recovery of GSH, which correlated with greater induction of glutamate-cysteine ligase, attenuated mitochondrial oxidative stress in female mice, as suggested by a lower GSSG/GSH ratio at 6h (3.8% in males vs. 1.4% in females) and minimal centrilobular nitrotyrosine staining. While c-jun N-terminal kinase (JNK) activation was similar at 2 and 4h post-APAP, it was 3.1 fold lower at 6h in female mice. However, female mice were still protected by the JNK inhibitor SP600125. 17 β -Estradiol pretreatment moderately decreased liver injury and oxidative stress in male mice without affecting GSH recovery. Conclusion: The lower susceptibility of female mice is achieved by the improved detoxification of reactive oxygen due to accelerated recovery of mitochondrial GSH levels, which attenuates late JNK activation and liver injury. However, even the reduced injury in female mice was still dependent on JNK. While 17 β -estradiol partially protects male mice, it does not affect hepatic GSH recovery.

Keywords

acetaminophen hepatotoxicity; gender difference; protein adducts; glutathione; c-jun N-terminal kinase; oxidant stress

For Correspondence: Dr. Hartmut Jaeschke, Department of Pharmacology, Toxicology & Therapeutics, University of Kansas Medical Center, 3901 Rainbow Blvd, MS 1018, Kansas City, KS 66160, Tel +1 913 588 7969, Fax +1 913 588 7501, hjaeschke@kumc.edu.

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Introduction

Acetaminophen (APAP) is a widely used analgesic and antipyretic drug. Although safe at therapeutic doses, it causes severe hepatotoxicity after an overdose, contributing to 70,000 hospitalizations and around 50% of all cases of acute liver failure in the US each year (Budnitz et al., 2011; Larson et al., 2005; Manthripragada et al., 2011; Nourjah et al., 2006). Despite recent substantial progress in understanding the pathogenesis of APAP hepatotoxicity in rodents (Jaeschke et al., 2011, 2012) and in humans (Antoine et al., 2012; Antoniadis et al., 2012; McGill et al., 2012a; Xie et al., 2014), many questions regarding the mechanisms of APAP-induced liver injury remain to be answered. It is well established that the hepatotoxicity of APAP is initiated by formation of a reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which can be detoxified by conjugation with glutathione (GSH) (Nelson, 1990). However, an excess of NAPQI after APAP overdose depletes GSH and binds to cellular proteins (Cohen et al., 1997). Current evidence suggests that it is the formation of mitochondrial protein adducts rather than total protein binding that plays a critical role in the initiation of the injury (Tirmenstein and Nelson, 1989; McGill et al., 2012b; McGill and Jaeschke, 2013). It is thought that the mitochondrial protein adducts are involved in the inhibition of mitochondrial respiration (Meyers et al., 1988) leading to formation of reactive oxygen (Jaeschke, 1990) and peroxynitrite in mitochondria (Cover et al., 2005). The resulting oxidative stress activates the c-jun *N*-terminal kinases 1/2 (JNK) by multiple pathways (Han et al., 2013). Activated JNK (phospho-JNK) then translocates to the mitochondrial membrane, further amplifies the mitochondrial oxidant stress, triggers the opening of the mitochondrial permeability transition (MPT) pore and leads to cell necrosis (Hanawa et al., 2008; Kon et al., 2004).

Gender can have an impact on the pharmacokinetics and pharmacodynamics of drugs (Morris et al., 2003; Tanaka and Hisawa, 1999). In addition, drug side effects and toxicity arising from gender differences must also be noted. Many studies have reported that female animals were less susceptible than males to toxin-induced liver injury, and that differences in metabolic activation of the hepatotoxins and hormonal levels were primarily responsible (Boelsterli and Lim, 2007; Miller, 2001; Zimmerman, 2000). In contrast, female C57Bl/6 mice are more resistant to APAP-induced liver injury with no difference in metabolic activation of APAP (Dai et al., 2006). Subsequently, it was shown that transgenic mice overexpressing glutamate cysteine ligase (GCL), the rate-limiting enzyme in GSH biosynthesis, had decreased susceptibility to APAP overdose in male mice, but not females, suggesting that the GCL-regulated GSH synthesis is limited in male mice (Botta et al., 2006). This finding was supported by a later study, which reported that pretreatment of CD-1 mice treated with L-buthionine sulfoximine (BSO), an inhibitor of GCL, reversed the gender difference in susceptibility to APAP hepatotoxicity, resulting in higher liver injury in female mice (Masubuchi et al., 2011). Moreover, co-treatment of GSH ethyl ester or N-acetylcysteine (NAC), a GSH biosynthesis precursor, increased the GSH level and afforded partial protection against APAP-induced liver injury in C57Bl/6 male mice, while neither significantly altered the GSH level and liver injury in females (McConnachie et al., 2007). These studies suggest that the recovery of hepatic GSH after its depletion may be a gender-dependent protective factor. Meanwhile, other gender-dependent factors, including the lower

expression of glutathione-s-transferase (Gst) Pi, which may catalyze the conjugation of NAPQI with GSH in female mice (Botta et al., 2006), the disposition of APAP- sulfate and glucuronide resulting from gender-dependent differences in conjugation and transporter expression (Lee et al., 2009), and the highly induced multidrug resistance-associated protein 4 (Mrp4) in female mice (Masubuchi et al., 2011) may also contribute to the gender difference in APAP hepatotoxicity in rodents. However, despite progress, many aspects of the mechanism of the gender difference are still unclear (Rohrer et al., 2014). In particular, the role of JNK activation and oxidant stress in female mice has not been investigated. Therefore, the objective of the present study was to assess the mechanism of the GSH recovery-dependent protection and the effect on JNK activation. In particular, we investigated whether JNK activation and mitochondrial translocation is still a critical event in female mice with reduced liver injury. In addition, we investigated tested the possibility that estrogen treatment of male mice could mimic the reduced injury observed in female mice in order to determine what role hormones play in the gender difference in APAP hepatotoxicity in mice.

Materials and Methods

Animals

Male and female C57Bl/6 mice used in the experiments (8-12 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). All animals were housed in environmentally controlled facilities with a 12h light/dark cycle. The animals had free access to food and water. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center and experimental operations followed the criteria of the National Research Council for the care and use of laboratory animals.

Experiment design

Mice were fasted overnight (12-15h) prior to APAP (Sigma, St Louis, MO) treatment. APAP was dissolved in warm saline and was administered intraperitoneally (i.p.) at a dose of 300 mg/kg. Mice were sacrificed at 30 min or 2 - 24h after APAP injection and then blood and livers were harvested. Some animals received 10 mg/kg of the JNK inhibitor SP600125 (LC Laboratories, Woburn, MA) dissolved in 8.3% DMSO in phosphate-buffered saline (PBS) (1 mg in 125 μ l of DMSO diluted with 1375 μ l of PBS) or the vehicle alone (15 ml/kg) 1h before treatment with 600 mg APAP/kg (Saito et al., 2010a). In another experiment, male mice received 0.2 mg/kg of 17 β -estradiol (Cayman, Ann Arbor, MI) dissolved in 0.1% DMSO in saline or the vehicle alone (10 ml/kg) 3h before treatment with 300 mg APAP/kg. Blood was drawn from the caudal vena cava into a heparinized syringe and centrifuged to obtain plasma for the determination of alanine aminotransferase (ALT) activity (ALT reagent kit, Pointe Scientific, MI). The liver was excised and sectioned. Portions from the left lobe were flash frozen for determination of APAP-protein adducts (APAP-CYS), real-time PCR and Western blotting, or fixed in 10% phosphate-buffered formalin for histology analyses; the portions from the median lobe were flash frozen for measuring glutathione (GSH and GSSG). The right and caudate lobes were used for isolating mitochondria as previously described (Xie et al., 2013).

Total liver and mitochondrial GSH and GSSG

Total liver GSH levels were determined using a modified Tietze assay (Jaeschke and Mitchell, 1990) and mitochondrial GSH levels were measured as previously described (Knight et al., 2002). In brief, frozen tissues (or mitochondria pellet) were homogenized on ice in 3% sulfosalicylic acid containing 0.1 mM EDTA. One aliquot of the homogenate was added to 0.01 N HCl, centrifuged and the supernatant was further diluted with 100mM potassium phosphate buffer (KPP); another aliquot was added to 10mM N-ethylmaleimide (NEM) in KPP to trap GSH. The residual NEM was removed with a C18 SepPack column and GSSG was determined by the Tietze assay using dithionitrobenzoic acid.

mRNA expression and Western Blotting

Expression of selected genes quantified by real-time PCR (RT-PCR) analysis was performed as described previously (Bajt et al., 2008). Briefly, total RNA was extracted from liver tissue using TRI reagent (Sigma, St Louis, MO), reversed transcribed into cDNA using random primers and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) at 0.1µg/µl. The cDNA was diluted 1/10 and 5µl was used as a template in each PCR reaction. SYBR green PCR Master Mix (Applied Biosystems) was applied as the detector. mRNA of glutamate cysteine ligase catalytic subunit (gclc) was evaluated by normalizing to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and then expressed as a fold increase relative to control (arbitrarily set as 1.0). Western blotting was performed as described in detail (Bajt et al., 2000). The antibodies used for JNK were: rabbit anti-JNK and anti-phospho-JNK antibodies (Cell Signaling Technology, Danvers, MA), horseradish peroxidase-coupled donkey anti-rabbit IgG (Santa Cruz).

Total liver and mitochondrial APAP-protein adducts

High-pressure liquid chromatography with electrochemical detection (HPLC-ECD) was used to measure APAP-protein adducts in liver tissues and mitochondrial pellets according to the method of Muldrew et al. (2002) with previously described modifications (Ni et al., 2012; McGill et al., 2012b).

Histology and immunohistochemistry

Formalin-fixed liver samples were embedded in paraffin and 5 µm thick sections were cut. Sections were stained with hematoxylin and eosin (H&E) for evaluation of tissue necrosis (Gujral et al., 2002). Replicate sections were also stained for nitrotyrosine (NT) protein adducts for assessment of peroxynitrite formation using the Dako LSAB peroxidase kit (Dako, Carpinteria, CA) and a rabbit polyclonal anti-nitrotyrosine antibody (Life Technologies, Grand Island, NY) (Knight et al., 2002).

Statistics

All data were expressed as mean ± SE. Statistical significance between two groups was assessed using the Student's t-test. Comparisons of three or more groups were done by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons. For non-normally distributed data, the Kruskal-Wallis Test (non-parametric ANOVA) was used, followed by Dunn's multiple comparisons. $P < 0.05$ was considered significant.

Results

Female mice are less susceptible than males to APAP hepatotoxicity

To evaluate gender-dependent APAP hepatotoxicity in C57Bl/6 mice, both male and female mice were fasted overnight and treated with 300 mg APAP/kg. Groups of animals were sacrificed at various time points between 0.5–24h after APAP treatment and liver injury was assessed by measuring plasma ALT activities and by histological analysis (Fig.1). Although ALT levels significantly increased for both male and female mice at 4h after APAP treatment and beyond, the increase in ALT activities was significantly reduced in female mice (Fig. 1A). The ALT results were confirmed by histology, which showed extensive centrilobular necrosis at 6 and 24h after APAP in male mice while the areas of necrosis were reduced in female mice (Fig. 1B).

No gender difference in metabolic activation of APAP

The key initiating events of APAP hepatotoxicity are reactive metabolite generation, GSH depletion and formation of APAP-protein adducts (Jaeschke et al., 2011). Male and female mice showed similar GSH depletion at 0.5 and 2h (Fig. 2A) and similar protein adduct formation as determined in the total liver and in mitochondria at 2h (Fig. 2B), which is the peak of adduct levels in mouse livers (McGill et al., 2013). Actually, mitochondrial adduct levels were slightly higher in female mice (Fig. 2B). Generally, a dose of 300 mg APAP/kg is completely metabolized by 2h after APAP administration and the hepatic GSH levels start to recover beyond that time (McGill et al., 2013). As shown in Fig. 2A, the recovery of hepatic GSH levels was significantly faster in female mice than in males at 4 and 6 h and, after re-feeding, at 24h.

APAP overdose causes less oxidative stress in female mice

Formation of reactive oxygen species and peroxynitrite in mitochondria is a critical event in APAP hepatotoxicity (Jaeschke et al., 2003). In support of this hypothesis the GSSG levels (not shown) and the GSSG-to-GSH ratio increased significantly at 4, 6 and 24h after APAP (Fig. 2C). In addition, centrilobular staining of nitrotyrosine (NT) protein adducts, a measure of peroxynitrite formation (Hinson et al., 1998), was observed at both time points (Fig. 2D). However, the GSSG-to-GSH ratio was lower and NT staining was reduced in females compared to male mice, indicating reduced oxidant stress and peroxynitrite formation (Fig. 2C,D).

Previous studies have shown the critical role of mitochondrial GSH in scavenging of the mitochondrial oxidant stress and peroxynitrite in male mice (Knight et al., 2002; Saito et al., 2010b). Assessment of mitochondrial GSH levels at 4h after APAP showed more than 2-fold higher mitochondrial GSH content in females than in males (Fig. 3A). Gcl is the rate-limiting enzyme in GSH biosynthesis, which is composed of the catalytic subunit gclc and the modifier subunit gclm (Lu, 2013). Although baseline mRNA levels of gclc were similar in both genders, the induction of gclc mRNA was faster and more extensive in females at all times after APAP treatment (Fig. 3B). The faster recovery of hepatic GSH levels (Fig. 2A) correlated with the higher induction of gclc in female mice (Fig. 3B).

Gender-dependent JNK activation

JNK activation has been shown to be critical in the initiation and exaggeration of APAP-induced liver injury. APAP overdose induces JNK activation (phosphorylation) in the cytosol, and the activated JNK (pJNK) translocates to the mitochondria and amplifies the mitochondrial oxidant stress (Hanawa et al., 2008; Saito et al., 2010a). Therefore, total JNK and pJNK expression were evaluated in the cytosolic and mitochondrial fractions up to 6h after APAP overdose (Fig. 4A). In control livers, there was no activated JNK in the cytosol and neither relevant amounts of JNK nor pJNK were present in mitochondria (Fig. 5A). As early as 2h after APAP, JNK was phosphorylated in the cytosol and pJNK translocated to the mitochondria in both sexes (Fig. 4A). However, whereas JNK activation in the cytosol and mitochondria was well maintained up to 6h in male mice, it was significantly reduced in the cytosolic fractions from female mice and pJNK almost completely disappeared from mitochondria in these animals by 6h (Fig. 4A). Although there was variation in pJNK levels and translocation between individual animals, it may have been the result of differences in individual injury between mice. The densitometric analyses of several blots and calculation of the pJNK-to-JNK ratio confirmed the significant difference between genders and the individual levels of pJNK correlated well with corresponding ALT values (Fig. 4A,B,C).

Previous studies have shown the protective effect of the JNK inhibitor SP600125 against APAP toxicity in male mice (Hanawa et al., 2008; Henderson et al., 2007; Saito et al., 2010a). In order to test if JNK is still important for toxicity in female mice despite the lower injury and shorter time course of JNK activation, they were treated under identical conditions as described for male mice (Saito et al., 2010a) with 10 mg SP600125/kg 1h before administration of 600 mg APAP/kg. The higher dose of APAP was required in order to overcome the protection by the necessary solvent component DMSO, which attenuated liver injury as indicated by plasma ALT activities in female mice by 40% compared to APAP alone (Fig. 5A). Importantly, the JNK inhibitor almost completely eliminated the remaining liver injury (Fig. 5A). These findings were confirmed by assessing necrosis in histological sections (Fig. 5B). The centrilobular necrosis observed in APAP and in APAP plus solvent groups was almost absent in the JNK inhibitor-treated animals (Fig. 5B). Consistent with the drastic protection, fully recovered hepatic GSH levels (Fig. 6A) and no increase in GSSG levels (Fig. 6B) and the GSSG-to-GSH ratio (Fig. 6C) were observed in SP600125-treated animals.

Effect of estradiol on APAP hepatotoxicity

To test whether estrogen plays a role in the gender-dependent GSH recovery in APAP hepatotoxicity, male mice were pretreated with 17 β -estradiol or its vehicle for 3h before the treatment with 300 mg/kg APAP. The mice were sacrificed at 3h and 6h post-APAP. Treatment with 17 β -estradiol moderately protected mice against APAP hepatotoxicity as indicated by significantly lower plasma ALT activities (Fig. 7A) and representative histological sections (Fig. 7B). Assessment of the APAP-protein adduct formation demonstrated that the protection was not caused by inhibition of the metabolic activation of APAP (data not shown). In addition, there was no significant difference in depletion and partial recovery of hepatic GSH levels between the groups (Fig. 7C). Consistent with the partial protection, the GSSG-to-GSH ratio was significantly attenuated in 17 β -estradiol-

treated animals (Fig. 7D). Furthermore, JNK activation and mitochondrial pJNK translocation were not different between groups (data now shown). These data suggest that the reduced injury in 17 β -estradiol-treated animals was not due to an effect on GSH recovery.

Discussion

The objective of this investigation was to gain further insight into the mechanism of gender differences in susceptibility to APAP hepatotoxicity. Our data confirmed that there was no significant difference in the metabolic activation of APAP, but clearly a faster recovery of hepatic and mitochondrial GSH levels which limited the oxidant stress and JNK activation and ultimately the injury. In addition, even the reduced liver injury in female mice is still dependent on JNK. However, the faster recovery of hepatic GSH levels after APAP overdose could not be mimicked by estrogen treatment of male mice.

Mitochondrial GSH and oxidant stress in female mice

Previous studies showed a faster recovery of hepatic GSH levels in female mice, which correlated with reduced liver injury (Masubuchi et al., 2011). When GSH synthesis was inhibited by either a Gclc inhibitor (Masubuchi et al., 2011) or by Gclm-deficiency (McConnachie et al., 2007), female mice became as susceptible to APAP toxicity as male mice. In addition, transgenic male mice overexpressing Gclc were protected against APAP toxicity (Botta et al., 2006). Together these studies strongly support the hypothesis that the capacity to respond to the stress on the GSH pool after an APAP overdose is a determining factor for the toxicity. However, the exact mechanism of protection remained unclear. GSH is the critical scavenger for the reactive metabolite of APAP to prevent protein binding and toxicity (Mitchell et al., 1973). In addition, more recent findings suggest that GSH is also vital for scavenging of reactive oxygen species and peroxynitrite after the metabolism phase (Knight et al., 2002; Saito et al., 2010b). Either mechanism could have explained why GSH re-synthesis could protect. Given that we did not find any difference in early GSH depletion (0.5 h) and protein adduct formation in the total liver or especially in mitochondria (2h), we conclude that the difference in toxicity between male and female animals was not caused by differences in metabolic activation. This is in agreement with a previous investigation assessing protein binding using radiolabeled APAP (Dai et al., 2006).

The main difference between male and female mice was accelerated recovery of hepatic GSH levels in the females, which included higher GSH levels in mitochondria between 2-6 h after APAP administration. This observation appears central to the protection because during that time reactive oxygen and peroxynitrite formation occurs specifically within mitochondria (Jaeschke, 1990; Cover et al., 2005). Although nitrotyrosine could also reflect tyrosyl radical formation by oxidants, potent oxidants known to cause this reaction are lipid alkoxyl (LO•) and lipid peroxyl radicals (LOO•), both of which are generated during lipid peroxidation (Radi, 2013). However, there is no relevant lipid peroxidation during APAP hepatotoxicity in mice (Knight et al., 2003) suggesting that nitrotyrosine protein adducts are less likely an indicator of tyrosyl radicals than of peroxynitrite formation.

When male mice are treated with GSH, which is rapidly degraded in circulation, or N-acetylcysteine (NAC) 1.5h after APAP, these precursors accelerate GSH synthesis and recovery of GSH levels in mitochondria (Knight et al., 2002; James et al., 2003; Saito et al., 2010b). The improved antioxidant levels are more effective in scavenging reactive oxygen and peroxynitrite which ultimately leads to reduced cell death and improved regeneration (Knight et al., 2002; Bajt et al., 2003; James et al., 2003). Interestingly, the protection against APAP hepatotoxicity by treatment with NAC works most effectively in male animals but not in females (McConnachie et al., 2007), suggesting that the GSH synthesis rate with lower expression of the rate-limiting enzyme Gclc in males can be accelerated by providing more substrate. The expression of the rate-limiting GSH biosynthesis enzyme Gcl is regulated by interleukin-4 (IL-4) (Ryan et al., 2012). Thus, male IL-4-deficient mice are much more susceptible to APAP than wild type animals due to the prolonged suppression of GSH synthesis (Ryan et al., 2012). In addition, IL-10/IL-4-double knockout mice are extremely sensitive to even moderate overdoses of APAP (Bourdi et al., 2007). In addition to the reduced GSH synthesis (IL-4^{-/-}), the absence of IL-10 promotes inducible nitric oxide synthase induction and thus nitric oxide and peroxynitrite formation (Bourdi et al., 2002). The role of IL-4 in regulation of GSH synthesis in female mice remains to be investigated.

It is well known that APAP is mostly subject to phase II metabolism and excretion of these metabolites by biliary transporters, e.g. Mrp2, and basolateral transporters such as Mrp4 (McGill and Jaeschke, 2013). Thus, differences in phase II metabolism and excretion (transporter expression) can increase reactive metabolite formation and enhance toxicity (Campion et al., 2008; Lai, 2009; Lee et al., 2006). A gender difference in metabolism and disposition of APAP has been evaluated in livers of C56Bl/6 mice (Lee et al., 2009). Based on these data, there is higher APAP-glucuronide formation and higher biliary as well as basolateral excretion in males compared to females. Although there was also higher biliary APAP-sulfate excretion in males, females excreted much more APAP-sulfate through the Mrp4-dependent basolateral pathway, which correlated with higher Mrp4 protein expression in females (Lee et al., 2009). However, despite these differences in phase II conjugation of APAP, GSH depletion and protein adduct formation, which are the critical initiating events in the pathway of toxicity, were not significantly different between male and female mice (Figure 2A,B). Thus, the data suggest that any differences in metabolism and disposition of APAP were not the determining factor for the gender differences in APAP toxicity.

Role of JNK activation in female mice

The activation of JNK is central to the pathophysiology of APAP-induced cell death in male mice (Gunawan et al., 2006; Henderson et al., 2007). APAP-induced JNK activation is thought to be an amplification mechanism in which the initial oxidant stress after GSH depletion and mitochondrial protein adduct formation cause JNK phosphorylation and pJNK translocation to mitochondria, leading to the amplification of the mitochondrial oxidant stress (Hanawa et al., 2008; Saito et al., 2010a). The enhanced mitochondrial oxidant stress eventually triggers the mitochondrial membrane permeability transition pore opening with collapse of the membrane potential and cessation of ATP synthesis (Kon et al., 2004; Masubuchi et al., 2005; Ramachandran et al., 2011a; LoGuidice and Boelsterli, 2011). The higher susceptibility to APAP of animals partially deficient in the mitochondrial antioxidant

enzyme Sod2 (Fujimoto et al., 2009; Ramachandran et al., 2011b) provides further support for the central role of the mitochondrial oxidant stress in APAP-induced cell necrosis. Thus, at least in male mice, oxidant stress regulates JNK activation, which in turn amplifies ROS and peroxynitrite formation and causes cell death.

Our data indicate that early JNK activation in the cytosol and subsequent translocation of pJNK to the mitochondria are similar in male and female mice. This finding is consistent with the observations that there are no differences in metabolic activation, protein binding or the initial oxidant stress between genders. However, whereas JNK activation and mitochondrial pJNK is maintained for up to 6h after APAP in male mice, the longer term JNK activation, and in particular the translocation of pJNK to mitochondria, is substantially reduced in females by 6h. These findings are consistent with the enhanced scavenging of reactive oxygen and peroxynitrite in female animals during the period between 2 and 6h after APAP treatment. Thus, the improved restoration of the mitochondrial antioxidant capacity in females attenuates the JNK-dependent amplification loop and consequently reduces cell death. Nevertheless, APAP-induced liver injury in females is still JNK-dependent. To demonstrate this effect, a higher dose of APAP had to be used due to the need for DMSO as solvent for the JNK inhibitor SP600125. DMSO is a well-established inhibitor of P450 enzymes (Park et al., 1988), even at very low doses and diluted with saline (Jaeschke et al., 2006). In support of this hypothesis, the solvent control had reduced ALT activities and necrosis. However, the JNK inhibitor-treated group of female mice was completely protected, similar to what had been reported under identical conditions for male mice (Gunawan et al., 2006; Saito et al., 2010a). Thus, JNK activation is equally important for the mechanism of APAP-induced cell death in both genders.

A caveat of this study is the specificity of the JNK inhibitor. SP600125 is specific for JNK1 and JNK2 with $IC_{50} = 0.04 \mu M$ *in vitro* (Bennett et al., 2001). However, SP600125 can also inhibit other MAP kinases such as MKK4 ($IC_{50} = 0.40 \mu M$) and MKK6 ($IC_{50} = 1.0 \mu M$) (Bennett et al., 2001). Because the actual concentrations of the inhibitor *in vivo* are unknown, it is possible that the effect of SP600125 is not only due to inhibition of JNK but may involve other kinases. However, some of the other kinases such as MKK4 are thought to be part of the kinase network, which results in phosphorylation of JNK (Han et al., 2012). This may explain the high efficacy of SP600125 in attenuating APAP hepatotoxicity in both male and female mice. Nevertheless, the critical role of JNK in APAP toxicity has also been shown by gene knockdown experiments (Gunawan et al., 2006) and by the use of different inhibitors (Henderson et al., 2007).

Role of estrogen in APAP hepatotoxicity

One possible hypothesis for the gender difference in GSH recovery and susceptibility to APAP overdose is that estrogen could be responsible for the effect. Previous studies showed that pretreatment with 17 β -estradiol attenuated APAP-induced liver injury (Chandrasekaran et al., 2011). Our experiments supported a moderate protection by 17 β -estradiol treatment and a reduced oxidant stress. However, this effect was not accompanied by improved recovery of GSH levels. Furthermore, estrogen treatment did not affect protein adduct formation or JNK activation. Thus, estrogen treatment of male mice did not mimic the

mechanism of protection observed in female mice. Further studies are needed to identify mediators that are responsible for the reduced susceptibility of female animals.

Clinical relevance of gender difference in APAP hepatotoxicity

Although the lower susceptibility of female mice to APAP overdose is well established, the clinical relevance of these animal findings remains unclear. There is evidence in patients that critical aspects of the mechanism of APAP toxicity such as protein adduct formation and mitochondrial dysfunction and damage are similar to mice (Davern et al., 2006; McGill et al., 2012a). In addition, more detailed analysis of intracellular signaling events in the metabolically competent human hepatocyte cell line HepaRG (McGill et al., 2011) and in freshly isolated human hepatocytes (Xie et al., 2014) highlight the many similarities between mice and humans in the response to an APAP overdose but also show differences in the time line of cell death. It is well recognized that a toxic dose in mice triggers maximal liver injury between 6 and 12h, but the injury in humans peaks around 48h after APAP ingestion (Larson, 2007). It is widely known that female patients dominate cases of APAP hepatotoxicity in both retrospective population-based studies (Kjartansdottir et al., 2012) and in prospective translational investigations (McGill et al., 2012a). The cause for this finding is probably more related to the preferred method of suicide of female patients in Western countries where APAP overdose is common (Hee Ahn et al., 2012) than to their susceptibility to APAP. In fact, although it was noted in one study that there were no differences in outcome between males and females with APAP-induced acute liver failure (Larson et al., 2005), there is no epidemiological study available that specifically addresses the question of gender-dependent susceptibility to APAP hepatotoxicity in humans which also takes into consideration critical factors such as dose and timing of ingestion. Whether metabolism and disposition may have an effect on the species differences between mouse and humans remains unclear at this point (Lai, 2009).

In summary, our study provided evidence for the similar metabolic activation of APAP in male and female C57Bl/6 mice, as indicated by the initial GSH depletion, protein adduct formation and JNK activation. However, enhanced recovery of hepatic and mitochondrial GSH levels, which correlated with higher induction of Gclc, enhanced the scavenging capacity for reactive oxygen and peroxynitrite in the liver of female mice after the metabolism of APAP was over. In addition, the reduced oxidant stress during the progression phase of the injury attenuated prolonged JNK activation and translocation to the mitochondria, which further reduced the amplification of the oxidant stress and consequently substantially limited liver injury in female mice. Translational studies evaluating the relevance of these findings in humans are warranted.

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List of Abbreviations

ALT alanine aminotransferase

APAP	acetaminophen
BSO	L-buthionine sulfoximine
DMSO	dimethyl sulfoxide
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
gclc	glutamate cysteine ligase catalytic subunit
GSH	glutathione
HPLC-ECD	high-pressure liquid chromatography with electrochemical detection
JNK	c-jun <i>N</i> -terminal kinase
pJNK	phospho-JNK
MPTP	mitochondrial membrane permeability transition pore
NAC	N-acetylcysteine
NAPQI	N-acetyl-p-benzoquinone imine
ROS	reactive oxygen species

Highlights

Female mice are less susceptible to acetaminophen overdose than males

GSH depletion and protein adduct formation are similar in both gender

Recovery of hepatic GSH levels is faster in females and correlates with Gclc

Reduced oxidant stress in females leads to reduced JNK activation

JNK activation and mitochondrial translocation is critical in females

Figure 1.

APAP-induced liver injury in C57Bl/6 male and female mice. Animals were treated with 300 mg APAP/kg after overnight fasting, and sacrificed 0-24h post-APAP. (A) Time course of plasma ALT values. (B) Representative H&E-stained liver sections (x50 magnification) of male mice (top row) and female mice (bottom row) at 6h and 24h post-APAP. Data are expressed as mean $\pm$ SE, $n = 4-7$ animals per group. * $P < 0.05$ (compared to controls, $t=0$). # $P < 0.05$ (compared to male mice).

Figure 2a

Figure 2b

Figure 2.

Hepatic GSH levels, APAP-protein adducts in total liver and mitochondria and parameters of oxidant stress in male and female mice. Animals were treated with 300 mg APAP/kg after overnight fasting and sacrificed 0-24h post-APAP. (A) Time course of GSH levels in total liver. (B) Total liver and mitochondrial APAP-cysteine adducts quantified by HPLC-ECD method. (C) Time course of GSSG-to-GSH ratio in total liver. (D) Representative liver

sections stained for nitrotyrosine protein adducts. Data are expressed as mean $\pm$ SE, n = 4-7 animals per group. *P<0.05 (compared to controls, t=0). #P<0.05 (compared to male mice).

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Figure 3.

Mitochondrial GSH and glutamate cysteine ligase catalytic unit (Gclc) mRNA expression in male and female mice. Gender differences in mitochondrial GSH levels (A) and mRNA levels of Gclc (B) in mice treated with APAP. Animals were treated with 300 mg APAP/kg body weight and were sacrificed 0-6h post-APAP. Mitochondrial GSH levels were measured using a modified Tietze assay. Hepatic mRNA of Gclc was determined by real-time RT-PCR. Data are expressed as mean $\pm$ SE, n = 4-7 animals per group. *P<0.05 (compared to controls, t=0). #P<0.05 (compared to male mice).

Figure 4a

Figure 4b

Figure 4.

JNK phosphorylation and mitochondrial translocation in livers from male and female mice. Total JNK and P-JNK were measured by western blotting using cytosolic and mitochondrial fractions from male and female mice treated with 300 mg APAP/kg (A). Densitometric analysis of total JNK and pJNK in the cytosol (B) and the mitochondria (C). Data are expressed as mean $\pm$ SE, $n = 4-7$ animals per group. * $P < 0.05$ (compared to controls, $t=0$). # $P < 0.05$ (compared to male mice).

Figure 5.

Effects of JNK inhibitor SP600125 on APAP-induced liver injury at 12h post-APAP in female mice. SP600125 (10 mg/kg) or DMSO (1.245 ml/kg) as the vehicle control were administered 1h before 600 mg/kg APAP or saline. (A) Plasma ALT values at 12h post-APAP. (B) Representative H&E-stained liver sections (x50 magnification). Data are expressed as mean $\pm$ SE, n = 4-6 animals per group. *P<0.05 (compared to controls). #P<0.05 (compared to vehicle control).

Figure 6.

Effects of JNK inhibitor on APAP-induced oxidative stress in female mice. Mice were pretreated with 10 mg/kg SP600125 or with DMSO for 1h then treated with 600 mg/kg APAP, or APAP alone for 12h. Hepatic GSH (A) and GSSG (B) levels were measured with a modified Tietze assay and the GSSG-to-GSH ratio was calculated (C). Data are expressed as mean $\pm$ SE, $n = 4-6$ animals per group. * $P < 0.05$ (compared to controls). # $P < 0.05$ (compared to the DMSO control).

Figure 7.

Effects of 17 β -estradiol on APAP-induced liver injury and oxidative stress in male mice.

Mice were pretreated with 0.2 mg/kg 17 β -estradiol or with 0.1% DMSO in saline (10 ml/kg) for 3h, then treated with 300 mg/kg APAP, and were sacrificed at 3h and 6h post-APAP. (A) Plasma ALT values at 3h and 6h post-APAP. (B) Representative H&E-stained liver sections at 6h post-APAP (x50 magnification). (C) Hepatic GSH levels measured with a modified Tietze assay. (D) GSSG-to-GSH ratio. Data are expressed as mean $\pm$ SE of n = 3-4 animals per group. *P<0.05 (compared to vehicle controls).