MITOCHONDRIAL OXIDATIVE STRESS AND BIOGENESIS DURING ACETAMINOPHEN HEPATOTOXICITY

BY

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KUO DU

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Abstract

Acetaminophen (APAP) overdose causes severe hepatotoxicity in animals and humans. Although numerous studies have established the existence of an extensive oxidative stress during APAP hepatotoxicity, its source, pathophysiological roles and therapeutic potentials have not been well clarified. In addition, little is known about the mechanisms of recovery of mitochondrial mass and function or the role of mitochondrial biogenesis (MB) in the injury and repair process. The primary purpose of this dissertation was to further investigate the role of mitochondria, especially mitochondrial oxidative stress and MB, in APAP hepatotoxicity.

In the first study, we explored the mechanisms underlying the gender differences in susceptibility to APAP overdose in mice. We demonstrated that female mice are less susceptible to APAP hepatotoxicity. The lower susceptibility of female mice was achieved by the improved detoxification of reactive oxygen due to accelerated recovery of mitochondrial GSH levels, which attenuates late JNK activation and oxidant stress. However, even the reduced injury in female mice was still dependent on c-Jun N-terminal kinase (JNK).

In the second study, we tested the therapeutic potential of targeting mitochondrial oxidant stress. We showed that mitochondria-targeted antioxidant Mito-Tempo (MT) protected against APAP hepatotoxicity in mice. It did not inhibit metabolic activation of APAP but dose-dependently attenuated mitochondrial oxidant stress and prevented the following mitochondrial dysfunction. Comparison of the protection by MT to its analog Tempo highlights the importance of mitochondrial oxidant stress in the development of APAP toxicity. Our study also demonstrated that MT as a treatment alone or together with NAC

offers a better protection than NAC alone, which supports MT as a therapeutic option for APAP overdosed patients.

In the third study, we demonstrated that metformin, a first-line drug to treat type 2 diabetes mellitus, protected against APAP hepatotoxicity in mice. It did not inhibit JNK activation or mitochondrial JNK translocation but significantly attenuated APAP-induced mitochondrial oxidant stress and the subsequent mitochondrial dysfunction, most likely through inhibition of mitochondrial complex I activity. In addition, metformin dose-dependently protected human HepaRG cells, a clinically relevant model for APAP overdose, against APAP induced cell injury, supporting metformin as a therapeutic option for treatment of APAP overdose or acute liver failure in patients.

In the last study, we investigated the role of MB after APAP overdose. We demonstrated that MB occurs selectively in hepatocytes surrounding necrotic areas after APAP hepatotoxicity. While induction of MB protected against the injury and promoted liver regeneration, its inhibition delayed the injury and compromised the regeneration process. Induction of MB may be another promising therapeutic approach for clinical APAP overdose in the future.

In summary, this dissertation further demonstrates that mitochondrial oxidant stress and dysfunction play a critical role in APAP hepatotoxicity, and targeting mitochondrial oxidant stress and MB can be promising therapeutic approaches for patients with APAP overdose.

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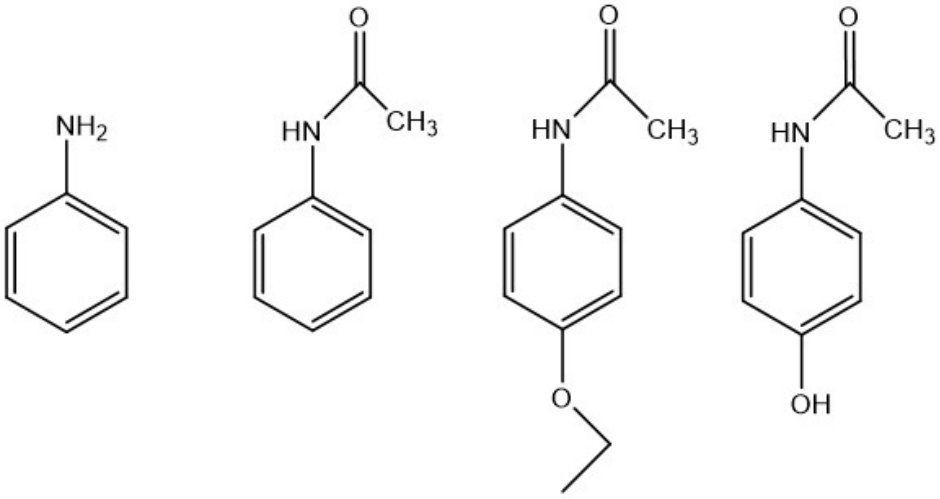
Chapter 1. Introduction

1.1 Background of Acetaminophen (APAP)

Despite the intense popularity of APAP, its discovery and wide spread application have been full of twists and turns. The history of APAP could go back to 1852, when its analog acetanilide, a structural derivative of aniline, was first synthesized and reported (Figure 1.1). In a lucky accident in 1886, acetanilide was mistakenly dispensed to a patient by a pharmacist instead of naphthalene, for treatment of intestinal worms. Interestingly, while it did not help to kill the worms, a significant antipyretic effect for the drug was found (Cahn and Hepp, 1886). Acetanilide was soon marketed as Antifebrin, and its analgesic effect was soon discovered by its continuous use. Around this time, another structural derivative of aniline, i.e. phenacetin, was also discovered and found to have strong analgesic and antipyretic effects. However, while effective, the usage of both was greatly limited by their significant adverse effects, including methemoglobinemia (David Josephy, 2005; Fürstenwerth, 2011).

The search for a safer alternative became urgent and APAP caught the attention of the chemists and pharmacists. Although APAP was synthesized in 1878, it was not used clinically until 10 years later. However, similar side effects including methemoglobinemia were also found in APAP-treated patients and APAP was thus not developed further. Fortunately, in the 1940's, studies of the mechanism of acetanilide-induced methemoglobinemia demonstrated that the adverse effect observed in earlier APAP clinical trials was actually due to an impurity in the formulations (small percentage of the drug was converted to aniline). More importantly, they also demonstrated that APAP is the metabolite responsible for the analgesic effect of



Aniline Acetanilide Phenacetin APAP

Figure 1.1: Structural derivatives of aniline.

the earlier drugs (Greenberg and Lester,1946; Lester and Greenberg, 1947; Greenberg and Lester, 1947). APAP was soon marketed by McNeil Laboratories in 1955 as Tylenol, an abbreviation of N-acetyl-para-aminophenol. Effective analgesia and antipyretic without risk of methemoglobinemia or adverse gastrointestinal effects quickly made APAP a popular drug in the US market, and soon spread throughout the world.

Currently, APAP has been used to treat fevers and pains such as headaches, muscle aches, arthritis, backaches, toothaches. Although the exact mechanisms of its pharmacological effects are still not well clarified, it is generally believed that APAP induces its analgesic effects by mainly inhibiting cyclooxygenase-2 (COX-2) (Flower and Vane, 1972). APAP reduces the oxidized form of the COX enzyme, thus decreasing the formation of inflammatory mediators (Bertolini et al., 2006). Recently, it was also suggested that N-acetyl-p-benzoquinone imine (NAPQI), the reactive metabolite of APAP, binds to TRPA1-receptors in the spinal cord to block signal transduction to alleviate pain (Andersson et al., 2011). The antipyretic activity of APAP is proposed to occur by the decrease in pyrogenic prostaglandin E2 in the CNS, which lowers the hypothalamic set-point in the thermoregulatory center (Aronoff and Neilson, 2001).

1.2 APAP metabolism and hepatotoxicity

1.2.1 APAP metabolism

APAP is a weak acid with pKa =9.5. Therefore, at physiological pH it is rapidly absorbed from the small intestine after oral administration. Thus, the plasma concentration of APAP is commonly used for measurement of gastric emptying following ingestion (Heading et al., 1973). In humans, the half-life of APAP at therapeutic doses in blood is 1.5-3h, but increased when it is overdosed (Nelson and Morioka, 1963; Cummings et al., 1967). Acetaminophen is metabolized in the liver, where the majority of the drug is either glucuronidated or sulfated and then excreted in the urine (McGill and Jaeschke, 2013; Xie et al., 2015a) (Figure 1.2). 50-70% of the drug is eliminated by glucuronidation (McGill and Jaeschke, 2013). The importance of glucuronidation in the metabolism and detoxification of APAP is supported by both rodent studies and clinical evidence. There are four families of uridine 5'-diphospho-glucuronosyltransferases (UGT1, UGT2, UGT3 and UGT8) in humans and rodents (Mackenzie et al., 2005). Interestingly, obese mice expressing higher level of UGTs have higher concentrations of APAP-glucuronide in plasma (Xu et al., 2012; Aubert et al., 2012). In contrast, Gunn rats, which are poor bilirubin glucuronidators, have a lower level of APAP-glucuronide and therefore an increased formation of downstream reactive metabolite NAPQI but with normal levels of UGT, and they were proved to be more susceptible to APAP hepatotoxicity than other rat strains (de Morais and Wells, 1989). In the clinic, Gilbert's syndrome patients that are deficient in UGT1A1 also demonstrated higher susceptibility to APAP hepatotoxicity, indicating that the UGT1A1 isoform is critical for APAP metabolism and detoxification (Monaghan et al., 1996;

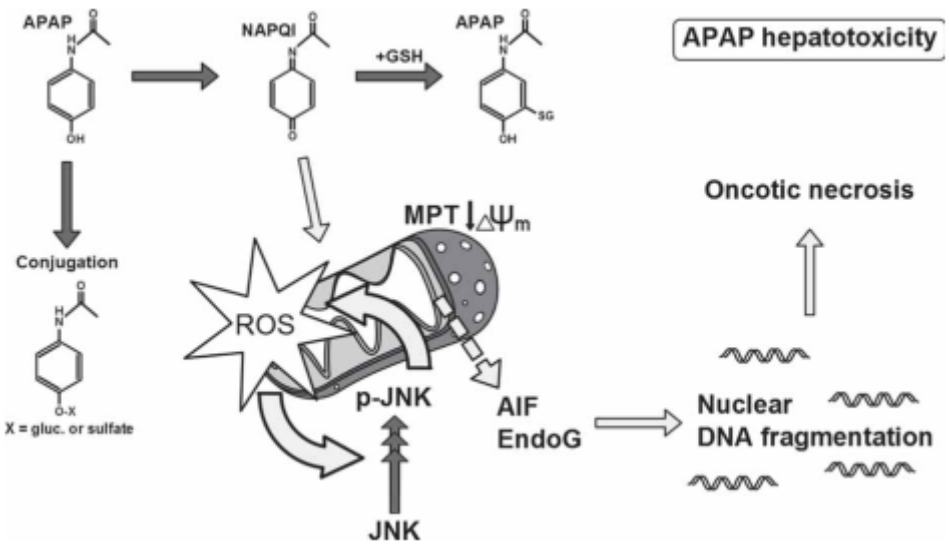


Figure 1.2. Mechanisms of APAP Hepatotoxicity.

At therapeutic doses, most APAP is conjugated and excreted. A small amount is converted into the reactive metabolite NAPQI, and this can be effectively detoxified by glutathione. However, after an overdose, excess NAPQI binds to proteins. Mitochondrial protein alkylation leads to oxidative stress which activates multiple signaling pathways converging on the JNK 1/2. Activated JNK (pJNK) then translocates into mitochondria and increases the oxidative stress. Eventually, the mitochondrial MPT occurs. Matrix swelling and lysis of the outer mitochondrial membrane, as well as translocation of Bax, facilitates release of the endonucleases AIF and EndoG from the intermembrane space. The endonucleases then translocate to the nucleus and fragment nuclear DNA. Ultimately, the cell dies by oncotic necrosis.

(AIF: Apoptosis-inducing factor: APAP: Acetaminophen; EndoG: Endonuclease G; GSH: Glutathione; JNK: c-Jun N-terminal kinases; MPT: Membrane permeability transition; NAPQI: N-acetyl-p-benzoquinone imine)

Clarke et al., 1997). Epidemiological evidence in later studies also demonstrated a strong correlation of another isoform UGT 1A6 to APAP susceptibility (de Morais et al., 1992; Court et al., 2001; Navarro et al., 2004).

Less work has been done for APAP sulfation (20-30% of the drug). Sulfation of xenobiotics is usually catalyzed by cytosolic SULTs, and at least thirteen SULT isoforms belonging to four families exist in humans (Lindsay et al., 2008). A number of studies have been performed to demonstrate the importance of sulfation in APAP metabolism. For example, mice lacking NaS_1, a kidney transporter involved in reabsorption of inorganic sulfate (SO_4^{2-}), are mo0-re susceptible to APAP hepatotoxicity (Lee et al., 2006). Using platelet preparations as surrogates for xenobiotic metabolism in the liver, SULT1A1 and 1A3/4 were found to catalyze APAP sulfation. Recently this list expanded to include SULT1E1 (Reiter and Weinshilboum, 1982; Adjei et al., 2008). Another piece of data is from steatotic livers from humans. These patients demonstrate a significantly higher SULT1A1 protein level and a subsequent higher APAP-sulfation conjugate in the plasma when compared to livers from normal patients (Hardwick et al., 2012).

At therapeutic dose, around 5-15% of APAP is converted by cytochrome P450 enzymes into a reactive intermediate N-acetyl-p-benzoquinone imine (NAPQI) through N-hydroxylation and dehydration (McGill and Jaeschke, 2013) (Figure 1.2). CYP2E1 is the major CYP450 enzyme that is responsible for the conversion of APAP to NAPQI. This is supported by numerous studies (reviewed in McGill and Jaeschke, 2013). For example, Cyp2e1 knockout mice are much less susceptible to APAP-induced acute liver injury (Lee et al., 1996; Cheung et al., 2005). In contrast, mice treated with Cyp2e1 inducers form higher levels of NAPQI and therefore are more susceptible to APAP toxicity (Lee et

al., 1996; McGill and Jaeschke, 2013). Other studies indicate that CYP1A2, 2D6 and 3A4 also metabolize APAP to NAPQI (Thummel et al., 1993; Patten et al., 1993; Dong et al., 2000). NAPQI binds to the cysteine thiol of GSH and APAP-GSH is initially excreted in bile, degraded in kidney and ultimately excreted in urine as a mercapturic acid or cysteine conjugate (McGill and Jaeschke, 2013). Enzymatic GSH conjugation is catalyzed by gluththione-S-transferases (GST). The isoform GST-Pi was suggested to be responsible for the enzymatic conjugation of NAPQI to GSH (Coles et al., 1988). A recent study also showed that GSTT and GSTM functional alteration can affect APAP hepatotoxicity (Lucena et al., 2008).

1.2.2 APAP hepatotoxicity

APAP is the most commonly used pain reliever and fever reducer in the US. It is available both as a single entity formulation and in combination with other medications. In 2008, 24.6 billion doses of APAP were sold (80% of which are in OTC products) and an estimated 48 million Americans used APAP-containing products every week (Krenzelok, 2009). Although it is considered safe at therapeutic doses, APAP overdose causes severe liver injury, contributing to around 70,000 hospitalizations and 50% of cases of acute liver failure each year in the US (Budnitz et al., 2011; Manthripragada et al., 2011). APAP alone and APAP-combination products have been listed as the fourth and sixth highest causes of poisoning-related fatalities, respectively (Mowry et al., 2013; Lancaster et al., 2014). Although many important questions regarding the mechanisms of APAP-induced liver injury remain to be answered, extensive studies performed in the last two decades using in

vitro and in vivo rodent models (Jaeschke et al., 2012; McGill et al., 2011) as well as data from humans (McGill and Jaeschke, 2014; Jaeschke, 2015), have resulted in significant progress in our understanding of key mechanistic steps.

It is well established that APAP overdose leads to formation of excess NAPQI, which depletes GSH and binds to proteins (Figure 1.2). APAP-protein adducts can be measured in liver tissues and plasma samples in both rodents and humans (McGill and Jaeschke, 2013). Recently, measurement of plasma APAP-protein adducts was established as a sensitive and convincing diagnosis marker for APAP overdose patients (McGill and Jaeschke, 2013). Protein binding in mitochondria is recognized as the initiating event in APAP toxicity (Nelson, 1990). Adduction to mitochondrial proteins disturbs the mitochondria electron transport chain and enhances the formation of reactive oxygen species (ROS) and peroxynitrite (Meyers et al., 1988; Jaeschke, 1990; Cover et al., 2005). The initial oxidant stress is amplified through c-jun-N-terminal kinase (JNK) signaling, leading to extensive mitochondrial dysfunction (reviewed in Du et al., 2015c), membrane permeability transition pore opening and collapse of the mitochondrial membrane potential (Kon et al., 2004). In addition, mitochondrial dysfunction causes release of mitochondrial nucleases including Apoptosis Inducing Factor (AIF) and Endonuclease G, which cleave nuclear DNA (Bajt et al., 2006). These signaling events induce cellular necrosis (Gujral et al., 2002). N-acetylcysteine (NAC) was introduced as the clinical antidote against APAP poisoning in the 1970s (Prescott et al., 1977). Multiple protective mechanisms are reported for NAC, including replenishment of the GSH pool to scavenge NAPQI during the metabolism phase (Corcoran et al., 1985; Corcoran and Wong, 1986) or later scavenging of ROS and peroxynitrite in the mitochondria (Knight et al., 2002;

Cover et al., 2005). However, although NAC is very effective during the early phase of the injury, many patients seek medical attention relatively late, when NAC is no longer effective (Larson, 2007). Therefore, a drug that is effective after the metabolism phase is needed.

1.3 Oxidative stress and antioxidant defense in liver

Oxidative stress reflects an imbalance between an increased production of oxidizing species and a compromised effectiveness of antioxidant defenses. The excessive peroxides and free radicals, including both the reactive oxygen species (ROS) and reactive nitrogen species (RNS), damage critical components of the cell (proteins, lipids and DNA), and cellular redox homeostasis disturbances alter redox signaling and even lead to cell death (Jaeschke et al., 2012). In humans, oxidative stress has been reported to be involved in the development of neurodegenerative diseases, cardiovascular disease, carcinogenesis and numerous other conditions (Barnham et al., 2004; Dhalla et al., 2002; Valko et al., 2006). The liver is a major organ that is constantly insulted by ROS/RNS due to its vital metabolic function and is constantly exposure to a number of toxic substances, such as environmental pollutants, alcohol and drugs. Not surprisingly, oxidant stress also plays a critical role in varied liver pathophysiologies including endotoxemia, bile duct ligation, acetaminophen overdose and ischemia-reperfusion injury (Jaeschke et al., 2012). Interestingly, however, ROS/RNS can be sometimes beneficial. For example, short-term and low level oxidative stress induces mitohormesis, which might be important in prevention of aging (Gems and Partridge, 2008). Moreover,

production of lethal oxidizing species is a central mechanism for the immune-mediated pathogen eradication (Segal, 2005).

The most invoked source of oxidative stress in the liver is the mitochondrion. Even under physiological conditions, about 2% of electrons passing through the electron transport chain is incompletely reduced to give the superoxide radical (O_2^-), mostly from Complex I and Complex III. O_2^- can undergo further single-electron reductions, giving rise to the formation of other ROS, including hydrogen peroxide (+2e) and hydroxyl radicals (+3e). Hydroxyl radicals can initiate lipid peroxidation (LPO), which produces a large number of fatty acid hydroperoxides and alkoxy radicals. O_2^- also reacts with nitric oxide and results in the formation of peroxynitrite, a highly reactive nitrogen species (RNS) (Jaeschke et al., 2012). The other intracellular sources of ROS include cytochondrome P450 and its reductases (superoxide and hydrogen peroxide), as well as various oxidases within the cell, such as xanthine oxidase in the cytosol and fatty acid oxidases in peroxisomes (Gonzalez, 2007). The most relevant non-parenchymal sources of ROS formation in the liver are resident macrophages (Kupffer cells) and infiltrating neutrophils and monocytes. The phagocytes express nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX2) that can synthesize and release O_2^- and hydrogen peroxide into the extracellular space and attacks the target cells when they are activated (Bedard and Krause, 2007). In particular, neutrophils also generates hypochlorous acid through myeloperoxidase-mediated peroxidation of chloride ions, which diffuse into target cells and oxidize target molecules and form chlorotyrosine protein adducts.

Multiple layers of sophisticated antioxidant defense systems evolved to protect against oxidative damage to the liver (Jaeschke et al., 2012). In each liver cell, O_2^- is readily

detoxified by superoxide dismutases (SOD1 in the cytosol; SOD2 in mitochondria) into oxygen and hydrogen peroxide. Several enzymatic defense mechanisms are used to detoxify hydrogen peroxide, including glutathione peroxidases (cytosol and mitochondria), catalase (peroxisomes), thioredoxins and peroxiredoxins (cytosol and mitochondria). In addition, liver cells also contain membrane-bound chain-breaking antioxidants vitamin E (α-tocopherol), water-soluble vitamin C (ascorbate) and millimolar concentrations of glutathione in almost all cellular compartments. Although these low molecular weight antioxidants are less specific than the above antioxidant enzymes to detoxify ROS/RNS, their large quantity and wide-spread existence account for the bulk of the total antioxidative capacity of the liver. Furthermore, since some free radical processes, such as lipid peroxidation (LPO), require redox-active transition metals (such as Cu^{+} and Fe^{2+}) to produce oxidizing species, another antioxidant strategy occupied by the liver is to sequester these metal ions with metalloproteins. Two such examples would be ferritin and transferrin, which store iron in the less redox-active ferric state and prevent its catalytic effect in the propagation of LPO. Due to this multilayer defense system, the liver can effectively detoxify ROS/RNS in spite of the sustained onslaught by various oxidants (Jaeschke and Ramachandran, 2011).

Unfortunately however, despite the wide-spread involvement of oxidative stress in numerous pathophysiologies including liver diseases, there is no drug available that specifically target ROS/RNS. This is likely due to the limited understanding of oxidative stress under specific circumstances. Frequently, in a large number of published papers, only evidence of general oxidant stress formation is provided without an assessment of other much more important questions regarding its pathophysiological relevance: (1)

what specific ROS/RNS are generated? (2) when are they generated? (3) where are they generated (source, location)? (4) Quantitatively, how much are they? (5) what specific signaling pathways are activated and/or what type of damage do they cause? (6) how do they cause the organelle dysfunction and overall cell injury? (Jaeschke et al., 2012). Inattention to these issues has led to misinterpretation of experimental results and ultimately jeopardized the translation of new therapeutic approaches to the human pathophysiology. To improve this, we ask for a more detailed and in-depth assessment of these questions in any future studies regarding the role of oxidative stress in human pathophysiology, including APAP hepatotoxicity.

1.4 Critical role of mitochondria in APAP-induced hepatotoxicity

The mitochondria are double membrane-bound organelles, which consist of a soluble matrix, an inner ion-impermeable membrane, and an outer membrane permeable to molecules < 5 kDa. It is well known that the mitochondrion is the cellular site for the Krebs cycle, fatty acid oxidation, heme biosynthesis, and most notably the site of the electron transport chain and oxidative phosphorylation to drive the synthesis of ATP (Scarpulla, 2008). Besides serving as the powerhouse of the cell, the mitochondrion also has important roles in other tasks, such as cell cycle regulation, cell survival and cell death signaling (Tait and Green, 2012; Kroemer et al., 2007; Green et al., 2011). Dysregulation of mitochondria in these tasks can disturb the normal cellular metabolism and cause diseases. Numerous pathophysiological processes, such as those associated with aging, diabetes, cardiac dysfunction and neurodegenerative diseases, involve

mitochondrial dysfunction (Rector et al., 2010; Ong and Gustafsson, 2012; Schapira, 2006). The liver is a mitochondria-rich organ. Numerous liver pathologies, including alcoholic liver disease, nonalcoholic fatty liver disease (NAFLD), ischemia/reperfusion (I/R) injury, drug-induced hepatotoxicity, viral hepatitis, and hepatocellular carcinoma (HCC), are characterized by mitochondrial dysfunction (Rolo et al., 2009; Pessayre et al., 2012; Serviddio et al, 2010; Degli et al., 2012). Accumulation of damaged mitochondria leads to abnormal ROS formation, glutathione depletion, and respiratory complex alterations, which subsequently result in lipid accumulation, apoptosis and/or necrosis, and inflammation (Degli et al., 2012).

Mitochondrial dysfunction is one of the major characteristics after APAP overdose in both mice and humans (Placke et al., 1987; Meyers et al., 1988; McGill et al., 2012a, b). The critical role of mitochondria in the initiation and exaggeration of APAP hepatotoxicity was first recognized by using the non-hepatotoxic isomer of APAP, 3'-hydroxyacetanilide (AMAP). Although AMAP and APAP caused comparable covalent protein binding in mice, AMAP did not generate liver toxicity (Tirmenstein and Nelson, 1989). Careful comparisons revealed the major differences between them: AMAP spared the GSH depletion and covalent binding in mitochondria, whereas APAP did not, which strongly indicated the importance of mitochondria as a target of APAP (Tirmenstein and Nelson, 1989). This finding was confirmed by data from our lab, which showed that rats, which are less susceptible to APAP-induced liver injury, have fewer mitochondrial APAP-protein adducts compared to mice (McGill et al., 2012b). A number of mitochondrial proteins have been identified to be adducted by NAPQI, such as glutamate dehydrogenase, aldehyde dehydrogenase, $Mn2^{+}$SOD (superoxide dismutase 2) and

subunits of the respiratory chain complexes (Qiu et al., 1998). Although mechanistic evidence is rare to prove that these currently known targets of NAPQI are directly responsible for the toxicity (Jaeschke et al., 2012), toxic doses of APAP alter mitochondrial morphology (Placke et al., 1987), inhibit mitochondrial respiration (Meyers et al., 1988) and cause oxidative stress within the organelle (Jaeschke, 1990; Cover et al., 2005) (Fig 1.4). Mitochondrial oxidative stress plays a vital role in APAP-induced liver injury (Bajt et al., 2003; James et al., 2003b; Knight et al., 2002; Saito et al., 2010b). The reactive oxygen species implicated in APAP toxicity in mice is superoxide (O_2^-), which is mainly produced at complexes I and III of the electron transport chain (ETC) (Fig 1.3). Superoxide reacts with nitric oxide (NO) to form the highly reactive oxidant peroxynitrite ($ONOO^-$) in mitochondria, which can severely impair mitochondrial protein function and damage mitochondrial DNA together with the ROS (Agarwal et al., 2011; Cover et al., 2005). A major link between oxidative stress and mitochondrial dysfunction during APAP hepatotoxicity is activation of c-Jun N-terminal Kinase (JNK) (Fig 1.3). Oxidative stress activates JNK through multiple pathways, and the subsequent translocation of activated JNK (P-JNK) to the mitochondria further amplifies the mitochondrial oxidant stress and triggers the opening of the mitochondrial permeability transition (MPT) pore (Kon et al., 2004; Hanawa et al., 2008). This results in severe impairment of aerobic energy metabolism, which leads to mitochondrial dysfunction and release of mitochondrial contents, such as apoptosis-inducing factor (AIF) and endonuclease G (EndoG), to the nucleus (Jaeschke and Bajt, 2006; Bajt et al., 2006). These endonucleases cause nuclear DNA fragmentation (Bajt et al., 2006; 2011). The result is necrotic cell death (Figure 1.4).

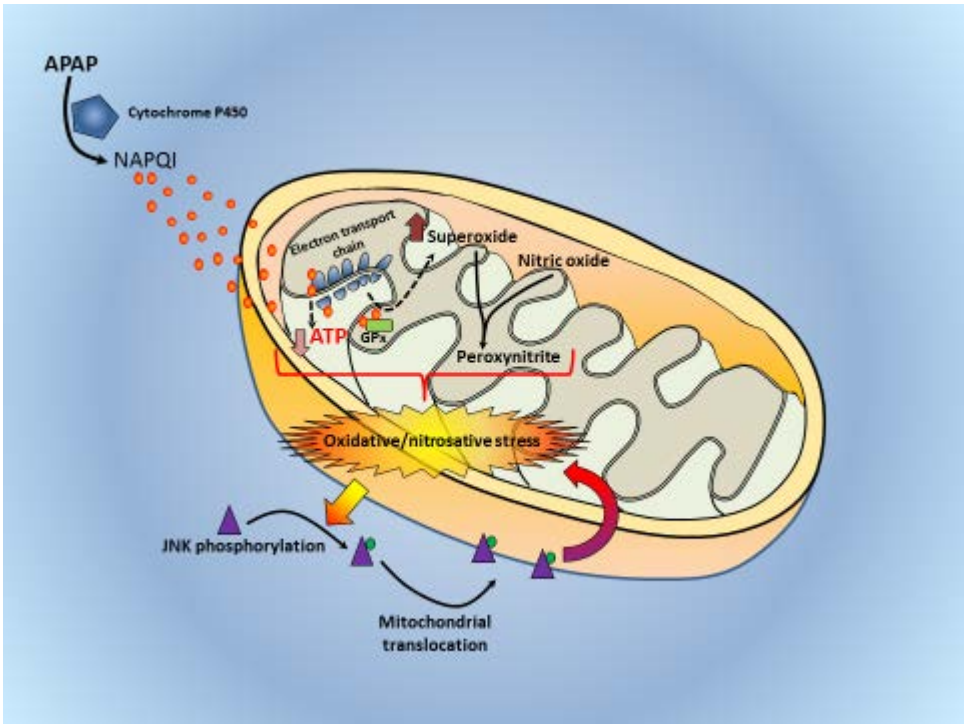


Figure 1.4. Mitochondrial oxidative stress and signaling in APAP hepatotoxicity.

Mitochondrial oxidative stress and signaling in APAP hepatotoxicity. Metabolism of APAP forms the reactive metabolite NAPQI, which targets proteins, especially mitochondrial proteins. Adduction of ATP synthase and glutathione peroxidase compromises generation of ATP through the electron transport chain and interferes with mitochondrial anti-oxidant capacity. The enhanced generation of superoxide results in its reaction with nitric oxide to produce peroxynitrite, which ultimately produces oxidative/nitrosative stress. This then activates the MAP kinase c-jun-N-terminal kinase (JNK), resulting in its phosphorylation and translocation to the mitochondria, which amplifies the initial oxidative stress.

With accumulating evidence supporting the critical role of mitochondria during the progress of APAP-induced liver injury, counteracting mitochondrial dysfunction emerges as a promising therapeutic strategy against these pathophysiological processes. To confront the serious consequences of mitochondrial lesions, cells have developed different mechanisms to ensure the integrity and function of mitochondria, such as mediating the signaling pathways to limit cell death, increasing disposal of damaged mitochondria by autophagy/mitophagy, and enhancing mitochondrial biogenesis to ensure energy metabolism (Degli et al., 2012). Interestingly, interventions aimed at regulating these mechanisms are highly effective against APAP-induced liver injury. For example, delayed treatment with GSH or NAC shortly after APAP exposure scavenges ROS, supports the mitochondrial energy metabolism and protects mice against APAP hepatotoxicity (Knight et al., 2002; Bajt et al., 2003; 2004; Saito et al., 2010b), while impairment of the mitochondrial antioxidant enzymes such as Mn^{2+} SOD in mice increases the susceptibility to the injury (Fujimoto et al., 2009; Ramachandran et al., 2011b). In addition, activation of autophagy to remove damaged mitochondria protects against APAP-induced hepatotoxicity, while inhibition of autophagy exacerbated the injury (Ni et al., 2012a). Inhibition of the JNK signaling pathway, which amplifies mitochondrial oxidant stress in APAP hepatotoxicity, has also proven to be a highly effective intervention against APAP overdose (Gunawan et al., 2006; Latchoumycandane et al., 2007; Saito et al., 2010a; Xie et al., 2014), and inhibition of the MPT prevents APAP-induced injury in rodent models both *in vitro* and *in vivo* (Kon et al., 2004; Masubuchi et al., 2005; Ramachandran et al., 2011a). Most recently, Ramachandran et al demonstrated that inhibition of mitochondrial fission attenuates APAP-induced cell death

in vitro (Ramachandran et al., 2013). Although targeting mitochondria biogenesis may possess therapeutic potential against APAP overdose as the interventions mentioned above, the effect of modulating mitochondria biogenesis on APAP hepatotoxicity has never been reported.

1.5 Regulation of mitochondrial biogenesis

Mitochondrial biogenesis is the forming and growth of new mitochondria. This process involves increases in mitochondrial number, size and mass (Clapier et al., 2008). Due to its endosymbiotic origin, the organelle has its own genetic system, including a compact circular DNA genome (mtDNA), a simple transcription system and a translation apparatus. Although most of the mtDNA-encoded genes were lost to the cell nucleus over evolutionary time, the remaining 37 genes encode 13 essential subunits of the respiratory chain and also the tRNAs and rRNAs required for protein translation within mitochondria. Correct mitochondrial biogenesis relies on the spatiotemporally coordinated synthesis and assembly of both nuclear-encoded proteins and mtDNA-encoded proteins (Baker et al., 2007). In addition, to ensure proper organization of the mitochondrial network during this process, mitochondrial DNA replication and mitochondrial fusion and fission mechanisms must also be coordinated (Chan, 2006a, b). Mitochondrial fission is driven by the dynamin-related protein 1 (DRP1) for the outer membrane and for the inner membrane, while mitochondrial fusion is controlled by optic atrophy 1 (OPA1) and mitofusins (Mfn) (Cipolat et al., 2004; Clapier et al., 2008).

Mitochondrial biogenesis is induced by numerous stimuli such as exercise, fasting, caloric restriction, low temperature and oxidative stress (Wright et al., 2007; Nisoli et al., 2005; Simon et al. 2011; Wu et al., 1999; Clapier et al., 2008). PGC-1α is the master regulator of mitochondrial biogenesis, and is a member of a family of transcriptional coactivators that also includes PGC-1β, and PGC-1 related coactivator (PRC) (Fernandez-Marcos and Auwerx, 2011). PRC is ubiquitously expressed, while PGC-1α and PGC-1β are primarily found in oxidative tissues, including the brain, heart, kidney, muscle, liver and brown adipose tissue (Lin et al., 2002). However, although all of them promotes mitochondrial biogenesis, only PGC-1α responds to metabolic challenges such as exercise, starvation or cold, while PGC-1β is more likely to serve as a constitutive regulator and PRC is essential during early embryonic development (Uldry et al., 2006; Handschin, 2009). PGC-1α mediates mitochondrial biogenesis by linking physiological signals to a key subset of transcription factors (NRF-1, NRF-2, PPARs, ERRs, and others) directing the coordinated expression of nuclear and mitochondrial genes (Scarpulla, 2008). Specifically, the activation of NRFs drives the expression of mitochondrial transcription factor A (Tfam), which is critical for both transcription and replication of the mitochondrial genome, key mitochondrial enzymes, a number of mitochondrial ribosomal proteins and tRNA synthetases. PPARs are involved in the regulation of enzymes, transporters and proteins for fatty acid oxidation. ERRs are implicated in the regulation of virtually all aspects of mitochondrial function, including fat and glucose metabolism (Scarpulla et al., 2012). Many signaling intermediates, such as eNOS, SIRTs, TORCs and AMPK, up-regulate the expression of PGC-1α (Jornayvaz and Shulman, 2010). Among these, AMPK and SIRT1 also activate PGC-1α via post-

translational phosphorylation or deacetylation, respectively (Lagouge et al., 2006; Reznick and Shulman, 2006). AMPK and SIRT1 are emerging as the critical kinases linking energy demands to mitochondrial biogenesis.

The importance of PGC-1α in regulation of mitochondrial biogenesis is demonstrated in numerous studies (Rasbach and Schnellmann, 2007; Funk et al., 2010; Rehman et al., 2013). For example, conditional overexpression of PGC-1α in cultured adipocytes, cardiac myocytes, and in tissue-specific transgenic mice, has been shown to be capable of driving many aspects of mitochondrial biogenesis, such as induction of respiratory chain and fatty acid oxidation genes, increased mitochondrial number, and augmentation of mitochondrial respiratory capacity (Puigserver et al., 1998; Lehman et al., 2000; Russell et al., 2004), while PGC-1α deficiency renders the mice unable to maintain their body temperature at low temperature and results in reduced cardiac output under pathophysiologic stress (Simon et al, 2011; Leone et al., 2006). Furthermore, abnormal regulation of PGC-1α expression and protein activity results in pathological consequences in most tissues (Handschin, 2009). Single nucleotide polymorphisms (SNPs) of PPARGC1A, the gene encoding PGC-1α, are associated with a diverse set of human diseases (https://geneticassociationdb.nih.gov).

Increased mitochondrial biogenesis is believed to be protective for cells and maintainence of normal mitochondrial biogenesis can determine cell survival or cell death (Jornayvaz and Shulman, 2010; Scarpulla, 2008). Consistent with this, impaired mitochondrial biogenesis is implicated in many pathologies, such as heart failure, neurodegenerative diseases and acute kidney injury, and its stimulation has been shown to be protective (Finck and Kelly, 2007; Rasbach and Schnellmann, 2007; St-Pierre et al., 2006; Funk et

al., 2010; Rehman et al., 2013). Recently, resveratrol (RSV), a natural polyphenolic compound mainly found in the skin of grapes, has been shown to significantly increase SIRT1 activity, leading to deacetylation of PGC-1α at multiple lysine sites and consequently increasing PGC-1α activity (Rodgers et al., 2005; Lagouge et al., 2006). Importantly, RSV treatment has been reported to enhance mitochondrial biogenesis in skeletal muscle and improve exercise tolerance, and protect mice against diet-induced-obesity and insulin resistance by improving PGC-1α activity and mitochondrial biogenesis (Rodgers et al., 2005; Baur et al., 2006). Similar to resveratrol, SRT1720 is also a small-molecule activator of the SIRT1, but is 1000x more potent. In animal studies SRT1720 improves insulin sensitivity and lowers plasma glucose levels in fat, muscle and liver tissue, and increases mitochondrial and metabolic function (Milne et al, 2007). Also, supporting mitochondrial biogenesis through SRT1720 activation rescues mitochondrial function after oxidant injury in renal proximal tubule cells and ischemia–reperfusion kidney injury in rats (Funk et al., 2010; Funk and Schnellmann, 2013).

1.6 Liver regeneration and recovery after APAP Overdose

Liver is the only human internal organ which has a remarkable capacity to regenerate after injury. Presumably, its regenerative ability evolved to protect animals against the catastrophic results of a loss of liver function caused by ingested toxins. Hepatocytes are normally in a quiescent state in the adult liver. However, during partial hepatectomy or liver damage, liver regeneration occurs to restore liver mass and function (Apte et al., 2002; Fausto, 2004; Mehendale, 2005; Michalopoulos, 2007). The regeneration process is

complex and highly regulated, involving the participation of growth factors, cytokines, matrix remodeling, and multiple feedback from stimulation and inhibition of growth related signals. The first step of this process is the priming of hepatocytes to increase their responsiveness to growth factors, which switch the cells from a resting, non-replicative phase (G0) to a preparatory phase for replication in the cell cycle. This stage is mediated by cytokines such as TNF-α, IL-6 and complement factors (Akerman et al., 1992; Cressman et al., 1996; Fausto et al., 1995; Strey et al., 2003). Hepatocytes then respond to growth factors such as hepatocyte growth factor or transforming growth factor-α and this results in the expression of cell cycle proteins (e.g., cyclins and cyclin-dependent kinases) (Fausto, 2000). The induction of cyclin D1 is a marker of cell cycle progression in hepatocytes (Albrecht and Hansen, 1999), which represents the entry of hepatocytes into G1-S check point and commitment of hepatocytes to DNA replication (Fausto, 2000). In addition, the activation of transcription factors such as NF-κB, STAT3, AP-1, Egr-1 and C/EBP-beta by cytokines, growth factors, or hormones also contributes to liver regeneration (Fausto, 2000). Interestingly, inhibitors of the cell cycle (e.g., p21 and p27) are induced together with the activators, which allows a sensitive regulation of liver regeneration (Albrecht et al., 1998; Fausto, 2000).

Liver regeneration was extensively studied in rodent models after partial hepatectomy or injury caused by toxicants, such as carbon tetrachloride, chloroform, thioacetamide and APAP (Mehendale, 2005; Fausto, 2004; Michalopoulos, 2007; Apte et al., 2009). In fact, the prognosis and outcome of liver injury is not only dependent on injury mechanisms but also by an opposing repair/regenerative response (Mehendale, 2005). Interestingly, improved prognosis was seen in two studies in patients who had a timely increase in

spontaneous liver regeneration following APAP overdose (Horn et al., 1999; Schmidt and Dalhoff, 2005). In addition, many interventions which reported to upregulate regeneration in rodents after APAP overdose improve outcome. For example, pre-treatment with the hepatotoxic chemical thioacetamide or subtoxic dose of APAP (Chanda et al., 1995; Dalhoff et al., 2001), or co-treatment and post-treatment with glutathione (Bajt et al., 2003) promote liver regeneration and limit liver injury. In contrast, some interventions such as prolonged treatment with N-acetylcystene or treatment with ethyl pyruvate, delay liver recovery after injury (Yang et al., 2009; 2012). A number of mediators and/or intracellular pathways have been demonstrated to promote tissue regeneration after APAP overdose, including IL-6 (James et al., 2003a), vascular endothelial growth factor (Donahower et al., 2006; Kato et al., 2010), plasminogen activation inhibitor-1 (Bajt et al., 2008b) and Wnt/b-catenin pathway (Apte et al., 2009).

Since most APAP-overdosed patients present relatively late – during or after the peak of injury (Singer et al., 1995), promoting liver regeneration and recovery may be a promising approach to the treatment of APAP-induced liver injury. Interestingly, muscle regeneration was shown to coincide with mitochondrial biogenesis, and pharmacologically blocking mitochondrial protein synthesis delays muscle regeneration (Wagatsuma et al., 2011). In addition, mitochondrial biogenesis is rapidly induced after hypoxic-ischemic brain injury, contributing to the endogenous repair mechanisms of the brain (Yin et al., 2008). Also, supporting mitochondrial biogenesis through PGC-1α activation accelerates injury recovery in both primary cell cultures and mice with kidney injury (Rasbach and Schnellmann, 2007; Tran et al., 2011). Given the promising findings of improved prognosis in those APAP overdose patients who have increased liver

regeneration and the potential benefits of mitochondrial biogenesis in tissue repair, in this dissertation we also evaluated the role of mitochondrial biogenesis in liver regeneration after APAP overdose.

Chapter 2. Lower Susceptibility of Female Mice to Acetaminophen Hepatotoxicity: Role of Mitochondrial Glutathione, Oxidant Stress and c-Jun N-Terminal Kinase

This section is adapted from Du et.al (2014), "Lower susceptibility of female mice to acetaminophen hepatotoxicity: Role of mitochondrial glutathione, oxidant stress and c-jun-N-terminal kinase", Toxicology and Applied Pharmacology, 281(1), 58-66, with permission from the publisher

2.1 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 the mitochondria at 4 h, and 2.5 and 3.3 fold higher in the total liver at 4 h and 6 h, 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 6 h (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 4 h post-APAP, it was 3.1 fold lower at 6 h in female mice. However, female mice were still protected by the JNK inhibitor SP600125. 17β-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 17β-estradiol partially protects male mice, it does not affect hepatic GSH recovery.

2.2 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 and Nourjah et al., 2006). Despite recent substantial progress in understanding the pathogenesis of APAP hepatotoxicity in rodents (Jaeschke et al., 2011 and Jaeschke et al., 2012) and in humans (Antoine et al., 2012, Antoniades et al., 2012, McGill et al., 2012a and 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 and 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 and Kon et al., 2004).

Gender can have an impact on the pharmacokinetics and pharmacodynamics of drugs (Morris et al., 2003 and Tanaka, 1999). In addition, drug toxicity arising from gender differences was also observed. Many studies have reported that females were more 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 and 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 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 and 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.

2.3 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 12 h 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 (12–15 h) 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 terminated at 30 min or 2–24 h 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 μl of DMSO diluted with 1375 μl of PBS) or the vehicle alone (15 ml/kg) 1 h before treatment with 600 mg APAP/kg (Saito et al., 2010a). In another experiment, male mice received 0.2 mg/kg of 17β-estradiol (Cayman, Ann Arbor, MI) dissolved in 0.1% DMSO in saline or the vehicle alone (10 ml/kg) 3 h 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 100 mM potassium phosphate buffer (KPP); another aliquot was added to 10 mM 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., 2008b). 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 0.1 μg/μl. The cDNA was diluted 1/10 and 5 μl 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., 2012a and 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.

2.4 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 terminated at various time points between 0.5–24 h after APAP treatment and liver injury was assessed by measuring plasma ALT activities and by histological analysis (Fig. 2.4.1). Although ALT levels significantly increased for both male and female mice at 4 h after APAP treatment and beyond, the increase in ALT activities was significantly reduced in female mice (Fig. 2.4.1A). The ALT results were confirmed by histology, which showed extensive centrilobular necrosis at 6 and 24 h after APAP in male mice while the areas of necrosis were reduced in female mice (Fig. 2.4.1B).

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 2 h (Fig. 2.4.2A) and similar protein adduct formation as determined in the total liver and in mitochondria at 2 h (Fig. 2.4.2B), 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. 2.4.2B). Generally, a dose of 300 mg APAP/kg is completely metabolized by 2 h after APAP administration and the hepatic GSH levels start to recover beyond that time (McGill et al., 2013). As shown in Fig. 2.2A, 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 24 h.

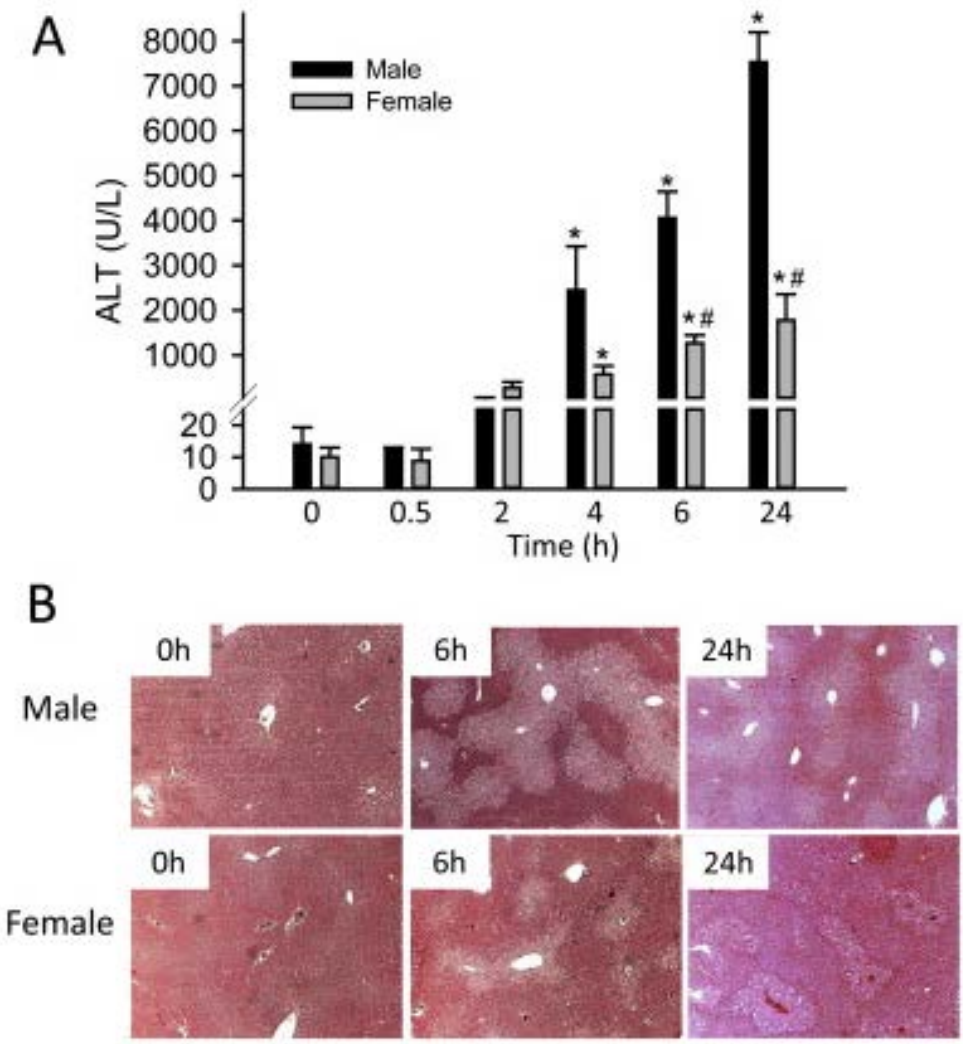


Figure 2.4.1 APAP-induced liver injury in C57Bl/6 male and female mice.

Animals were treated with 300 mg APAP/kg after overnight fasting, and terminated 0–24 h post-APAP. (A) Time course of plasma ALT values. (B) Representative H&E-stained liver sections (× 50 magnification) of male mice (top row) and female mice (bottom row) at 6 h and 24 h post-APAP. Data are expressed as mean ± SE, n = 4–7 animals per group. *P < 0.05 (compared to controls, t = 0). #P < 0.05 (compared to male mice).

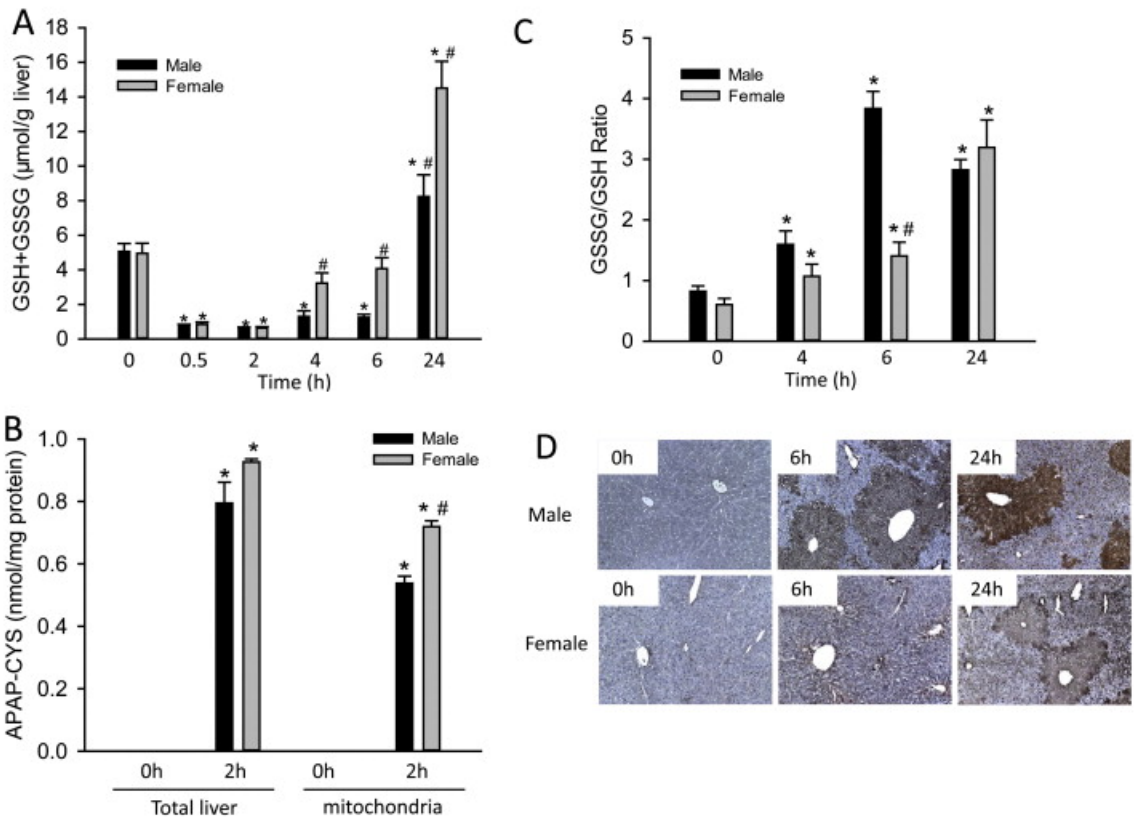


Figure 2.4.2 Hepatic GSH levels, APAP–protein adducts in total liver and mitochondria and parameters of oxidant stress in male and female mice.

Animals were treated with 300 mg APAP/kg after overnight fasting and terminated 0–24 h 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 ± SE, n = 4–7 animals per group. *P < 0.05 (compared to controls, t = 0). #P < 0.05 (compared to male mice).

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 24 h after APAP (Fig. 2.4.2C). 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. 2.4.2D). 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 (Figs. 2.4.2C, 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 and Saito et al., 2010b). Assessment of mitochondrial GSH levels at 4 h after APAP showed more than 2-fold higher mitochondrial GSH content in females than in males (Fig. 2.4.3A). 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. 2.4.3B). The faster recovery of hepatic GSH levels (Fig. 2.4.2A) correlated with the higher induction of gclc in female mice (Fig. 2.4.3B).

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 and Saito et al., 2010a). Therefore, total JNK and pJNK expression were evaluated in the cytosolic and mitochondrial fractions up to 6 h after APAP overdose (Fig. 2.4.4A). In control livers, there was no activated JNK in the cytosol and neither relevant amounts of JNK nor pJNK were present in mitochondria (Fig. 2.4.5A). As early as 2 h after APAP, JNK was phosphorylated in the cytosol and pJNK translocated to the mitochondria in both sexes (Fig. 2.4.4A). However, whereas JNK activation in the cytosol and mitochondria was well maintained up to 6 h 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 6 h (Fig. 2.4.4A). 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 (Figs. 2.4.4A,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 and 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 1 h 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%

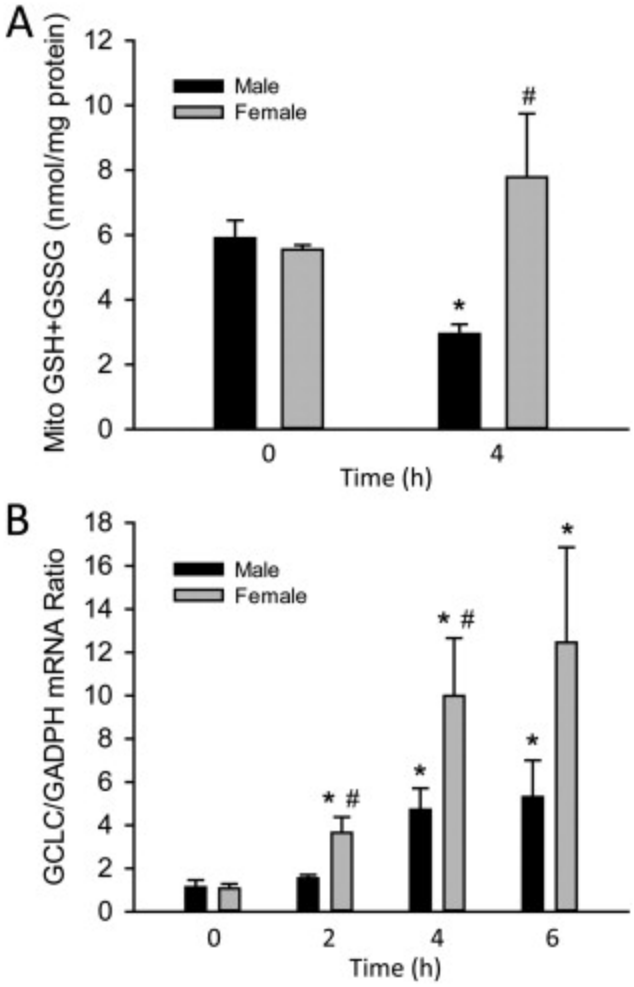


Figure 2.4.3 Mitochondrial GSH and glutamate cysteine ligase catalytic unit (Gclc) mRNA expression in male and female mice.

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 terminated 0–6 h 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 ± SE, n = 4–7 animals per group. *P < 0.05 (compared to controls, t = 0). #P < 0.05 (compared to male mice).

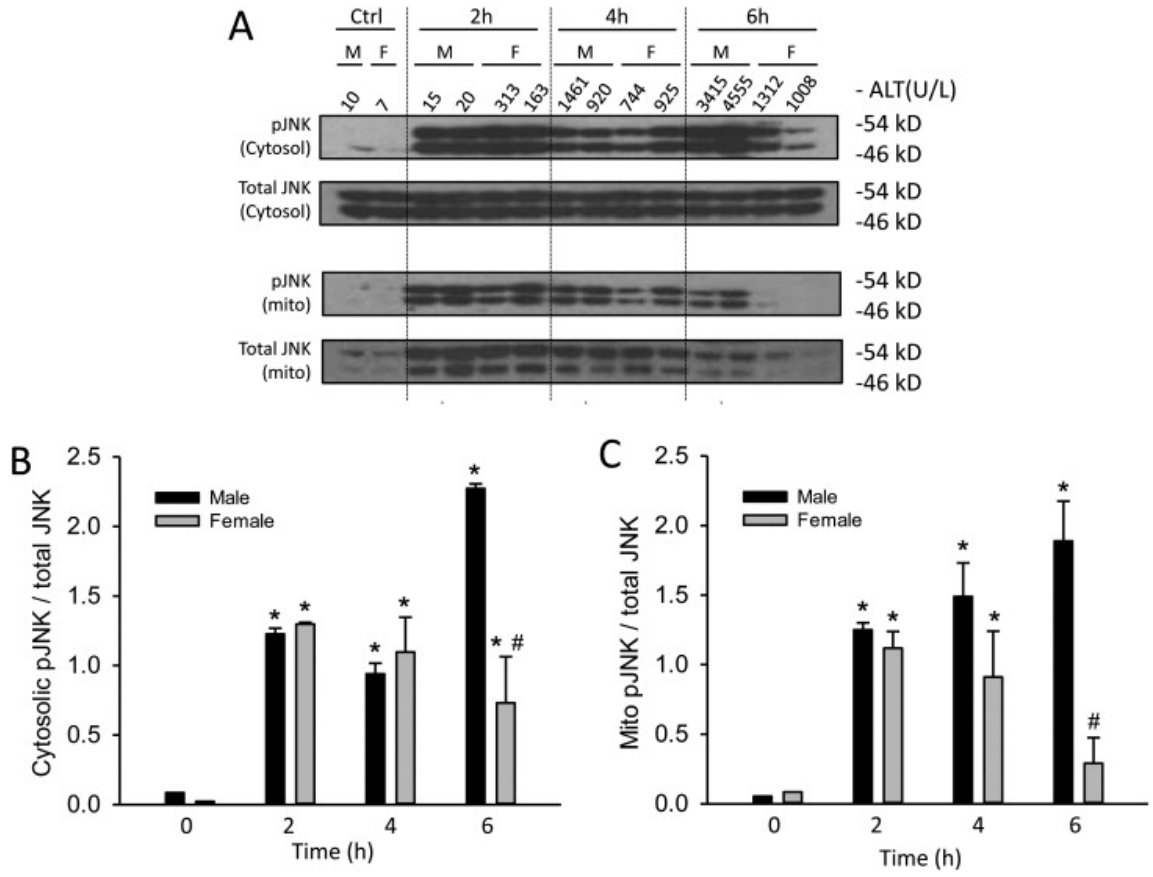


Figure 2.4.4 JNK phosphorylation and mitochondrial translocation in livers from male and female mice.

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 ± SE, n = 4–7 animals per group. *P < 0.05 (compared to controls, t = 0). #P < 0.05 (compared to male mice).

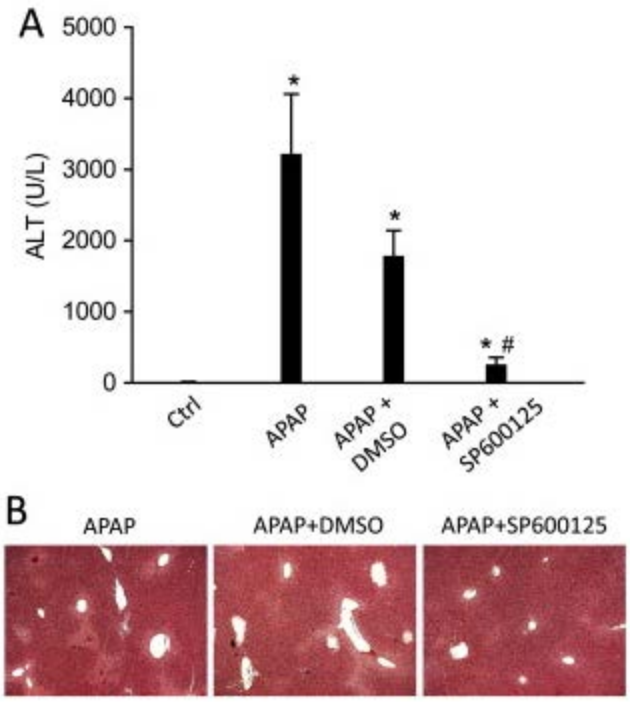


Figure 2.4.5 Effects of JNK inhibitor SP600125 on APAP-induced liver injury at 12 h post-APAP in female mice.

SP600125 (10 mg/kg) or DMSO (1.245 ml/kg) as the vehicle control was administered 1 h before 600 mg/kg APAP or saline. (A) Plasma ALT values at 12 h post-APAP. (B) Representative H&E-stained liver sections (× 50 magnification). Data are expressed as mean ± SE, n = 4–6 animals per group. *P < 0.05 (compared to controls). #P < 0.05 (compared to vehicle control)

compared to APAP alone (Fig. 2.4.5A). Importantly, the JNK inhibitor almost completely eliminated the remaining liver injury (Fig. 2.4.5A). These findings were confirmed by assessing necrosis in histological sections (Fig. 2.4.5B). The centrilobular necrosis observed in APAP and in APAP plus solvent groups was almost absent in the JNK inhibitor-treated animals (Fig. 2.4.5B). Consistent with the drastic protection, fully recovered hepatic GSH levels (Fig. 2.4.6A) and no increase in GSSG levels (Fig. 2.4.6B) and the GSSG-to-GSH ratio (Fig. 2.4.6C) 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 17β-estradiol or its vehicle for 3 h before the treatment with 300 mg/kg APAP. The mice were terminated at 3 h and 6 h post-APAP. Treatment with 17β-estradiol moderately protected mice against APAP hepatotoxicity as indicated by significantly lower plasma ALT activities (Fig. 2.4.7A) and representative histological sections (Fig. 2.4.7B). 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. 2.4.7C). Consistent with the partial protection, the GSSG-to-GSH ratio was significantly attenuated in 17β-estradiol-treated animals (Fig. 2.4.7D). Furthermore, JNK activation and mitochondrial pJNK translocation were not different between groups (data now shown). These data suggest that the reduced injury in 17β-estradiol-treated animals was not due to an effect on GSH recovery.

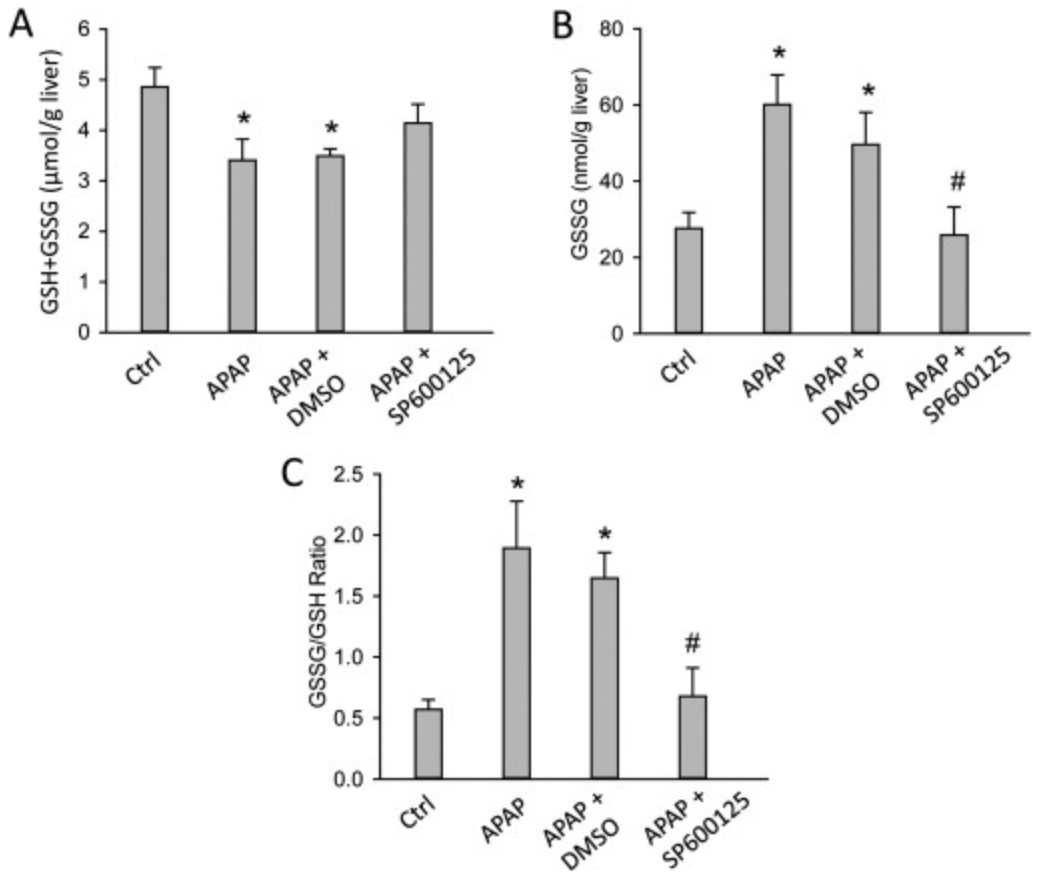


Figure 2.4.6 Effects of JNK inhibitor on APAP-induced oxidative stress in female mice.

Mice were pretreated with 10 mg/kg SP600125 or with DMSO for 1 h then treated with 600 mg/kg APAP, or APAP alone for 12 h. 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 ± SE, n = 4–6 animals per group. *P < 0.05 (compared to controls). #P < 0.05 (compared to the DMSO control).

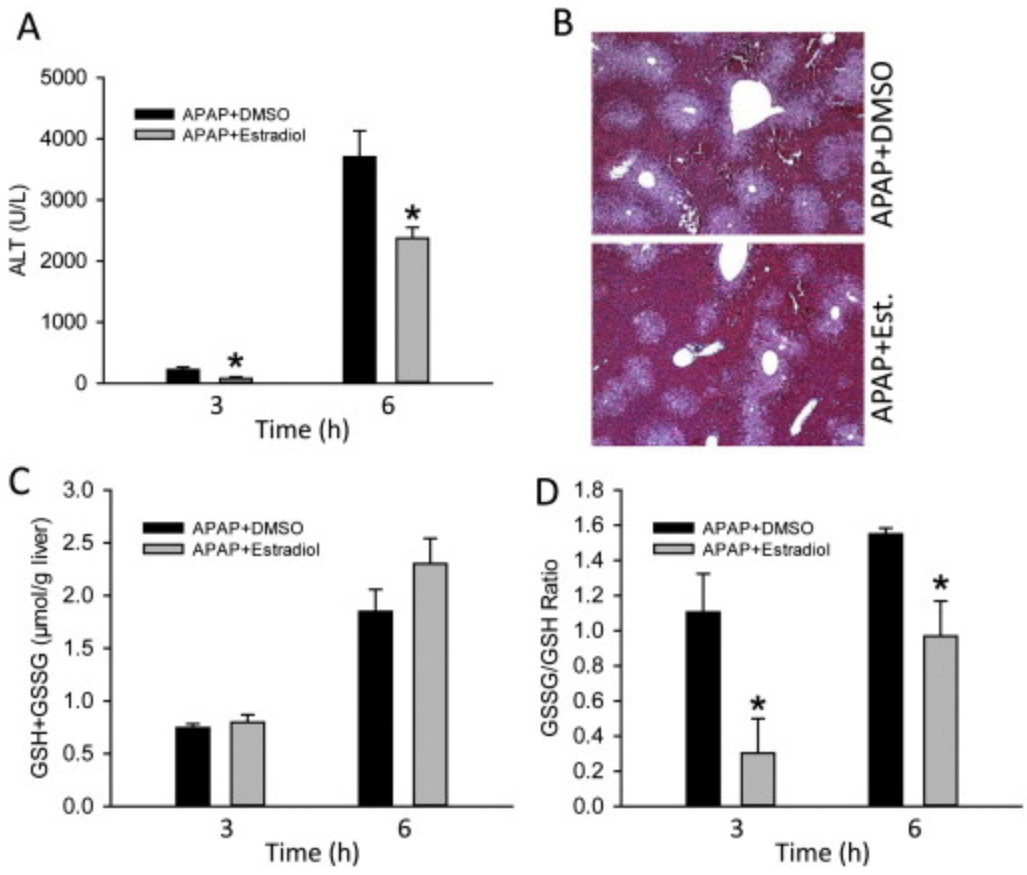


Figure 2.4.7 Effects of 17β-estradiol on APAP-induced liver injury and oxidative stress in male mice.

Mice were pretreated with 0.2 mg/kg 17β-estradiol or with 0.1% DMSO in saline (10 ml/kg) for 3 h, then treated with 300 mg/kg APAP, and were terminated at 3 h and 6 h post-APAP. (A) Plasma ALT values at 3 h and 6 h post-APAP. (B) Representative H&E-stained liver sections at 6 h post-APAP (× 50 magnification). (C) Hepatic GSH levels measured with a modified Tietze assay. (D) GSSG-to-GSH ratio. Data are expressed as mean ± SE of n = 3–4 animals per group. *P < 0.05 (compared to vehicle controls).

2.5 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 and 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 (2 h), 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 and 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 (LOOradical dot), 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) 1.5 h after APAP, these precursors accelerate GSH synthesis and recovery of GSH levels in mitochondria (Knight et al., 2002, James et al., 2003b and 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 and James et al., 2003b). 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 and 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 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 (Figs. 2.4.2A,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 and 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 and 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 and LoGuidice and Boelsterli, 2011). The higher susceptibility to APAP of animals partially deficient in the mitochondrial antioxidant enzyme Sod2 (Fujimoto et al., 2009 and 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 6 h 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 6 h. These findings are consistent with the enhanced scavenging of reactive oxygen and peroxynitrite in female animals during the period between 2 and 6 h 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 and 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 IC50 = 0.04 μM in vitro (Bennett et al., 2001). However, SP600125 can also inhibit other MAP kinases such as MKK4 (IC50 = 0.40 μM) and MKK6 (IC50 = 1.0 μM) (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., 2013). 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 17β-estradiol attenuated APAP-induced liver injury (Chandrasekaran et al., 2011). Our experiments supported a moderate protection by 17β-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 and 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 12 h, but the injury in humans peaks around 48 h 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.The aim of this investigation was to elucidate intracellular mechanisms of cell death after APAP in primary human hepatocytes. Importantly, we used freshly isolated cells. The hepatocytes were exposed to APAP immediately after isolation from fresh tissue and adherence to the tissue culture plates. Focusing on known events in murine hepatocytes, we observed similarities but also relevant differences between human and mouse cells which need to be considered when new therapeutic strategies are discussed.

Chapter 3. Mitochondria-targeted antioxidant Mito-Tempo protects against acetaminophen hepatotoxicity

This section is adapted from Du et.al (2017), "Mitochondria-targeted antioxidant Mito-Tempo protects against acetaminophen hepatotoxicity", Archives of Toxicology, doi:10.1007/s00204-016-1692-0. This article is published under the terms of the Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND), with permission from the publisher.

3.1 Abstract

Acetaminophen (APAP) hepatotoxicity is characterized by an extensive mitochondrial oxidant stress. However, its importance as a drug target has not been clarified. To investigate this, fasted C57BL/6J mice were treated with 300 mg/kg APAP and the mitochondria-targeted antioxidant Mito-Tempo (MT) was given 1.5 h later. APAP caused severe liver injury in mice, as indicated by the increase in plasma ALT activities and centrilobular necrosis. MT dose-dependently reduced the injury. Importantly, MT did not affect APAP-protein adducts formation, glutathione depletion or c-jun N-terminal kinase activation and its mitochondrial translocation. In contrast, hepatic glutathione disulfide and peroxynitrite formation were dose-dependently reduced by MT, indicating its effective mitochondrial oxidant stress scavenging capacity. Consequently, mitochondrial translocation of Bax and release of mitochondrial intermembrane proteins such as apoptosis-inducing factor were prevented, and nuclear DNA fragmentation was eliminated. To demonstrate the importance of mitochondria-specific antioxidant property of MT, we compared its efficacy with Tempo, which has the same pharmacological mode of action as MT but lacks the mitochondria targeting moiety. In contrast to the dramatic protection by MT, the same molar dose of Tempo did not significantly reduce APAP hepatotoxicity. In contrast, even a 3 h post-treatment with MT reduced 70 % of the injury, and the combination of MT with N-acetylcysteine (NAC) provided superior protection than NAC alone. We conclude that MT protects against APAP overdose in mice by attenuating the mitochondrial oxidant stress and preventing peroxynitrite formation and the subsequent mitochondrial dysfunction. MT is a promising therapeutic agent for APAP overdose patients.

3.2 Introduction

Acetaminophen (APAP) hepatotoxicity is the leading cause of acute liver failure in the USA and many Western countries (Budnitz et al. 2011; Manthripragada et al. 2011). Its toxicity is initiated by the formation of a reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI) (Nelson 1990). Excess NAPQI after APAP overdose depletes glutathione (GSH) and binds to cellular proteins (Cohen et al. 1997). Mitochondrial protein adducts disturb mitochondrial respiration and enhance the formation of reactive oxygen species (ROS) and peroxynitrite (Cover et al. 2005; Donnelly et al. 1994; Jaeschke 1990; Meyers et al. 1988). The oxidative stress activates MAP kinases ultimately resulting in the phosphorylation of c-jun N-terminal kinase (JNK), which then translocates to mitochondria and further amplifies the mitochondrial oxidant stress (Hanawa et al. 2008; Saito et al. 2010a). The enhanced mitochondrial oxidant stress triggers the opening of the mitochondrial permeability transition (MPT) pore and cell necrosis (Kon et al. 2004; LoGuidice and Boelsterli 2011; Ramachandran et al. 2011a). N-acetylcysteine (NAC) was introduced in the 1970s as the clinical antidote for APAP poisoning (Prescott et al. 1977), and until today, it is still the only available treatment for APAP overdose. NAC promotes the re-synthesis of GSH, which can scavenge more NAPQI and prevent protein adduct formation (Corcoran et al. 1985). Consequently, NAC is most effective for early presenting APAP overdose patients. However, in reality most APAP overdose patients seek medical attention relatively late; i.e., around or after the peak of the injury (Larson 2007). Therefore, a drug that is effective for late-presenting patients is needed.

Mitochondrial oxidant stress has been suggested to be critical in the progression of the injury (Jaeschke et al. 2012). Evidence for a selective APAP-induced mitochondrial oxidant stress was the specific increase in mitochondrial GSSG levels (Jaeschke 1990; Knight et al. 2001) and the selective formation of nitrotyrosine protein adducts in mitochondria in vivo (Cover et al. 2005). In addition, the specific MitoSox Red staining in isolated hepatocytes supports the conclusion of a mitochondrial oxidant stress (Yan et al. 2010). The pathophysiological relevance of this oxidant stress was documented by the protective effect of promoting hepatic GSH synthesis (Du et al. 2014; James et al. 2003b; Knight et al. 2002; Saito et al. 2010b), which will result in enhanced mitochondrial GSH levels and an improved capacity to scavenge ROS and peroxynitrite. Furthermore, mice with partial MnSOD-deficiency or inactivation of MnSOD resulted in increased liver injury, suggesting that impairment of mitochondrial antioxidant defense enhances the hepatocyte's susceptibility to oxidative injury (Agarwal et al. 2011; Fujimoto et al. 2009; Ramachandran et al. 2011b). Together, these data support the critical role of a mitochondrial oxidant stress in APAP hepatotoxicity. However, a therapeutic approach that specifically targets mitochondrial ROS has not been tested in this model.

Mito-Tempo (MT) was recently reported as a mitochondria-targeted antioxidant (Trnka et al. 2008). This compound combines the antioxidant piperidine nitroxide (Tempo) with the lipophilic cation triphenylphosphonium (TPP^+) (Trnka et al. 2008). Tempo is a SOD mimetic that dismutases superoxide in the catalytic cycle, while TPP is a membrane permeant cation that accumulates several hundred folds within mitochondria driven by the membrane potential (Trnka et al. 2008). This combination creates a mitochondria-targeted chemical with effective superoxide scavenging properties. Interestingly, this

compound has been shown to protect against oxidative injury in various pathologies, such as endotoxin-induced liver injury, sepsis-induced acute kidney injury, hypertension or colitis (Choumar et al. 2011; Dikalova et al. 2010; Patil et al. 2014; Wang et al. 2014). Therefore, the objective of the present study was to test whether MT could also protect against APAP hepatotoxicity by alleviating mitochondrial oxidant stress and dysfunction, which are critical events in the pathophysiology of APAP hepatotoxicity. In addition, we also assessed its therapeutic potential for APAP overdose by comparing its effect with NAC treatment, the current standard of care antidote.

APAP is a safe analgesic and antipyretic drug at therapeutic doses. However, APAP overdose can cause severe liver damage and even liver failure. Currently, it is the most prevalent cause of acute liver failure in the US and UK (Lee, 2012). Mechanistic investigations over the past several decades have revealed key events contributing to toxicity (Jaeschke et al., 2012 and Jaeschke et al., 2013). Bioactivation of APAP results in the formation of the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI). At therapeutic doses or mild overdoses, NAPQI can be detoxified by glutathione (GSH) with minimal protein binding (McGill et al., 2013). However, after a more severe overdose, there is extensive hepatic GSH depletion and greater protein adduct formation (McGill et al., 2013). Interestingly, binding to mitochondrial proteins correlates with injury (Tirmenstein and Nelson, 1989 and McGill et al., 2012b). Mitochondrial protein binding precedes mitochondrial dysfunction (Meyers et al., 1988), mitochondrial oxidant stress (Jaeschke, 1990, Tirmenstein and Nelson, 1990 and Knight et al., 2001) (Knight et al., 2001) and peroxynitrite formation (Cover et al., 2005). An initial oxidant stress activates and phosphorylates c-jun-N-terminal kinase (JNK), which then translocates to

mitochondria (Hanawa et al., 2008) where it amplifies the oxidative stress (Saito et al., 2010a) and triggers opening of the mitochondrial permeability transition pore (Kon et al., 2004, Ramachandran et al., 2011a and LoGuidice and Boelsterli, 2011), resulting in cell necrosis (Gujral et al., 2002).

3.3 Materials and Methods

Animals

Male C57BL/6J mice (8–12 weeks old) purchased from Jackson Laboratories (Bar Harbor, ME) were kept in an environmentally controlled room with a 12-h light/dark cycle. All animals were accustomed at least 3 days before experiments with free access to food and water. All experimental protocols followed the criteria of the National Research Council for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center.

Experimental design

Mice were fasted overnight and treated i.p. with 300 mg/kg APAP (Sigma-Aldrich, St. Louis, MO) dissolved in warm saline. Mito-Tempo (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride) (10 or 20 mg/kg) or Tempo (2,2,6,6-tetramethyl-1-piperidine 1-oxyl) (6.1 or 100 mg/kg) (Sigma-Aldrich) dissolved in saline (10 ml/kg) was i.p. administered 1.5 h after APAP. To test the therapeutic potential of MT, some mice were treated i.p. with MT or NAC (500 mg/kg dissolved in saline) or both at 3 h post-APAP. The number of mice used in our experiments was based on our extensive experience with the APAP model. In our study, we used 3–7

mice/group. Generally, more mice were applied when liver injury was expected to be more variable (e.g., NAC-treated mice), while less mice were used when the results were consistent and the difference was obvious (e.g., MT-treated mice). Mice were euthanized at 3, 6 or 12 h post-APAP; under isoflurane anesthesia, blood was drawn into a heparinized syringe and livers were harvested. Blood was centrifuged to obtain plasma for ALT determination. The liver tissue was cut into pieces and then used for mitochondrial isolation as described (Du et al. 2015a), or fixed in phosphate-buffered formalin for histology analyses, or was flash frozen in liquid nitrogen and subsequently stored at −80 °C.

Biochemical assays

Plasma ALT activities were measured using an ALT assay kit (Pointe Scientific, MI). GSH and GSSG measurements were taken using a modified method of the Tietze assay as described in detail (Jaeschke and Mitchell 1990; McGill and Jaeschke 2015). Hepatic APAP-protein adducts were measured as described previously (Muldrew et al. 2002) with modifications as described (Ni et al. 2012a). In short, liver homogenates were filtered through Bio-Spin 6 columns (Bio-Rad, Hercules, CA) that were pre-washed with 10 mM sodium acetate buffer (pH 6.5) to separate cellular proteins from small molecules. The protein samples were digested overnight with proteases to liberate APAP-CYS from APAP-protein adducts. The debris was then pelleted with 40 % trichloroacetic acid, and supernatants were filtered. APAP-CYS was measured using an HPLC method with electrochemical detection (Muldrew et al. 2002; Ni et al. 2012a).

Histology and Western blotting

Formalin-fixed tissue samples were embedded in paraffin, cut in 5 µm sections and stained with hematoxylin and eosin (H&E) for necrosis evaluation (Gujral et al. 2002). Cell necrosis was identified by morphological criteria in high-resolution microscopic fields: cell swelling, vacuolization, karyorrhexis and karyolysis. The percentage of necrosis was estimated by comparing the necrosis area to the entire section. The pathologist (A. Farhood) evaluated all histological sections in a blinded manner. Nitrotyrosine staining was performed to assess nitrotyrosine (NT) protein adducts as described (Knight et al. 2002), using the Dako LSAB peroxidase kit (Dako, Carpinteria, CA) and a rabbit polyclonal anti-nitrotyrosine antibody (Life Technologies, Grand Island, NY; Cat. # A-21285). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed for DNA strand break assessment with the In Situ Cell Death Detection Kit, AP (Roche Diagnostics, Indianapolis, IN) following manufacturer's instructions. The positive NT and TUNEL cells were identified by the dark brown staining around the centrilobular areas. In general, the positive staining was first estimated at low power (100×); questionable areas were evaluated at higher magnification (200× or 400×). Western blotting was performed as described (Bajt et al. 2000). The primary antibodies (1:1000 dilutions) included a rabbit anti-JNK antibody (Cat. # 9252) and a rabbit anti-phospho-JNK antibody (Cat. # 4668), rabbit anti-Bax polyclonal antibody (Cat. # 2772), rabbit anti-AIF antibody (Cell Signaling Technology, Danvers, MA; Cat # 5318) and mouse anti-Smac/DIABLO (BD Biosciences, San Diego, CA; Cat # 612245). A horseradish peroxidase-coupled anti-rabbit or anti-mouse IgG (Santa Cruz) was used as secondary antibody (1:5000 dilutions). Proteins were visualized

by enhanced chemiluminescence (GE Bioscience) and quantified by densitometric analysis of the X-ray film (Kodak, Rochester, NY).

Statistics

All results were expressed as mean ± SEM. Statistical significance between two groups was evaluated using the Student's t test, while comparisons of multiple groups were assessed by one-way analysis of variance (ANOVA), followed by Student–Newman–Keul's test. For non-normally distributed data, ANOVA was performed on ranks, followed by Dunn's multiple comparisons. $p < 0.05$ was considered significant.

3. 4 Results

Mito-Tempo protects against APAP-induced liver injury

Mice treated with 300 mg/kg APAP developed severe liver injury at 3 h and 6 h post-APAP, as indicated by increased plasma ALT activities (Fig. 3.4.1A) and extensive centrilobular necrosis in H&E-stained histology sections (Fig. 3.4.1C). Quantification of the areas of necrosis by the pathologist (A.F.) in a blinded manner at 6 h confirmed these results (Fig. 3.4.1B). Treatment with MT (10 or 20 mg/kg) 1.5 h post-APAP dose-dependently attenuated the increase in ALT activities (Fig. 3.4.1A) and significantly reduced the areas of necrosis at both time points (Fig. 3.4.1B, C), suggesting that MT effectively protected against APAP-induced liver injury.

Mito-Tempo does not inhibit the metabolic activation of APAP

To determine whether MT affects the metabolic activation of APAP, APAP-protein adducts were measured at 3 h post-APAP. MT did not affect APAP-protein adducts formation at either dose (Fig. 3.4.2A), indicating that MT did not inhibit reactive metabolite formation when it was given 1.5 h after APAP, i.e. when APAP metabolic activation is almost completed. Consistent with this, GSH depletion at 3 h was not inhibited by MT (Fig. 3.4.3B). In addition, there was no significant difference in GSH recovery among the groups at 6 h post-APAP (Fig. 3.4.2B), suggesting that the protection by MT is neither caused by an effect on protein adduct formation nor hepatic GSH recovery.

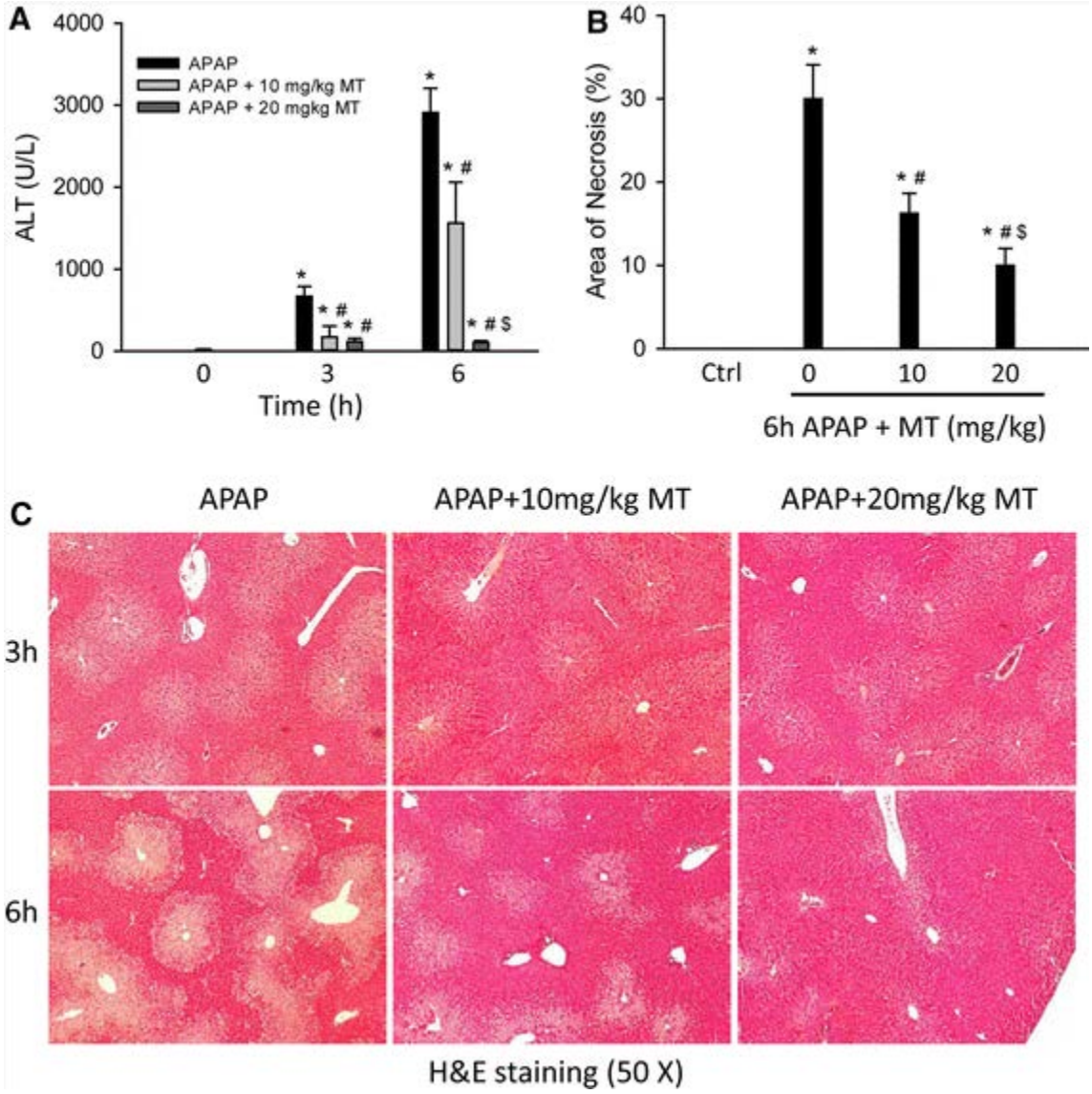


Figure. 3.4.1 MT protected against APAP hepatotoxicity.

Mice were treated with 300 mg/kg APAP, and 10 or 20 mg/kg MT or saline (10 ml/kg) was given 1.5 h later. Blood and liver tissue were harvested at 3 or 6 h post-APAP. a Plasma alanine aminotransferase (ALT) activity. b Area of necrosis at 6 h (%). c H&E-stained liver sections. Bars represent mean ± SEM for n = 4–7 mice. *p < 0.05 versus 0 h, #p < 0.05 versus APAP, $p < 0.05 versus APAP + 10 mg/kg MT.

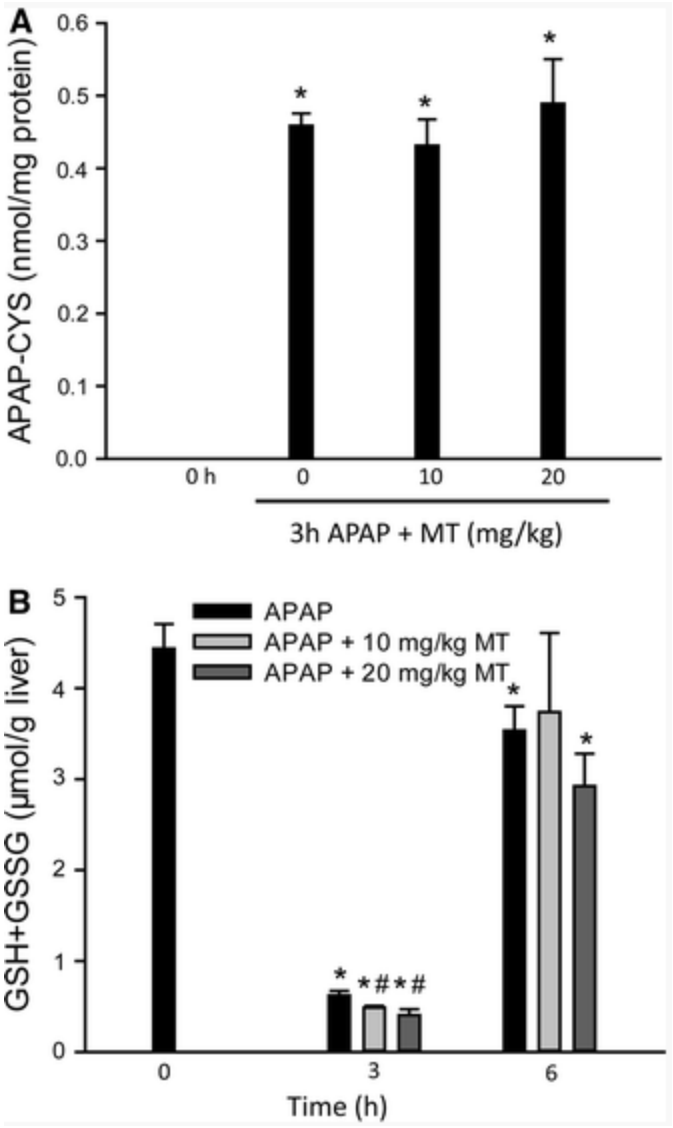


Figure 3.4.2 MT did not inhibit APAP metabolic activation.

Mice were treated with 300 mg/kg APAP, and 10 or 20 mg/kg MT or saline (10 ml/kg) was given 1.5 h later. **A** Total liver APAP-cysteine adducts. **B** Time course of hepatic GSH levels. *Bars* represent mean ± SEM for$n$ = 4–7 mice. *p < 0.05 versus 0 h, #p < 0.05 versus APAP

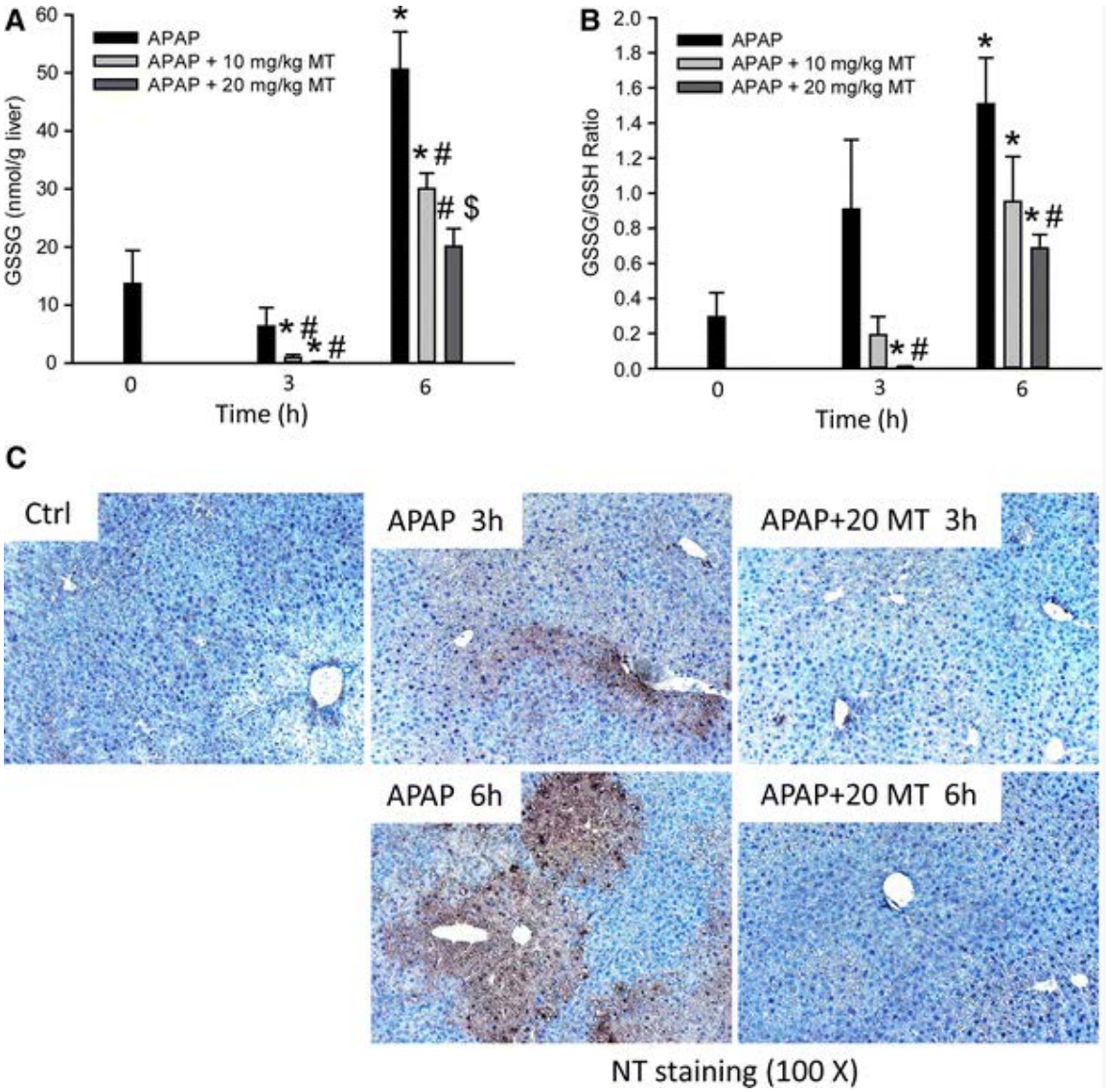


Figure 3.4.3 MT dose-dependently attenuated mitochondrial oxidant stress.

Mice were treated with 300 mg/kg APAP, and 10 or 20 mg/kg MT or saline (10 ml/kg) was given 1.5 h later. A Total liver GSSG levels. B GSSG-to-GSH ratios. C Nitrotyrosine staining of representative liver sections. Bars represent mean ± SEM for n = 4–7 mice. *p < 0.05 versus 0 h, #p < 0.05 versus APAP, $p < 0.05 versus APAP + 10 mg/kg MT.

Mito-Tempo attenuates APAP-induced mitochondrial oxidant stress in a JNK-independent manner

Next, we tested whether MT can alleviate the mitochondrial oxidant stress as was hypothesized based on the mode of action of the drug. Formation of reactive oxygen species was assessed by GSSG levels and the GSSG-to-GSH ratio (Fig. 3.4.3A, B). In support of the protection by MT (Fig. 3.4.1), GSSG levels and the GSSG-to-GSH ratio were significantly lower in MT-treated mice in a dose-dependent manner at both 3 h and 6 h (Fig. 3.4.3A, B). Superoxide reacts with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), a highly potent oxidant and nitrating species that has been shown to be formed during APAP toxicity (Hinson et al. 1998). Consistent with previous findings (Hinson et al. 1998; Knight et al. 2001), there was extensive staining for nitrotyrosine protein adducts in the centrilobular areas (Fig. 3.4.3C). Importantly, MT treatment almost eliminated APAP-induced nitrotyrosine staining (Fig. 3.4.3C), consistent with the hypothesis that the effective dismutation of superoxide can prevent peroxynitrite formation.

It has been hypothesized that the initial oxidant stress after APAP overdose is responsible for JNK activation, and it translocates to mitochondria where it further amplifies the mitochondrial oxidant stress (Hanawa et al. 2008; Saito et al. 2010a). Consistent with the previous studies, our Western blots showed that APAP overdose induced JNK activation (phosphorylation) in the cytosol, and activated P-JNK translocated to the mitochondria as early as 3 h (Fig. 3.4.4A); cytosolic JNK activation and to a lesser degree mitochondrial JNK translocation were maintained up to 6 h (Fig. 3.4.4B). However, neither JNK activation nor the mitochondrial translocation was affected by MT treatment (Fig. 3.4.4A,

B). Densitometric analysis of these Western blots further confirmed these findings (Fig. 3.4.4C, D). This indicated that the protection by MT is independent of the activation of the JNK signaling pathway.

Mito-Tempo attenuates APAP-induced mitochondrial dysfunction

Bax was shown to translocate to mitochondria and to form pores in the mitochondrial outer membrane, resulting in the release of intermembrane proteins such as Smac and AIF after APAP overdose (Bajt et al. 2006; 2008a). Consistent with these previous studies, we observed Bax translocation to mitochondria after APAP, as indicated by the appearance of Bax in the mitochondrial fraction and a reduction in the cytosolic fraction (Fig. 3.4.5A), which was confirmed by the corresponding densitometric analysis (Fig. 3.4.5B, C). MT almost completely prevented Bax translocation (Fig. 3.4.5A–C). As a result, the release of mitochondrial intermembrane proteins such as Smac and AIF was also completely eliminated (Fig. 3.4.5A, D, E). The corresponding DNA fragmentation as indicated by the TUNEL assay (Fig. 3.4.5F) is caused by release of mitochondrial endonucleases such as AIF and endonuclease G (Bajt et al. 2006, 2011; Cover et al. 2005). Consistent with the elimination of mitochondrial intermembrane protein release, MT treatment also prevented DNA fragmentation as indicated by the elimination of TUNEL-positive cells (Fig. 3.4.5F).

Protection by Tempo was less effective than Mito-Tempo

To investigate whether the mitochondria targeting property of MT is critical for its protection, we compared its efficacy with its analog Tempo, which retains the ROS scavenging moiety piperidine nitroxide of MT but lacks the part of the molecule (TPP^+)

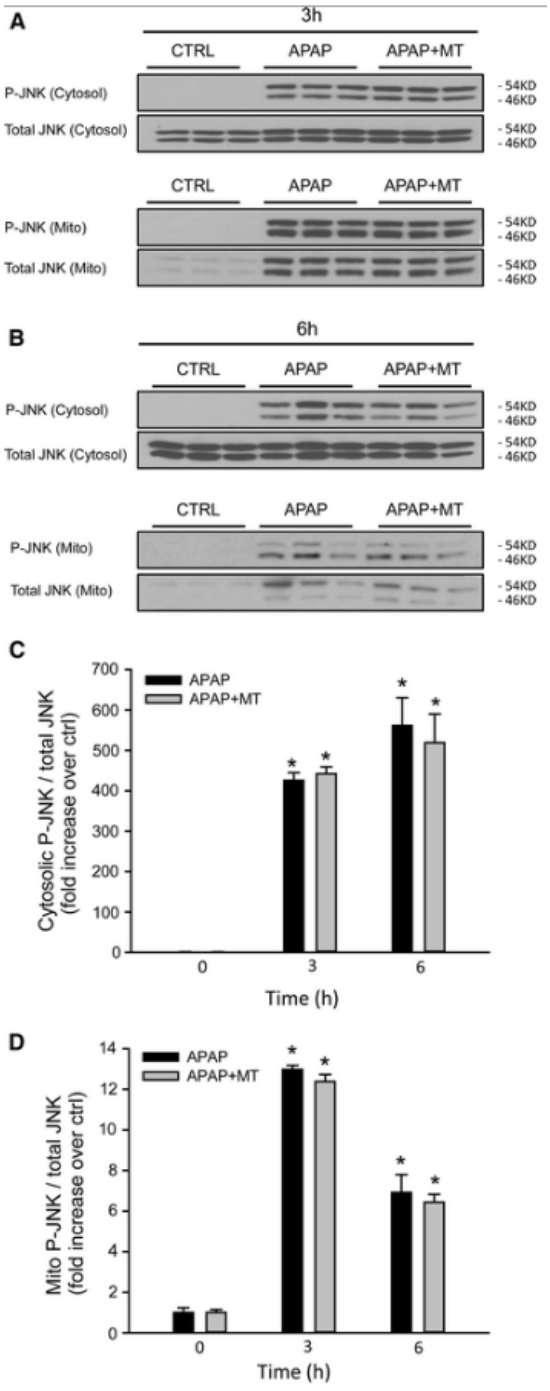


Figure 3.4.4 MT did not inhibit JNK activation or mitochondrial translocation.

Mice were treated with 300 mg/kg APAP, and 20 mg/kg MT or saline (10 ml/kg) was given 1.5 h later. Total JNK and P-JNK were measured in cytosolic and mitochondrial liver fractions at 3 h (**A)** or 6 h (**B)** post-APAP. Densitometric analysis of P-JNK/JNK in the cytosolic (**C**) and mitochondrial (**D**) fractions. *Bars* represent mean ± SEM for 3 mice. *$p < 0.05$ versus Ctrl

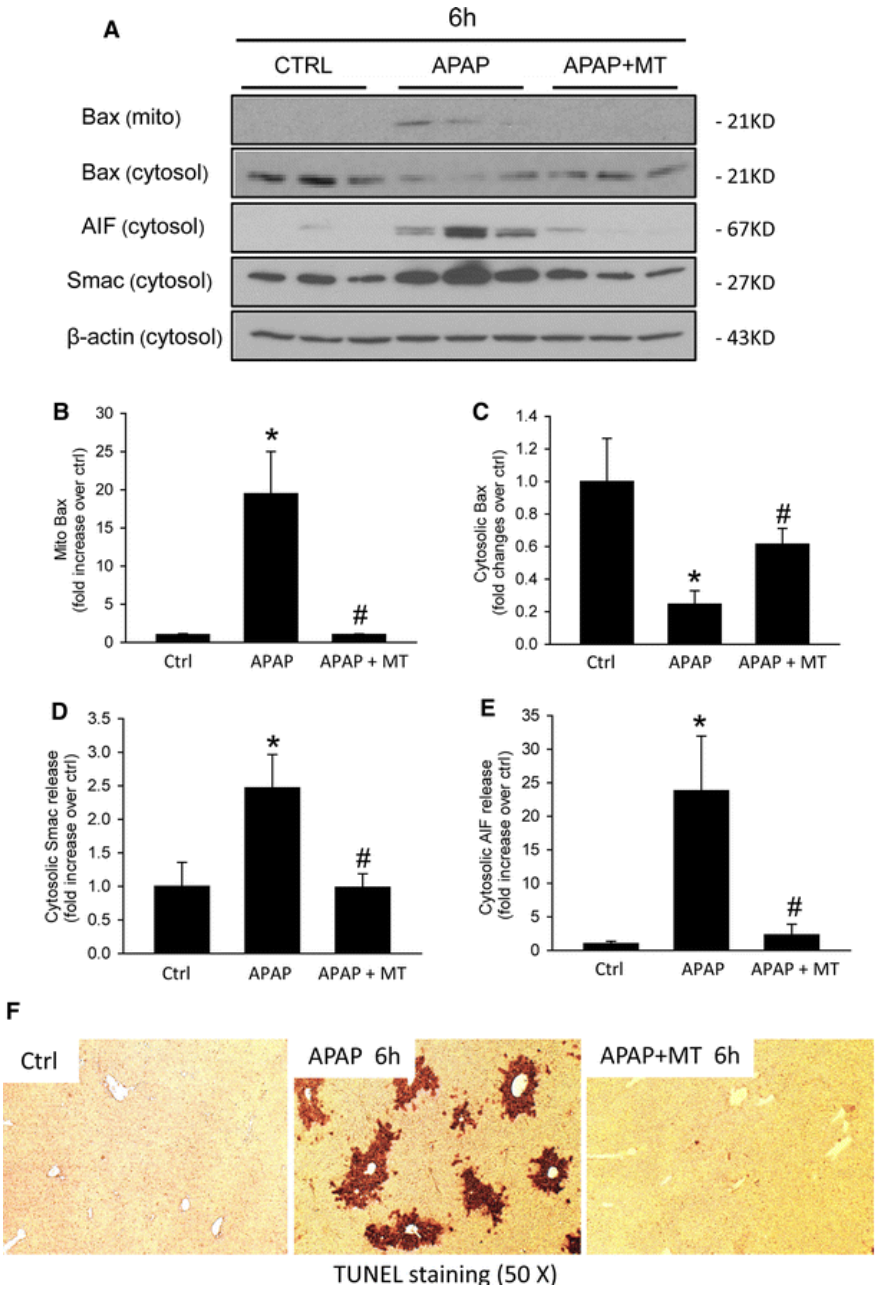


Figure 3.4.5 MT protected against mitochondrial dysfunction.

Mice were treated with 300 mg/kg APAP, and 20 mg/kg MT or saline (10 ml/kg) was given 1.5 h later. A Mitochondrial Bax and cytosolic Bax, Smac, AIF and β-actin measured by Western blot at 6 h post-APAP. Densitometric analysis of mitochondrial Bax (B) and cytosolic Bax (C), Smac (D) and AIF (E) with β-actin as the loading control. f TUNEL staining of representative liver sections. Bars represent mean ± SEM for 3 mice. *$p < 0.05$ versus Ctrl, #$p < 0.05$ versus APAP

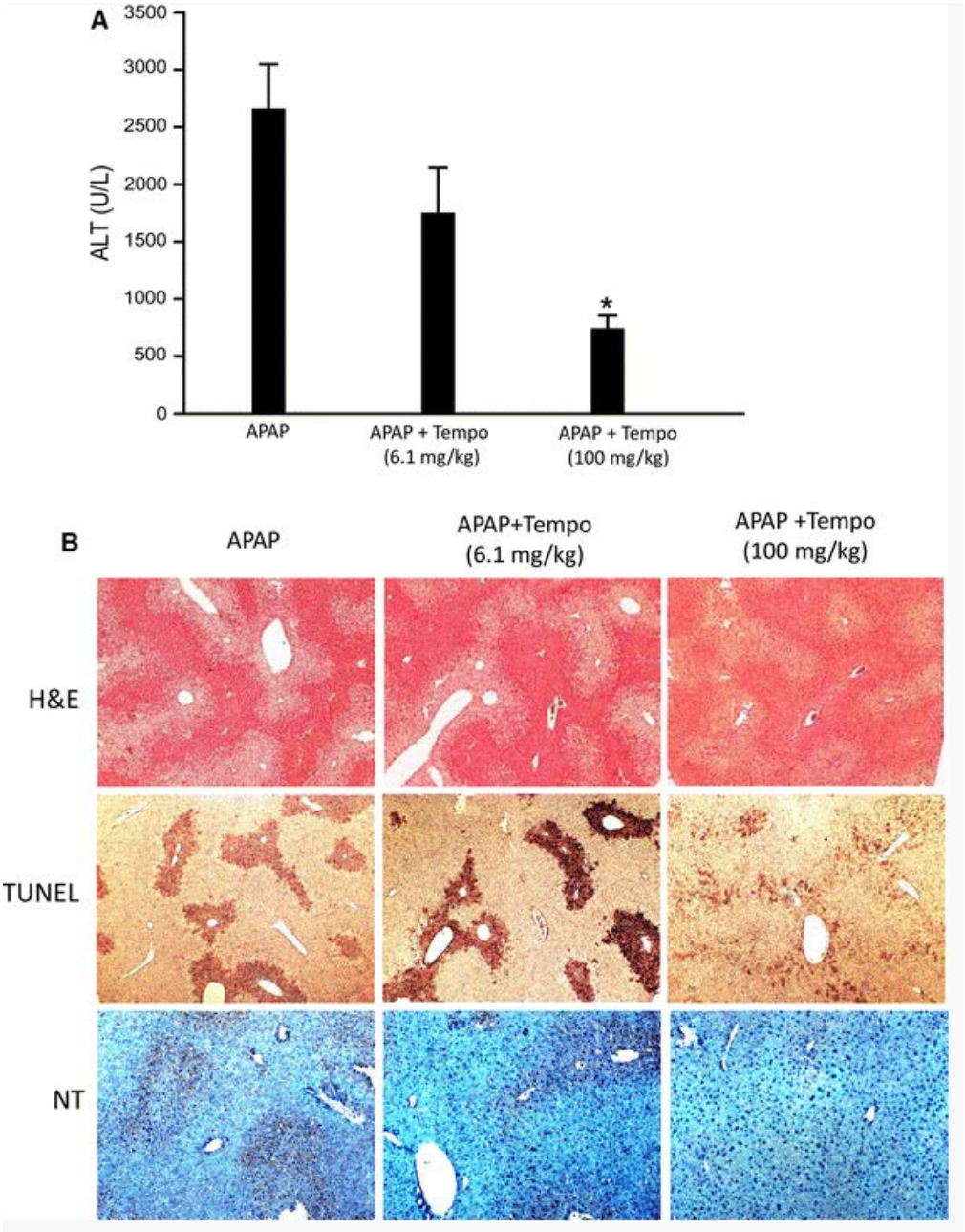


Figure 3.4.6 Protection by Tempo was less effective than MT.

Mice were treated with 300 mg/kg APAP, and 6.1 or 100 mg/kg Tempo or saline (10 ml/kg) was given 1.5 h later. A Plasma ALT activity at 6 h post-APAP. B Representative liver Sects. (6 h APAP) were stained with H&E (50X), TUNEL (50X) and nitrotyrosine (100X). Bars represent mean ± SEM for n = 4 mice. *p < 0.05 versus APAP

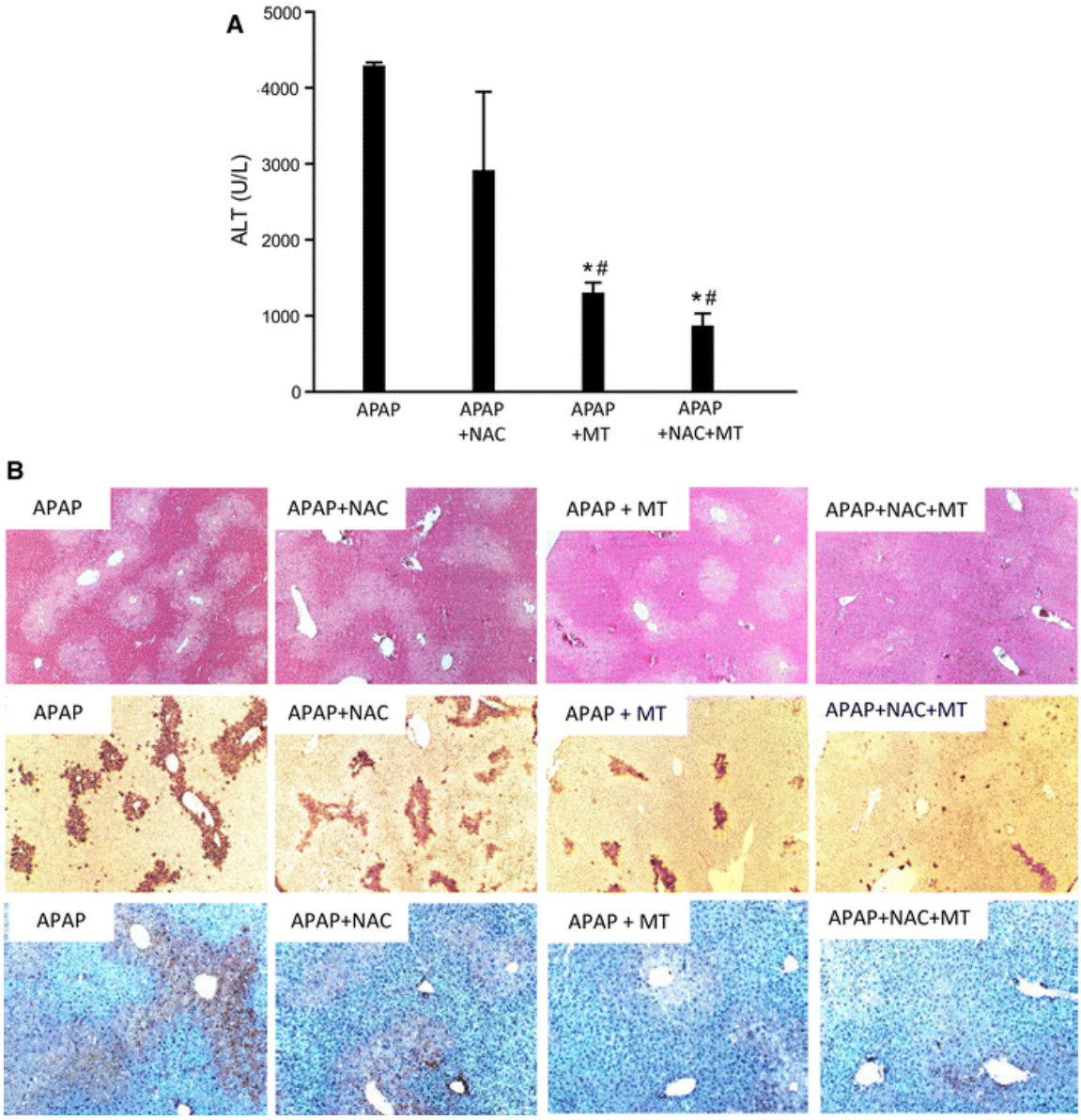


Figure 3.4.6 MT seemed to be a promising therapeutic agent for APAP poisoning.

Mice were treated with 300 mg/kg APAP, and saline (10 ml/kg), 20 mg/kg MT or 500 mg/kg NAC or MT + NAC was given 3 h later, plasma and liver tissue were harvested at 12 h post-APAP. A Plasma ALT activity. B Representative liver sections (12 h APAP) were stained with H&E (50X), TUNEL (50X) and nitrotyrosine (100X). Bars represent mean ± SEM for n = 3–7 mice. *p < 0.05 versus APAP, #p < 0.05 versus APAP + NAC.

that is responsible for targeting mitochondria. Considering the differences in molecular weight between MT (510.03 g/mol) and Tempo (156.25 g/mol), the amount of Tempo in 20 mg/kg MT is 6.1 mg/kg on a molar basis, which was subsequently used in our experiments. In contrast to the dramatic protection by MT, 6.1 mg/kg Tempo did not afford significant protection to APAP toxicity, as indicated by the plasma ALT activities (Fig. 3.4.6A) and H&E-stained sections and the TUNEL assay (Fig. 3.4.6B). NT staining was also not affected by the lower dose of TEMPO (Fig. 3.4.6B). Interestingly, Tempo did significantly protect against APAP toxicity and reduced NT staining when a much higher dose (100 mg/kg) was administered (Fig. 3.4.6A, B), although this protection was still less when compared to a much lower dose of MT (20 mg/kg) (Fig. 3.4.1A, B).

Therapeutic potential of Mito-Tempo against APAP hepatotoxicity

Until today, N-acetylcysteine (NAC) is the only available clinically approved antidote against APAP poisoning. However, it has to be given shortly after APAP overdose to achieve its greatest effect, while in reality most APAP overdose patients present for medical care relatively late (Larson 2007). Therefore, a pharmacological intervention that still works after the onset of liver injury would greatly benefit the late-presenting patients. Our data showed that NAC as a 3 h post-treatment provided only marginally lower average ALT values at 12 h after APAP treatment (Fig. 3.4.7A, B). In contrast, MT administration 3 h after APAP treatment significantly reduced liver injury as indicated by 70 % lower ALT activities, less necrosis and less TUNEL-positive cells (Fig. 3.4.7A, B). Interestingly, the mice receiving both MT and NAC showed greater protection than those with NAC alone (Fig. 3.4.7A, B). These results indicate that MT can improve the beneficial effect of NAC, the current standard of care in the clinic. The significant

protection by MT against APAP hepatotoxicity, especially when given as a late post-treatment, indicates its therapeutic potential for APAP overdose patients.

3.5. Discussion

The objective of this study was to evaluate the efficacy of the mitochondria-targeted antioxidant Mito-Tempo in a murine model of APAP hepatotoxicity. Our data demonstrated that MT attenuated the mitochondrial oxidant stress and subsequent mitochondrial dysfunction and thus protected against APAP hepatotoxicity. In addition, MT did not affect the metabolic activation of APAP, GSH recovery or the JNK signaling pathway. More importantly, MT showed a superior protective effect compared to NAC as a late post-treatment alone or together with NAC, suggesting that MT has therapeutic potential for APAP overdose patients.

Critical role of mitochondrial oxidant stress in APAP hepatotoxicity

Our current study demonstrated that MT, when administered 1.5 h after an overdose of APAP, dose-dependently attenuated liver injury. The protection was independent of any impact on the metabolic activation of APAP, the critical initiating step in the pathophysiology, as indicated by the lack of an effect on APAP-protein adducts formation. This result is not surprising as a dose of 300 mg/kg is largely metabolized, and the peak of adduct formation is reached 1.5 h after APAP treatment (McGill et al. 2013). In addition, MT did not affect JNK activation and the translocation of activated (phosphorylated) JNK to the mitochondria. Since the initial mitochondrial dysfunction is thought to be responsible for activation of MAP kinases, which ultimately result in JNK

activation (reviewed in Du et al. 2015c), the fact that MT did not prevent JNK activation suggests that MT did not affect these early signaling events. In contrast, MT eliminated NT staining and attenuated GSSG formation. It is known that NT protein adducts (Cover et al. 2005) and GSSG (Jaeschke 1990) are accumulating almost exclusively in mitochondria after APAP overdose. Thus, the elimination of NT staining is consistent with the effective promotion of superoxide dismutation by MT and therefore the prevention of nitrotyrosine formation. On the other hand, dismutation of superoxide yields oxygen and hydrogen peroxide, which can be detoxified by glutathione peroxidase, resulting in formation of GSSG. This explains why MT was more effective in preventing the formation of NT adducts than GSSG. Nevertheless, since peroxynitrite is the more potent oxidant compared to hydrogen peroxide, the shift away from the more aggressive oxidant resulted in effective attenuation of the injury. These data are consistent with previous findings where the accelerated recovery of hepatic and mitochondrial GSH levels improved the scavenging capacity for peroxynitrite and hydrogen peroxide with similar protection (James et al. 2003b; Knight et al. 2002; Saito et al. 2010b). In addition, mice with partial deficiency of SOD2, which resides in mitochondria, showed enhanced toxicity after APAP overdose (Fujimoto et al. 2009; Ramachandran et al. 2011a, b). Since the mitochondrial oxidants generated during APAP toxicity inactivate SOD2 (Agarwal et al. 2011), this explains why an improved SOD activity in the mitochondria by MT treatment effectively prevents mitochondrial dysfunction and cell necrosis.

The mitochondria-targeted antioxidant is most effective against APAP hepatotoxicity

When MT was compared to Tempo, the identical antioxidant lacking a mitochondrial target signal, it was obvious that on a molar basis, MT was considerably more effective in protecting against the mitochondrial oxidant stress during APAP overdose. This supports the conclusion that the active transport of MT into mitochondria achieves a higher concentration in the target organelle and thus higher efficacy. It actually required 20 times the dose of Tempo to cause significant but still less protection than MT. Together, these results indicate the high efficacy of a SOD mimetic that can be targeted to mitochondria in protecting against the selective mitochondrial oxidant stress during APAP-induced liver injury.

MT prevents mitochondrial dysfunction and DNA fragmentation

DNA fragmentation is a hallmark of APAP hepatotoxicity (Lawson et al. 1999; Ray et al. 1990). Previous studies have shown that nuclear DNA damage is related to mitochondrial dysfunction (Cover et al. 2005), which causes the release and nuclear translocation of mitochondrial intermembrane proteins AIF and endonuclease G (Bajt et al. 2006). The release of AIF and endonuclease G is caused initially by formation of a Bax pore in the outer mitochondrial membrane and later, after the MPT, by rupture of the outer membrane due to matrix swelling (Bajt et al. 2008a). Since MT prevented mitochondrial Bax translocation, the release of intermembrane proteins such AIF and nuclear DNA strand breaks as indicated by the TUNEL assay, these findings are consistent with the attenuation of mitochondrial dysfunction by MT. Overall, the data suggest that preventing peroxynitrite formation by MT prevents the oxidant stress-mediated MPT and as a consequence all downstream events such as intermembrane protein release, their nuclear translocation and nuclear DNA damage. Ultimately, the severe mitochondrial

dysfunction and the nuclear DNA fragmentation are the cause of necrotic cell death (Jaeschke et al. 2012).

MT is more effective than N-acetylcysteine

NAC is currently the only approved clinical antidote against APAP overdose (Polson et al. 2005). NAC treatment supports hepatic GSH synthesis and protects against APAP toxicity by providing GSH to scavenge the reactive metabolite and preventing protein adduct formation (Corcoran et al. 1985). Thus, NAC is most effective when administered during the metabolism phase of APAP (Xie et al. 2015; Smilkstein et al. 1988). Unfortunately, many patients present after the metabolism is over and the injury is already in progress (Larson 2007). During this time, NAC is still somewhat effective (Smilkstein et al. 1988). However, GSH is now being used to scavenge peroxynitrite and reactive oxygen in mitochondria (James et al. 2003b; Knight et al. 2002; Saito et al. 2010b). Furthermore, some of the excess NAC is being converted to Krebs cycle intermediates supporting mitochondrial energy metabolism (Saito et al. 2010b). Although the mouse model of APAP overdose mimics closely events in humans (McGill and Jaeschke 2014), overall the time to liver injury is shorter in mice. Thus, the therapeutic window for NAC in mice is between 0 and 2 h after APAP and the protective effect of NAC disappears by 3 h (James et al. 2003b; Knight et al. 2002). Interestingly, MT administered 3 h after APAP was still highly effective making it a better therapeutic intervention than NAC at later time points. Furthermore, there was some additive effect between MT and NAC. This is possible because the mechanism of protection is different. MT prevents peroxynitrite formation, and NAC, after GSH synthesis in the cytosol and uptake into mitochondria, can scavenge peroxynitrite and hydrogen peroxide.

NAC is administered intravenously (i.v.) in clinical practice. In the mouse model of APAP hepatotoxicity, both i.v. injection and i.p. injection of NAC have been shown to effectively protect against APAP toxicity (Saito et al. 2010b; James et al. 2003b). Since i.p. injection is less stressful to the animal, both NAC and MT were injected i.p. in our study, which may need to be considered when translating these findings to the human pathophysiology.

Another issue to consider is the difference in the time course of APAP hepatotoxicity in mice and humans. It is known that the development of liver injury after APAP overdose is much more delayed in humans compared to mice. This fact has to be taken into account when defining “late post-treatment” therapy. In humans, the liver injury is typically first seen around 24 h with peak injury at 48–96 h after APAP overdose (Larson 2007; McGill et al. 2012a; Rumack 1983); this time course of cell death can also be reproduced in primary human hepatocytes exposed to APAP (Xie et al. 2014). In mice, the injury normally begins at 3 h and peaks around 12 h post-APAP (McGill et al. 2013). The reason for this delay in humans may be related to the delayed mitochondrial protein binding and delayed JNK activation as we have shown in primary human hepatocytes (Xie et al. 2014). Consequently, NAC is very effective in patients when administered within 8 h of APAP ingestion (before the injury starts) (Smilkstein et al. 1988). Again, this can be reproduced in human hepatocytes where NAC is 100 % effective at 6 h and partially effective at 15 h after APAP exposure (Xie et al. 2014). In contrast, NAC is only highly effective up to 2 h after APAP in mice (James et al. 2003b; Knight et al. 2002; Saito et al. 2010b). When administered at 3 h after APAP, the “protection” (if any) is very marginal and variable because the injury is already in progress. In this scenario,

treatment of NAC or MT at 3 h post-APAP can be considered as a “late post-treatment” in mice comparable to a 24-h time point in humans. Since MT is still effective in mice when given at 3 h post-APAP while NAC is not, MT could be regarded as a promising therapeutic agent for late-presenting patients in the clinic.

3.6. Summary and conclusions

In summary, we demonstrated that MT protected against APAP hepatotoxicity in mice. It did not inhibit metabolic activation of APAP but dose-dependently attenuated mitochondrial oxidant stress and prevented the following mitochondrial dysfunction. Comparison of the protection by MT to its analog Tempo highlights the importance of mitochondrial oxidant stress in the development of APAP toxicity. Our study also demonstrated that MT as a treatment alone or together with NAC offers a better protection than NAC alone, which supports MT as a therapeutic option for treatment of APAP overdose or acute liver failure in patients.

Chapter 4. Metformin Protects against Acetaminophen Hepatotoxicity by Attenuation of Mitochondrial Oxidant Stress and Dysfunction

This section is adapted from Du et.al (2016), "Metformin protects against acetaminophen hepatotoxicity by attenuation of mitochondrial oxidant stress and dysfunction", Toxicological Sciences, 154(2), 214-226, with permission from the publisher

4.1 Abstract

Overdose of acetaminophen (APAP) causes severe liver injury and even acute liver failure in both mice and human. A recent study by Kim et al. (2015) showed that metformin, a first-line drug to treat type 2 diabetes mellitus, protected against APAP hepatotoxicity in mice. However, its exact protective mechanism has not been well clarified. To investigate this, C57BL/6J mice were treated with 400 mg/kg APAP and 350 mg/kg metformin was given 0.5h pre- or 2h post-APAP. Our data showed that pretreatment with metformin protected against APAP hepatotoxicity, as indicated by the over 80% reduction in plasma ALT activities and significant decrease in centrilobular necrosis. Metabolic activation of APAP, as indicated by glutathione depletion and APAP-protein adducts formation, was also slightly inhibited. However, 2h post-treatment with metformin still reduced liver injury by 50%, without inhibition of adduct formation. Interestingly, neither pre- nor post-treatment of metformin inhibited c-jun N-terminal kinase (JNK) activation or its mitochondrial translocation. In contrast, APAP-induced mitochondrial oxidant stress and dysfunction were greatly attenuated in these mice. In addition, mice with 2h post-treatment with metformin also showed significant inhibition of complex I activity, which may contribute to the decreased mitochondrial oxidant stress. Furthermore, the protection was reproduced in JNK activation-absent HepaRG cells treated with 20 mM APAP followed by 0.5 or 1 mM metformin 6h later, confirming JNK-independent protection mechanisms. Thus, metformin protects against APAP hepatotoxicity by attenuating the mitochondrial oxidant stress and subsequent mitochondrial dysfunction, and may be a potential therapeutic option for APAP overdose patients.

4.2 Introduction

Acetaminophen (APAP) is a safe and effective analgesic and antipyretic at therapeutic doses. However, an overdose leads to the excessive formation of a reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI), which depletes glutathione (GSH) and binds to mitochondrial proteins (Nelson, 1990). The well-documented inhibition of mitochondrial respiration (Meyers et al., 1988; Donnelly et al., 1994), mitochondrial oxidant stress and mitochondrial peroxynitrite formation (Jaeschke, 1990; Cover et al., 2005) after APAP overdose are thought to be initiated by formation of APAP adduct on proteins of the electron transport chain (ETC). The subsequent mitochondrial oxidant stress triggers the mitochondrial membrane permeability transition (MPT) pore opening, resulting in the collapse of mitochondrial membrane potential, release of mitochondrial endonucleases and finally cell necrosis (Hanawa et al., 2008; Kon et al., 2004; Bajt et al., 2006). Currently, APAP overdose is the primary cause of acute liver failure in the US and many Western countries (Budnitz et al., 2011; Manthripragada et al., 2011). N-acetylcysteine (NAC) was introduced as a clinical antidote for APAP overdose patients in the 1970s (Prescott et al., 1977). Until today, it is still the only available antidote despite its limited effectiveness, with early presenting patients benefitting most (Larson, 2007). Therefore, novel therapeutic agents are needed, especially for patients presenting late after an overdose. However, despite the large number of potential therapeutic agents that has been identified in animal models or hepatocytes over the last several decades, the enormous costs of developing a new drug specifically for a disease with a limited number of patients is prohibitive. Therefore, the best chance of getting a new antidote against APAP overdose is by re-purposing an existing drug.

Despite some controversies, c-jun N-terminal kinase (JNK) has emerged as a promising therapeutic target against APAP toxicity (Du et al., 2015c). The majority of studies suggested that JNK activation is detrimental in APAP overdose, and JNK deficiency or inhibition protected against APAP hepatotoxicity in murine models (Gunawan et al., 2006; Hanawa et al., 2008; Henderson et al., 2007; Saito et al., 2010a) and in primary human hepatocytes (Xie et al., 2014). More recently, metformin, a first-line drug for type 2 diabetes, was shown to protect against APAP hepatotoxicity in mice by inhibiting JNK activation (Kim et al. 2015). This is particularly interesting given that metformin is a marketed drug, which could be readily used to treat APAP overdose patients if its effectiveness is demonstrated. However, several serious concerns exist regarding the interpretation of the data in this study (Kim et al. 2015). The effect of metformin on metabolic activation of APAP, which needs to be ruled out before claiming any other protective mechanisms, was not carefully addressed (Kim et al. 2015). This is a very important issue as a number of recently identified potential therapeutic agents such as the purinergic receptor antagonist A438079, the gap junction inhibitor 2-aminoethoxy-diphenyl-borate and the toll-like receptor 4 antagonist benzyl alcohol turned out to be simply inhibitors of the metabolic activation of APAP and both the mechanistic interpretation and the clinical potential are highly questionable (Xie et al., 2013; Du et al., 2013; 2015a). Also, since previous studies proposed that JNK activation serves to amplify the APAP-induced mitochondrial oxidant stress and to aggravate subsequent mitochondrial dysfunction (Hanawa et al., 2008; Saito et al., 2010a), effects of metformin on these events need to be investigated. In addition, considering some differences

between the mouse model and the human pathophysiology, it is important to further test the effectiveness of metformin in the human HepaRG cell line, where APAP-induced cell death closely resembles the human pathophysiology (McGill et al., 2011).

4.3 Materials and Methods

Animals

Male C57BL/6J mice (Jackson Lab, Bar Harbor, ME) of the age of 8-12 weeks were housed in environmentally controlled rooms with a 12h light/dark cycle and allowed free access to food and water. Experiments followed the criteria of the National Research Council for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center.

Experimental design

After overnight fasting (16h), mice were treated (i.p.) with 400 mg/kg APAP (Sigma-Aldrich) dissolved in warm saline. Metformin (350 mg/kg) (Sigma-Aldrich) or its vehicle saline (20 mL/kg) was administered (p.o.) 0.5h before or 2h after APAP. Mice were euthanized at 0 - 24h post-APAP and blood and livers were harvested. Blood was drawn into heparinized syringes for measurement of plasma ALT activity. The liver tissue was sectioned into pieces for mitochondrial isolation as described (Du et al., 2015a), fixed in 10% phosphate-buffered formalin for histology, or flash frozen for determination of glutathione (GSH), APAP-cysteine adducts on proteins and Western blotting.

Biochemical assays. ALT activity was measured using the ALT (SGPT) Reagent Kit (Pointe Scientific, MI, A7526-625) and lactate dehydrogenase (LDH) activity was measured as described in detail (Du et al., 2013). Liver GSH and GSSG measurements were performed using a modified method of the Tietze assay (Jaeschke and Mitchell 1990; McGill and Jaeschke, 2015). APAP-protein adducts in liver tissues and mitochondrial pellets were measured as described (Ni et al., 2012a; McGill et al., 2012b). Complex I activity was measured in isolated mitochondria as described (Janssen et al., 2007). MitoSOX fluorescence in isolated mitochondria was measured as described (Johnson-Cadwell et al., 2007). Briefly, 250 μg freshly isolated mitochondria were incubated with isolation buffer containing respiratory substrates including 5 mmol/L malate, glutamate and succinate. 5 μmol/L MitoSOX Red (Invitrogen) was added and fluorescence was read at 510/580 nm for 30 min (excitation/emission).

Histology and western blotting

Formalin-fixed tissue sections were stained with hematoxylin and eosin (H&E) for necrosis assessment (Gujral et al., 2002). Nitrotyrosine staining was performed as described (Knight et al., 2002), using a rabbit polyclonal anti-nitrotyrosine antibody (Life Technologies, Grand Island, NY) and the Dako LSAB peroxidase kit (Dako, Carpinteria, CA). DNA strand breaks were assessed by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using the In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN). Western blotting was performed using the following antibodies from Cell Signaling Technology (Danvers, MA) unless stated otherwise: rabbit anti-Bax polyclonal antibody (#27742), rabbit anti-AIF antibody (#5318), rabbit anti-JNK antibody (#9252) and a rabbit anti-phospho-JNK antibody (#

9251), MitoProfile® OXPHOS Cocktail for CI-NDUFB8 (#110413) (Abcam, Cambridge, MA), mouse anti-phospho-JNK antibody (#6254) (Santa Cruz, Dallas, TX), and mouse anti-Smac/DIABLO antibody (# 612245) (BD Biosciences, San Diego, CA), as described (Bajt et al., 2000).

HepaRG cell culture

HepaRG cells were obtained from Biopredic International (Rennes, France) and cultured as described previously (McGill et al., 2011). The cells were treated with 20 mM APAP and 0.5 or 1 mM metformin was added 6h post-APAP. Cells were harvested at 24h for the Seahorse XF assay or at 48h for LDH activity measurement, as described (Bajt et al., 2004).

Seahorse XF-assay

Oxygen consumption rate (OCR) was measured in real-time using the Seahorse XF24[3] extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA, USA) as per manufacturer guidelines. Briefly, HepaRG cells were seeded in 24-well Seahorse microplates at 1×10^4 cells/well. The XF24 sensor cartridge was hydrated with 1 ml calibration buffer per well overnight at 37°C. The sensor cartridge was loaded with oligomycin (1 μM, port A), carbonyl cyanide 4-trifluoromethoxy phenylhydrazone (FCCP, 0.5 μM, port B) and rotenone plus antimycin-A (1 μM each, port C) to measure the bioenergetics profile. Cells were washed twice with pre-warmed XF assay medium containing 25 mM glucose and incubated in XF assay medium at 37°C without CO_2. Once the sensor cartridge was equilibrated, the calibration plate was replaced with the cell plate to measure OCR.

Statistics

All results were expressed as mean ± SEM. Statistical significance between two groups was evaluated using the Student's t-test, while comparisons of multiple groups was assessed by one-way analysis of variance (ANOVA), followed by Student-Newman-Keul's test. For non-normally distributed data, Kruskal-Wallis Test (non-parametric ANOVA) was used followed by Dunn's Multiple Comparisons Test. $p < 0.05$ was considered significant.

4.4 Results

Pretreatment with metformin protected against APAP-induced liver injury

APAP overdose (400 mg/kg) caused severe liver injury at 6h and 24h post-APAP in mice, as indicated by the increased plasma ALT activities (Fig. 4.4.1A) and extensive centrilobular necrosis in H&E-stained slides (Fig. 4.4.1B, C). Pre-treatment with metformin (350 mg/kg) attenuated the increase in ALT activities by 80% (Fig. 4.4.1A) and significantly decreased areas of necrosis at both time points (Fig. 4.4.1B, C), demonstrating that metformin effectively protected against APAP hepatotoxicity.

Metformin inhibited APAP metabolic activation but did not affect JNK activation

Metabolic activation of APAP forms the reactive metabolite NAPQI, which depletes GSH, binds to cellular proteins and forms APAP-protein adducts (Mitchell et al., 1973; Nelson, 1990). To assess whether metformin interferes with APAP metabolic activation, APAP-protein adducts were measured 2h post-APAP, which is close to the peak levels of adducts (McGill et al., 2013). Our data showed that metformin significantly reduced APAP-protein adducts formation in both the total liver homogenate (-32.5%) and in mitochondria (-27.9%) (Fig. 4.4.2A). Consistent with these findings, GSH depletion at 0.5h and 2h was also significantly inhibited (Fig. 4.4.2B), suggesting that metformin as a pretreatment moderately inhibited the metabolic activation of APAP. In support of the protection by metformin, GSH recovery was almost 2-fold higher at 24h in mice with metformin treatment though no significant difference was seen at 6h (Fig. 4.4.2B). Interestingly, in contrast to the reduction of JNK activation by metformin reported by Kim et al. (2015), neither JNK activation nor its translocation to mitochondria was

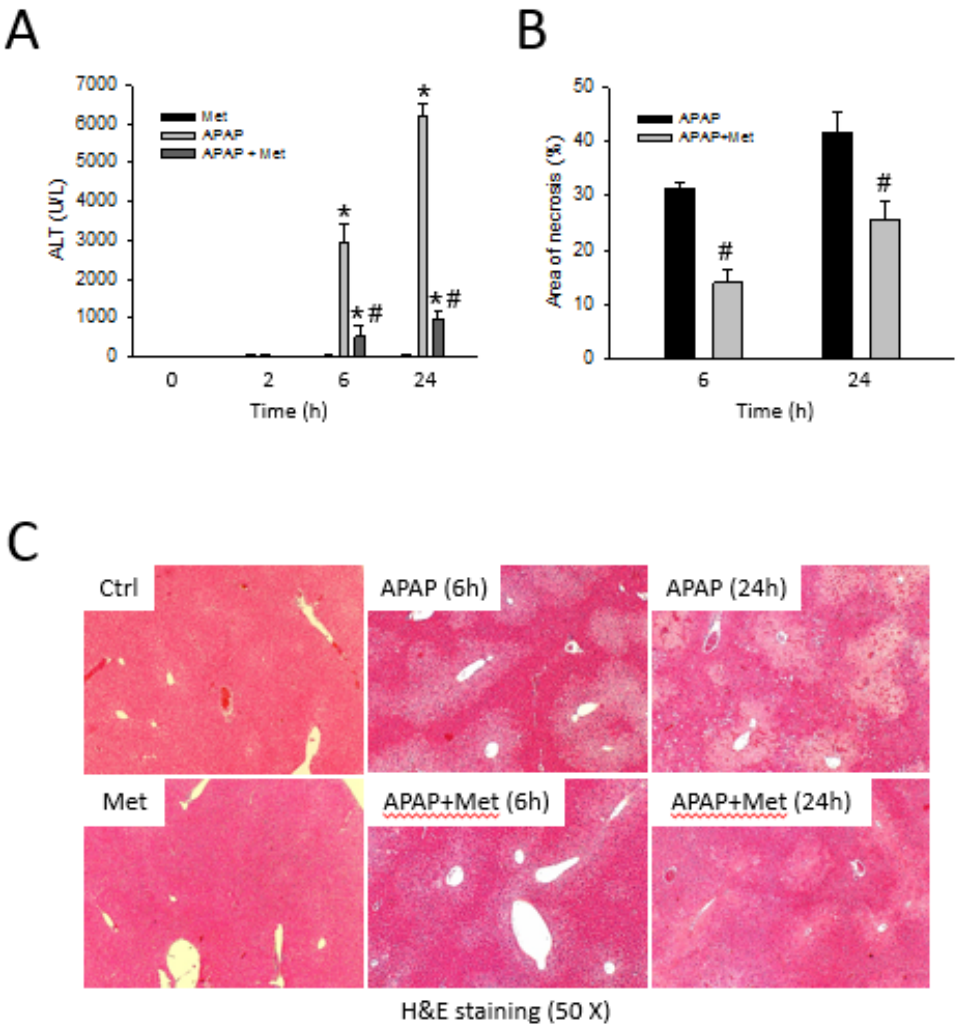


Figure 4.4.1 Metformin protected against APAP hepatotoxicity.

Mice were treated with 350 mg/kg metformin followed by 400 mg/kg APAP 0.5h later. Blood and liver tissue were obtained at 0, 2, 6 or 24h post-APAP. (A) Plasma alanine aminotransferase (ALT) activity. (B) Areas of necrosis (%). (C) H&E staining of representative liver sections. Bars represent means ± SEM for n = 3 - 6 mice. *$p<0.05$ compared to 0h #$p<0.05$ compared to respective APAP group.

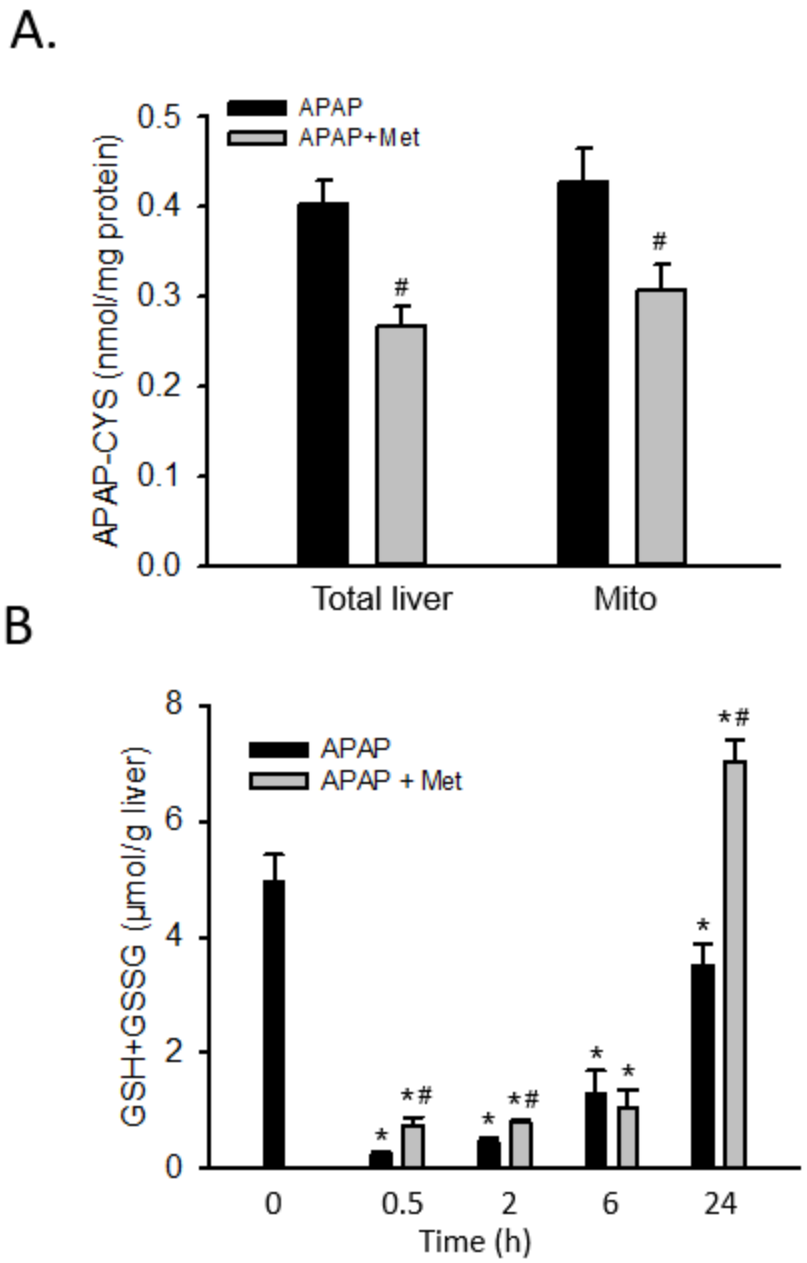


Figure 4.4.2 Metformin slightly inhibited APAP metabolic activation.

Mice were treated with 350 mg/kg metformin followed by 400 mg/kg APAP 0.5h later. (A) Total liver and mitochondrial APAP-cysteine adducts at 2h. (B) Time course of hepatic GSH levels in total liver. Bars represent means ± SEM for n = 3 - 6 mice. *p<0.05 compared to 0h #p<0.05 compared to respective APAP group.

affected at 2h or 6h in our study (Fig. 4.4.3A, B). Densitometric analysis of these western blots further confirmed these findings (Fig. 4.4.3C, D, E). Similar results were obtained using a P-JNK antibody from a different vender, which was used by Kim et al. (2015) (Fig. 4.4.3F).

Metformin attenuated APAP-induced mitochondrial oxidant stress and dysfunction

Covalent binding of NAPQI to mitochondrial proteins disturbs mitochondrial respiration and causes mitochondrial oxidant stress (Meyers et al., 1988; Donnelly et al., 1994; Cover et al., 2005). GSSG levels and the GSSG-to-total GSH (oxidized + reduced form of glutathione) ratio are excellent indicators of oxidant stress (Jaeschke, 1990). Our data showed that metformin-treated mice had significantly lower GSSG levels at 6h and a trend to lower GSSG / total GSH at both 6h and 24h (Fig. 4.4.4A, B). The lack of lower GSSG levels at 24h in metformin-treated mice could be due to the fact that these mice had much lower injury and thus consumed more food when the diet was returned at 6h. This would allow them to better replenish their GSH pool (Fig 4.4.2B) and efficiently scavenge ROS, resulting in increased GSSG formation. Consistent with the GSSG data showing protection against oxidant stress by metformin at 6h, specific measurement of mitochondrial superoxide production, and nitrotyrosine (NT) protein adducts, which are a footprint of peroxynitrite formation, were also substantially reduced in mice with metformin treatment (Fig. 4.4.4C, D). Overwhelming mitochondrial oxidant stress causes collapse of the mitochondrial membrane potential and release of mitochondrial proteins including AIF and Smac to the cytosol and translocation to the nucleus, resulting in

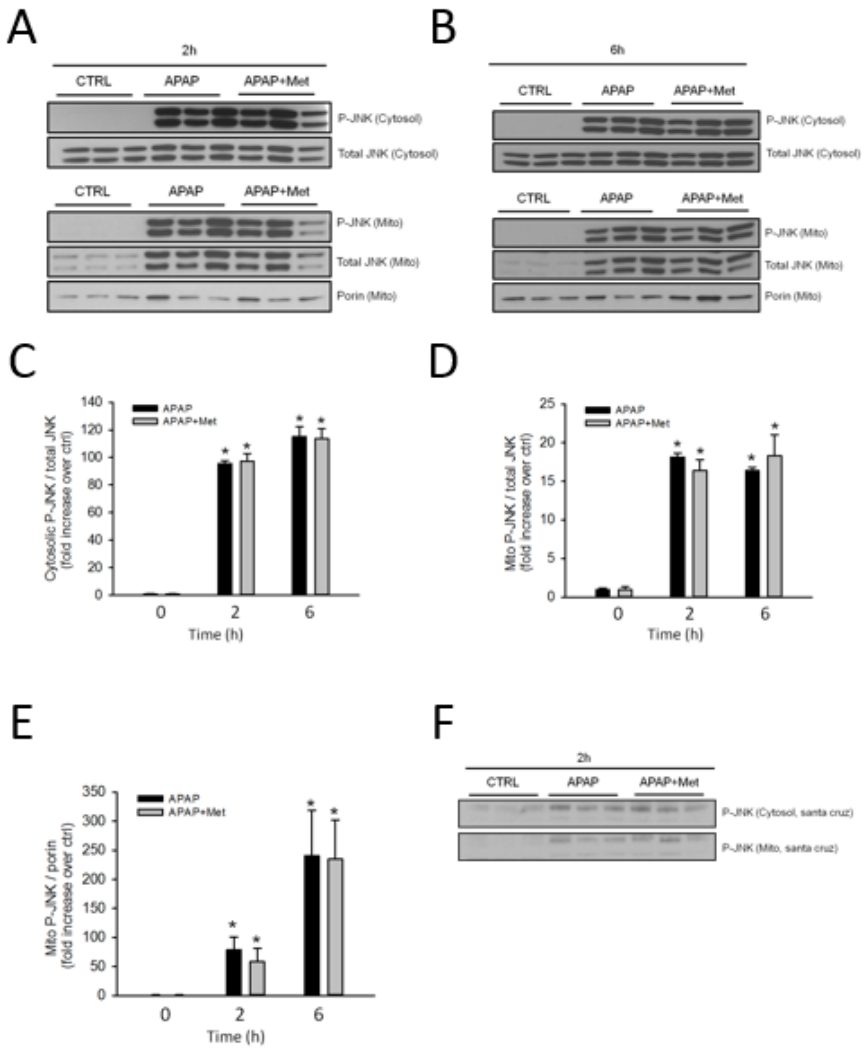


Figure 4.4.3 Metformin did not inhibit JNK activation or mitochondrial JNK translocation.

Mice were treated with 350 mg/kg metformin followed by 400 mg/kg APAP 0.5h later. Total JNK and P-JNK were measured in cytosolic and mitochondrial liver fractions at 2h (A) or 6h (B) post-APAP. Porin was used as loading control for the mitochondrial fraction. Data of densitometric analysis of cytosolic P-JNK / JNK(C), mitochondrial P-JNK / JNK (D) and mitochondrial P-JNK / porin (E) are shown. Cytosolic and mitochondrial P-JNK at 2h detected by antibody from Santa Cruz (F). Bars represent means ± SEM for 3 mice. *p<0.05 compared to Ctrl.

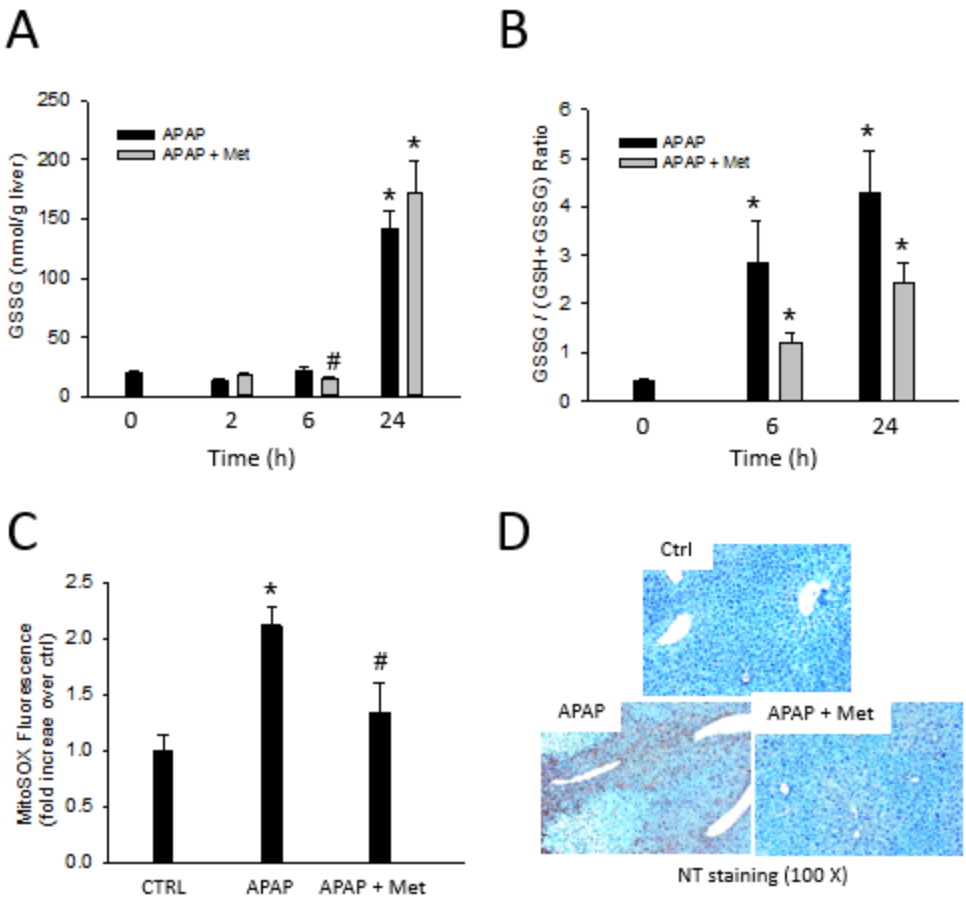


Figure 4.4.4 Metformin attenuated APAP-induced mitochondrial oxidant stress.

Mice were treated with 350 mg/kg metformin followed by 400 mg/kg APAP 0.5h later. (A) Total liver GSSG levels. (B) Hepatic GSSG-to-total GSH ratios. (C) MitoSOX fluorescence. (D) Nitrotyrosine staining of representative liver sections. Bars represent means ± SEM for n = 3 - 6 mice. *p<0.05 compared to 0h #p<0.05 compared to respective APAP group.

nuclear DNA fragmentation (Kon et al., 2004; Bajt et al., 2006). Bax was also shown to translocate to mitochondria and form pores in the mitochondrial outer membrane, contributing to the release of mitochondrial intermembrane proteins at the early phase of liver injury (Bajt et al., 2008a). Consistent with the previous investigations, in our study we observed Bax translocation to mitochondria at both 2h and 6h after APAP (Fig. 4.4.5A, B). However, metformin did not affect this translocation at either time point (Fig. 4.4.5A, B). In contrast, the release of mitochondrial proteins such as Smac and AIF was substantially reduced in metformin-treated mice (Fig. 4.4.5A, B). The corresponding DNA fragmentation was also extensively attenuated (Fig. 4.4.5C). These data clearly indicate that metformin attenuated the APAP-induced mitochondrial oxidant stress and the subsequent mitochondrial dysfunction.

Posttreatment with metformin still protected against APAP-induced liver injury

To assess the effect of metformin on APAP-induced liver injury without inhibiting its metabolic activation, metformin was given 2h post-APAP, at which time the metabolic activation of APAP is almost completed (McGill et al., 2013). Interestingly, metformin still reduced about 50% of the liver injury at 8h, as indicated by the plasma ALT activities and areas of necrosis in H&E-stained slides (Fig. 4.4.6A, B, C). Also, we confirmed that metformin as a 2h posttreatment did not inhibit APAP metabolic activation, as indicated by similar APAP-protein adducts at both 4h and 8h (Fig. 4.4.6D). In addition, neither JNK activation nor mitochondrial translocation was affected, as shown by western blotting (Fig. 4.4.6E) and confirmed by the densitometric analysis

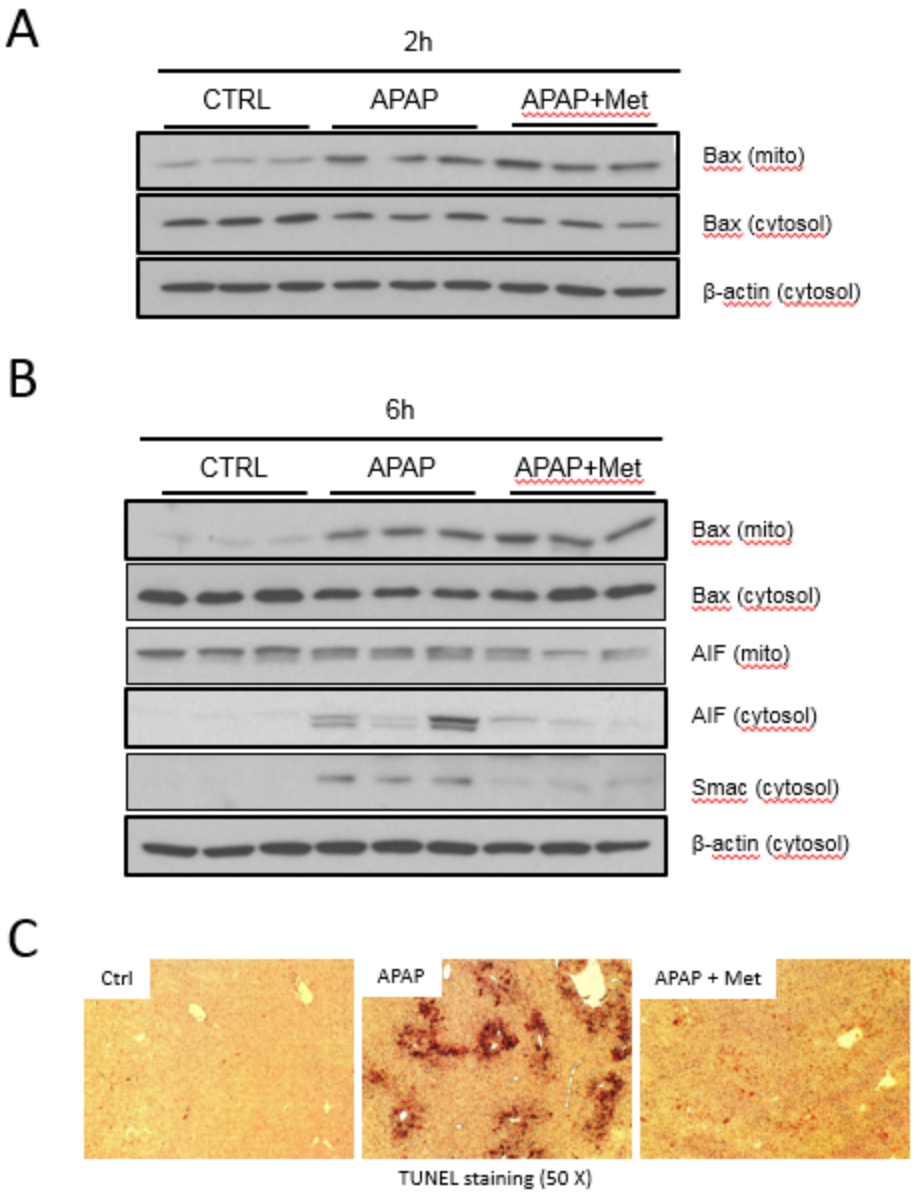


Figure 4.4.5 Metformin attenuated APAP-induced mitochondrial dysfunction.

Mice were treated with 350 mg/kg metformin followed by 400 mg/kg APAP 0.5h later. (A) Total liver GSSG levels. (B) Hepatic GSSG-to-total GSH ratios. (C) MitoSOX fluorescence. (D) Nitrotyrosine staining of representative liver sections. Bars represent means ± SEM for n = 3 - 6 mice. *$p<0.05$ compared to 0h #$p<0.05$ compared to respective APAP group.

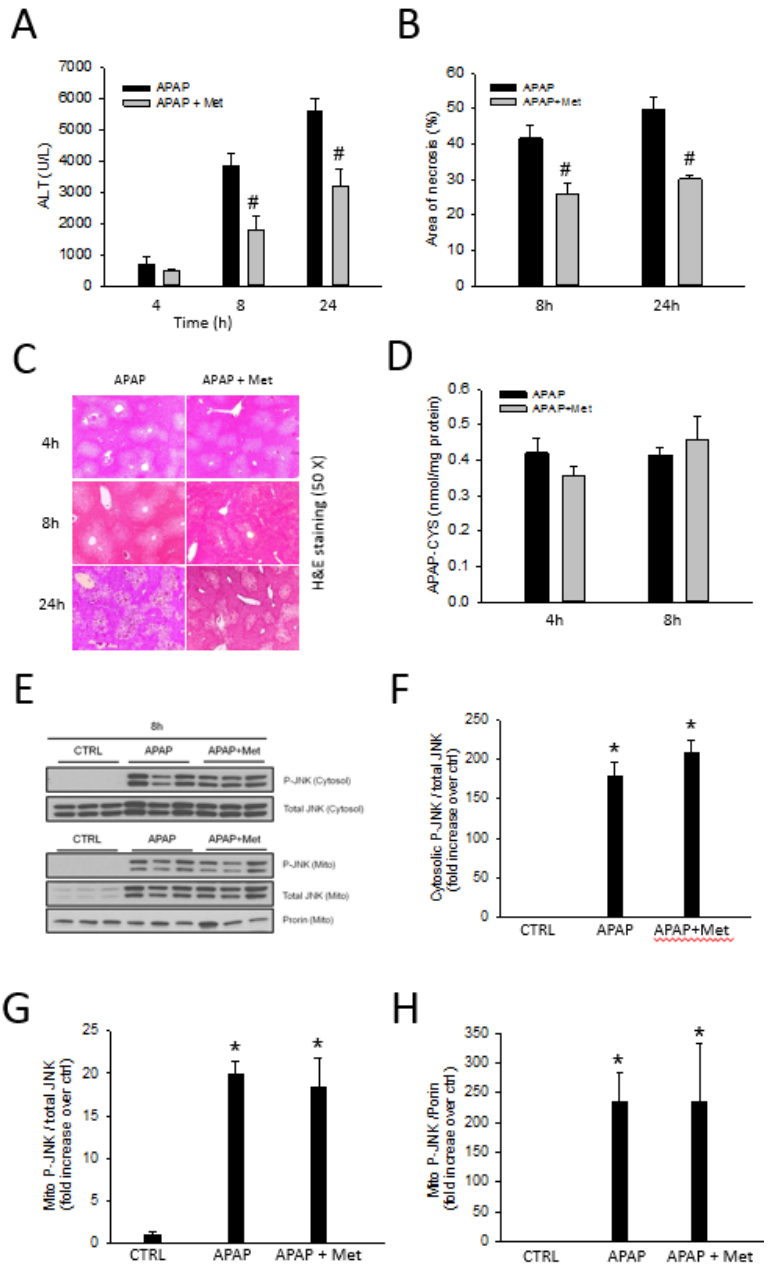


Figure 4.4.6 Post-treatment with metformin still protected against APAP hepatotoxicity without inhibition of APAP metabolic activation or JNK activation.

Mice were treated with 400 mg/kg APAP and 350 mg/kg metformin was given 2h later. Blood and liver tissue were obtained at 0, 4 or 8h post-APAP. (A) Plasma ALT activities. (B) Areas of necrosis (%). (C) H&E stained liver sections. (D) Total liver APAP-cysteine adducts at 8h. (E) Cytosolic and mitochondrial total JNK and P-JNK at 8h. Densitometric analysis of cytosolic P-JNK / JNK (F), mitochondrial P-JNK / JNK (G) and P-JNK / porin (H). Bars represent means ± SEM for n = 3 - 4 mice. *$p<0.05$ compared to Ctrl #$p<0.05$ compared to respective APAP group.

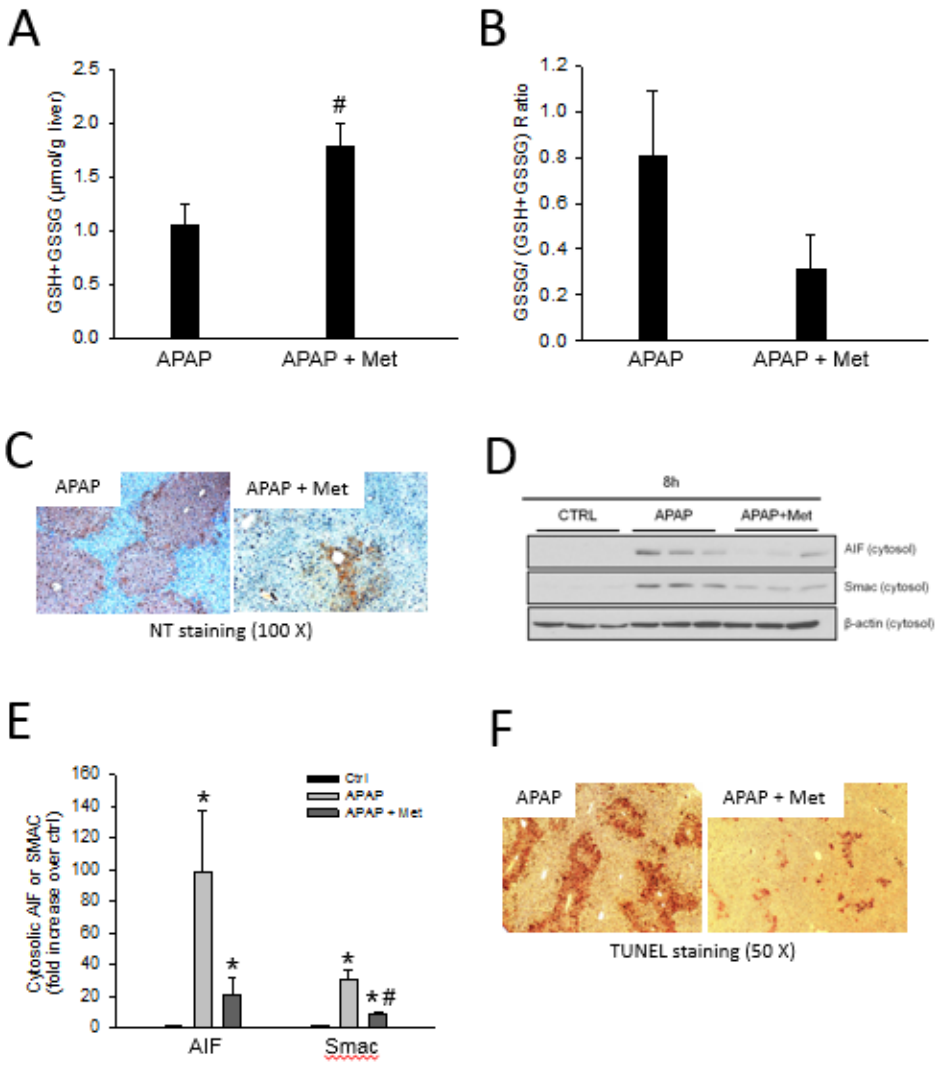


Figure 4.4.7 Post-treatment with metformin still protected against APAP-induced mitochondrial oxidant stress and dysfunction.

Mice were treated with 400 mg/kg APAP and 350 mg/kg metformin was given 2h later. Liver tissues were harvested at 8h post-APAP. (A) Total liver GSH levels. (B) Hepatic GSSG-to-total GSH ratios. (C) Nitrotyrosine staining of representative liver sections. (D) Cytosolic Smac, AIF and β-actin measured by western blot and densitometric analysis (E). (F) TUNEL staining of representative liver sections. Bars represent means ± SEM for n = 3 - 4 mice. *$p<0.05$ compared to Ctrl #$p<0.05$ compared to respective APAP group.

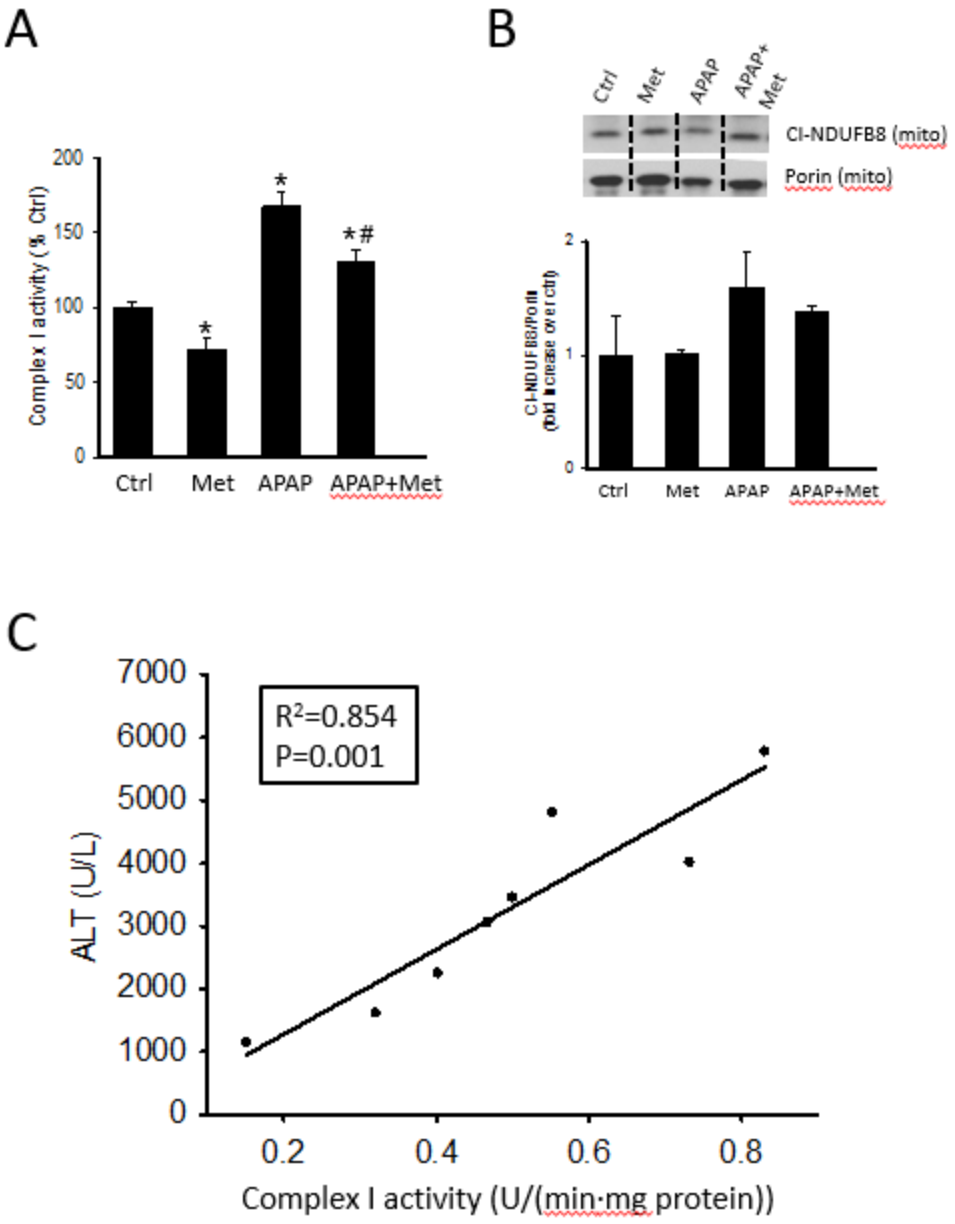


Figure 4.4.8 Metformin inhibited mitochondrial complex I activity.

Mice were treated with 400 mg/kg APAP and 350 mg/kg metformin was given 2h later. Mitochondria were isolated from liver tissue at 8h post-APAP. (A) Complex I activity; (B) Complex I-NDUFB8 subunit protein expression and corresponding densitometric analysis using porin as the loading control; (C) Linear correlation between ALT activity and complex I activity (minus control) in APAP-treated mice. Bars represent means ± SEM for n = 3 - 4 mice. *p<0.05 compared to Ctrl #p<0.05 compared to APAP group.

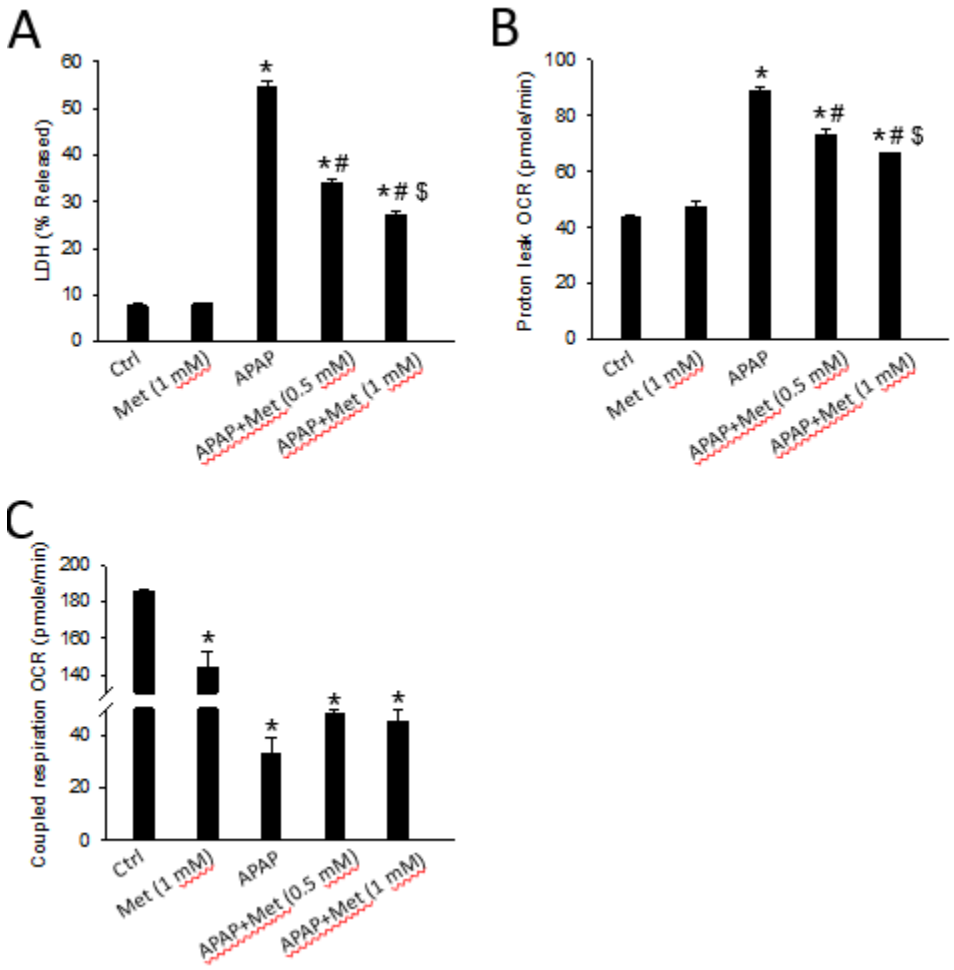


Figure 4.4.9 Metformin protected against APAP-induced cell injury in HepaRG cells.

The cells were treated with 20 mM APAP and 0.5 or 1 mM metformin was added 6h post-APAP. Cells were harvested at 48h for LDH activity measurement (A), or at 24h for the XF assay: proton leak (B) and coupled respiration (C). Bars represent means ± SEM for n = 4 independent cell preparations. *$p<0.05$ compared to Ctrl $^{\#}p<0.05$ compared to APAP $^{\$}p<0.05$ compared to APAP+Met (0.5mM).

Table 1:

Induction of antioxidant genes by acetaminophen and metformin

Gene	Ctrl	Met (6h)	APAP (6h)	APAP+Met(6h)	APAP (24h)	APAP+Met (24h)	APAP(8h)	APAP+Met(8h)
Gclc	1.10±0.34	0.76±0.01	2.85±0.48	1.80±0.17	3.73±0.41*	3.54±0.29*	3.83±0.14*	4.21±0.50*
MT-1	1.18±0.45	1.00±0.10	8.85±1.17*	6.20±0.79*	2.31±0.42	3.23±0.52*	9.73±1.25*	13.96±0.74*#
MT-2	1.08±0.26	0.55±0.08	9.26±1.12*	5.90±0.57*	1.98±0.37	2.87±0.17*	8.83±1.63*	12.77±0.65*#
NQO1	1.04±0.20	0.92±0.18	1.23±0.21	1.05±0.09	0.44±0.07*	0.57±0.11*	1.47±0.03	1.76±0.27
Mn-SOD	1.03±0.17	0.98±0.05	0.92±0.11	0.78±0.03	0.76±0.04	0.74±0.09	0.90±0.04	0.61±0.03#
HO-1	1.06±0.26	0.87±0.16	49.98±8.15*	26.42±0.76*#	19.79±1.67*	24.24±1.76*	65.23±7.17*	58.73±9.13*

Mice were treated with 400 mg/kg APAP and 350 mg/kg metformin was given 0.5h before APAP or 2h after APAP. Liver tissues were harvested at 0h (ctrl), 6h or 24h post-APAP for pretreatment or 8h for posttreatment. Fold inductions of mRNAs compared to control was expressed as mean ± SE for n=3–5 mice. *$p<0.05$ compared to Ctrl; #$p<0.05$ compared to APAP.

(Fig. 4.4.6F, G, H). In contrast, APAP-induced mitochondrial oxidant stress, as assessed by GSSG-to- total GSH ratio (Fig. 4.4.7A, B) and NT staining (Fig. 4.4.7C), was reduced in metformin-treated mice. As a result, the release of mitochondrial proteins such as AIF and Smac, as shown by western blotting (Fig. 4.4.7D) and quantified by densitometry (Fig 4.4.7E), and the resulting DNA fragmentation, as measured by TUNEL staining (Fig. 4.4.7F), were also largely reduced. Interestingly, there was no evidence that metformin treatment promoted the antioxidant capacity of hepatocytes as none of the relevant enzymes were induced (Table 4.1). This suggested that metformin did not improve the capacity to scavenge ROS but more likely affected the capacity to generate ROS, as is evident by the direct measurement of mitochondrial superoxide formation (Fig. 4.4.4C). In support of this, metformin treatment significantly decreased complex I activity in mitochondria from controls and from APAP-treated mice (Fig. 4.4.8A), and this seemed not due to an effect on protein expression (Fig. 4.4.8B). In these animals, there was a strong correlation between complex I activity and liver injury (Fig 4.4.8C). Thus, reducing complex I activity by metformin may lower the leakage of electrons from the electron transport chain and therefore contribute to the reduced mitochondrial oxidant stress in metformin-treated mice.

Metformin protected against APAP-induced cell death in HepRG cells

Our data so far indicated that metformin protected against APAP-induced liver injury in mice by a JNK-independent mechanism. To further corroborate our results, we tested the effectiveness of metformin against APAP toxicity in human HepaRG cells. The justification for use of these cells are two folds; a) they are human cells and would hence

closer recapitulate the human condition, and b) while these cells show cellular necrosis and mitochondrial oxidant stress in response to APAP as in human patients (McGill et al., 2011), the process is independent of JNK activation (Xie et al., 2014). Thus, if metformin protected against APAP-induced injury in these cells, it would confirm the JNK-independent nature of the effect and also its relevance to humans. HepaRG cells were first treated with 20 mM APAP and metformin was given 6h post-APAP, at which APAP-protein adducts formation peaks (McGill et al., 2011), to avoid its potential effect on the metabolic activation of APAP. Our data showed that metformin alone (1mM) did not cause any cell death while APAP (20mM) resulted in massive cell death at 48h post-APAP, as indicated by the percentage of LDH release (Fig. 4.4.9A). More importantly, metformin dose-dependently protected against APAP-induced cell death (Fig. 4.4.9A), indicating its potential benefit for APAP overdose patients. Interestingly, metformin also significantly decreased the APAP-induced proton leakage from the ETC and increased coupled respiration, which was impaired by APAP (Fig. 4.4.9B, C). This may decrease the mitochondrial oxidant stress and contribute to the protection.

4.5 Discussion

The primary objective of this study was to investigate the protective mechanisms of metformin in APAP hepatotoxicity. Our data demonstrated that both pretreatment and posttreatment with metformin protected against APAP hepatotoxicity in mice. The mechanism did not involve inhibition of JNK activation but was caused in part by inhibition of APAP protein adducts formation and mostly by attenuation of the APAP-

induced mitochondrial oxidant stress and subsequent mitochondrial dysfunction, likely by inhibiting mitochondrial complex I activity.

Role of JNK in APAP hepatotoxicity

Although controversies and conflicting data exist, most studies argue for a detrimental role of JNK activation in APAP hepatotoxicity, and JNK deficiency or pharmacological inhibition was shown to protect against APAP hepatotoxicity in both mice and primary human hepatocytes (reviewed in Du et al., 2015c). In support of this, metformin was proposed to protect mice against APAP hepatotoxicity by inhibiting JNK activation (Kim et al., 2015). However, careful analysis of the data in this paper raised some serious concerns. Although the authors did not differentiate between the effect of metformin on JNK1 and JNK2, it was obvious that metformin mainly reduced JNK2 activation based on the representative western blots (Kim et al., 2015), while the injury induced by APAP was almost completely prevented during this time period (0 - 5h). However, previous studies demonstrated that deficiency of JNK2 alone in mice was only moderately protective at most (Gunawan et al. 2006; Henderson et al., 2007). This leaves the possibility that other protective mechanisms may still exist. Also, the effect of metformin on the metabolic activation of APAP was not fully investigated, which is an important omission that questions the claim for any other downstream protective mechanisms in APAP hepatotoxicity. This is particularly critical when considering the fact that negligence of this issue has led to many misleading conclusions from recent studies investigating the protective mechanisms of drugs and chemicals. In our study, we found that pre-treatment of metformin extensively reduced the injury at both 6h and 24h.

Interestingly, metformin seemed to slightly inhibit metabolic activation of APAP, which is indicated by the moderate inhibition of the early GSH depletion and reduction of APAP-protein adducts formation in both total liver and mitochondria in metformin-treated mice. In contrast to the previous report (Kim et al., 2015), we did not observe any inhibition of JNK activation or mitochondrial JNK translocation by metformin either as a pretreatment or post-treatment. The JNK-independent mechanism of metformin protection in APAP hepatotoxicity was confirmed in HepaRG cells, which recapitulate key mechanisms of toxicity such as protein adducts formation, GSH depletion, oxidant stress, mitochondrial dysfunction and necrotic cell death (McGill et al., 2011), without JNK activation (Xie et al., 2014). The protective effect of metformin in the HepaRG cells, where JNK activation is absent and a JNK inhibitor is not protective (Xie et al., 2014), strongly supports the conclusion that the protective effect of metformin is independent of JNK signaling. We also confirmed this by using the same antibody as acquired by Kim et al. (2015), ruling out the possibility that this discrepancy was due to the different JNK antibodies used. Although elucidating the exact reason for this discrepancy needs further investigation, our studies provide no evidence for JNK as the therapeutic target of metformin.

Metformin protects against APAP-induced mitochondrial oxidant stress and dysfunction

APAP hepatotoxicity is characterized by extensive mitochondrial oxidant stress and severe mitochondrial dysfunction (Jaeschke et al., 2012). It is suggested that the reactive metabolite NAPQI binds to mitochondrial proteins, impairs the function of the electron

transport chain (ETC) and causes leakage of electrons and thus results in formation of reactive oxygen species (Donnelly et al., 1994; Jaeschke, 1990). Electrons released from the ETC react with O_2 to form superoxide, which can be metabolized by superoxide dismutases (SOD) to form hydrogen peroxide and molecular oxygen or react with NO to produce peroxynitrite, which is a potent oxidant and nitrating species in APAP hepatotoxicity (Hinson et al., 1998; Knight et al., 2002; Cover et al., 2005). The importance of a mitochondrial oxidant stress in the development of APAP hepatotoxicity has been demonstrated in numerous studies. For example, it was shown that Mn-SOD, the mitochondrial antioxidant enzyme to detoxify O_2^-, was nitrated and partially inactivated by peroxynitrite after APAP (Agarwal et al., 2011), and mice partial deficient in this enzyme ($MnSOD^{+/-}$) were more susceptibility to APAP overdose while mice treated with the SOD-mimetic Mito-Tempo were protected from the toxicity (Fujimoto et al., 2009; Ramachandran et al., 2011; Du et al., 2017). In addition, accelerated recovery of hepatic GSH in female mice or by treatment with exogenous GSH or its precursor amino acids decreased mitochondrial oxidant stress and protected against APAP hepatotoxicity (Du et al., 2014; James et al., 2003b; Knight et al., 2002; Saito et al., 2010b). Together these reports established the critical role of the mitochondrial oxidant stress in the development of APAP-induced cell death. In our study, we observed that metformin either as a pre-treatment or post-treatment dramatically decreased the mitochondrial oxidant stress, as indicated by the lower GSSG-to-total GSH ratio in the liver, the reduced MitoSOX fluorescent intensity in mitochondria and less nitrotyrosine staining in mice when compared to APAP-treated animals. Interestingly, metformin did not increase the induction of Nrf-2 regulated-antioxidant genes, including gclc, catalase,

NQO1, HO-1, and MnSOD (Table I). It has also been shown that metformin cannot scavenge superoxide radicals and thus is unlikely to engage in direct scavenging activity (Bonnefont-Rousselot et al., 2003; Khouri et al., 2003). Thus, the protective effect of metformin may be due to a decrease in ROS generation, probably by preventing proton leak as seen in HepRG cells, rather than increase in ROS scavenging. Mitochondrial complex I is a crucial site of ROS formation in mitochondria, and can exist in an active or a deactivated form (Grivennikova et al., 2001). Biguanides were shown to selectively "lock" complex I in its deactivated form leading to decreased superoxide production capacity (Matsuzaki and Humphries, 2015). In rats fed a high fat diet, metformin was shown to protect against hepatic ischemia reperfusion injury by inhibiting complex I and lowering mitochondrial ROS production (Cahova et al., 2015). In various cell culture models, metformin was reported to inhibit complex I activity, mitochondrial permeability transition and cell death (Bridges et al., 2014; Drahota et al., 2014; Kelly et al., 2015). Consistent with these reports, our study showed that metformin inhibited complex I activity, and decreased mitochondrial superoxide production, which prevents mitochondrial oxidant stress and subsequent mitochondrial dysfunction, as measured by the inhibition of mitochondrial protein release (AIF and Smac) and reduction in nuclear DNA fragmentation. It is interesting that metformin treatment prevented AIF release, while having no effect on JNK activation, even though AIF-deficient Harlequin mice were protected from APAP hepatotoxicity with reduced mitochondrial oxidant stress and less JNK activation (Bajt et al., 2011). However, these results are caused by different mechanisms. In the current experiment, AIF release mainly is caused by mitochondrial dysfunction (MPT) and matrix swelling; in the previous experiments, the reduced JNK

activation in AIF-deficient Harlequin reflects the physiological role of AIF in regulating leakage of electrons from the ETC (Bajt et al., 2011).

Metformin as a potential therapeutic agent for APAP overdose

NAC was introduced as the clinical antidote for APAP poisoning in the late 1970s (Prescott et al., 1977), and even today it is still the only available therapeutic agent in the clinic. However, NAC has to be given early (before injury is present) to achieve its greatest efficacy. Unfortunately, in clinical practice, many patients arrive relatively late for medical care after taking an overdose of APAP (around or after the peak of injury) (Larson, 2007; Xie et al., 2015). At this later time point, the efficacy of NAC is significantly diminished (Smilkstein et al., 1988). Actually, NAC is only highly effective up to 2h after APAP in mice and within 8h of APAP ingestion in patients (before initiation of injury) (Knight et al. 2002; Smilkstein et al., 1988). Therefore, an intervention that is still beneficial after the onset of the injury is needed. However, due to the high costs of drug development, even late acting drugs may not be developed for human use. In contrast, metformin as a marketed drug has a higher potential to be used in a clinical trial. We demonstrated that metformin given as late as 2h after APAP overdose in mice still reduced ~ 50% of the injury at 8 and 24h. Whether metformin is still beneficial beyond 2h post-APAP requires further experiments. In addition, a comparison of the protection by NAC to that by metformin, as well as an evaluation of their potential synergistic effects is needed to further test the feasibility of metformin as a therapeutic agent for overdose patients. Interestingly, human HepaRG cells treated with metformin 6h after APAP, at the time when the metabolic activation of APAP is almost completed

and cell injury is in development, are still dose-dependently protected from APAP-induced cell injury. Since the HepaRG cell model has been shown to closely resemble the human pathophysiology for APAP overdose (McGill et al., 2011), it also strongly supports the potential effectiveness of metformin for patients with APAP overdose.

A caveat for the clinical use of metformin, however, is the reported increase in incidences of lactic acidosis in metformin-treated patients according to some case reports and its contraindication in patients with liver disease. However, these instances are uncommon (9/100,000 person-years) and not different from that in the general population (Stang et al., 1999). A systematic review also concluded that no data exist to definitively link metformin to lactic acidosis (Salpeter et al., 2003) and the beneficial effect of metformin on varied liver disease have been noted (Bhat et al., 2015). None of the attributed side effects of metformin such as diarrhea, kidney injury or liver toxicity were noted in the mice at the dose we used (data not shown) and neither was any cell injury evident from metformin alone in HepRG cells. Nevertheless, before metformin can be considered as a realistic antidote against APAP hepatotoxicity in patients, studies need to assess the effective therapeutic dose in humans and any potential side effects of this new treatment after APAP overdose.

4.6 Summary and Conclusions

We showed that metformin did not inhibit JNK activation or mitochondrial JNK translocation but significantly reduced APAP protein adducts formation in mitochondria and in particular attenuated the mitochondrial oxidant stress and subsequent

mitochondrial dysfunction, most likely through inhibition of mitochondrial complex I activity. In addition, metformin dose-dependently protected human HepaRG cells, a clinically relevant model for APAP overdose, against APAP-induced cell injury, supporting metformin as a potential therapeutic option for treatment of APAP overdose-induced acute liver failure in patients.

Chapter 5. Induction of Mitochondrial Biogenesis via PPARγ Co-Activator 1α Signaling Protects against Acetaminophen Hepatotoxicity

5.1 Abstract

Mitochondrial biogenesis (MB) is an adaptive response to maintain metabolic homeostasis after mitochondrial dysfunction. Although compromised mitochondrial function is a critical step in the pathophysiology of acetaminophen (APAP) hepatotoxicity in both mice and humans, induction of MB during APAP hepatotoxicity has not been studied. To investigate this, mice were treated with toxic doses of APAP and euthanized between 0 and 96h later. At early time points, APAP caused both mitochondrial dysfunction and reduction of mitochondrial mass, indicated by reduced activity of electron transport chain (ETC) complexes I and IV and depletion of mitochondrial DNA (mtDNA), respectively. Both ETC activity and mtDNA gradually recovered after 12 h, suggesting that MB occurs at late time points after APAP overdose. Immunofluorescent staining of mitochondria with mitochondrial protein Tom 20 further demonstrated that MB occurs selectively in hepatocytes surrounding necrotic areas. MB signaling mediators including PPARγ co-activator 1-α (Pgc-1α), nuclear respiratory factor-1 (Nrf-1) and mitochondrial fission protein dynamin-related protein-1 (Drp-1) were also induced. Interestingly, Pgc-1α was selectively increased in hepatocytes surrounding necrotic areas. We also found that the time course of MB induction coincides with increased liver regeneration in mice, indicating that MB could be important for recovery after APAP. Consistent with the latter, post-treatment with the known MB inducer SRT1720 increased Pgc-1α expression and liver regeneration, resulting in protection against late liver injury after APAP overdose, while inhibition of MB by chloramphenicol prolonged the injury and impaired the injury resolution. Together, these data suggest that induction of MB is an important feature during APAP hepatotoxicity and it plays a role in liver regeneration.

5.2 Introduction

Acetaminophen (APAP)-induced liver injury is the leading cause of acute liver failure in the United States and many other Western countries (Lee, 2008). Numerous studies have established the critical role of mitochondria in the initiation and progression of APAP hepatotoxicity in both mice and humans (Placke et al., 1987; Meyers et al., 1988; Jaeschke, 1990; Kon et al., 2004; Reid et al., 2005; Ramachandran et al., 2011; McGill et al., 2012a; 2014; Du et al., 2016a). There is considerable evidence that the reactive metabolite of APAP binds to mitochondrial proteins (Tirmenstein and Nelson, 1989; McGill et al., 2012b; Xie et al., 2014) leading to altered mitochondrial morphology (Placke et al., 1987), inhibition of mitochondrial respiration (Meyers et al., 1988), mitochondrial oxidative stress (Jaeschke, 1990; Cover et al., 2005), loss of mitochondrial membrane potential (Kon et al., 2004; McGill et al., 2011; Xie et al., 2014) and release of mitochondrial proteins into the cytosol and plasma (Bajt et al., 2006; McGill et al., 2012a; 2014). In addition, several interventions aimed at preventing or reducing mitochondrial dysfunction protect against APAP-induced liver injury, including post-treatment with the antidote N-acetylcysteine (NAC) or SOD-mimetic Mito-Tempo to scavenge ROS, inhibition of the mitochondrial membrane permeability transition (MPT) and activation of autophagy to remove damaged mitochondria (Satio et al., 2010b; Du et al., 2017; Kon et al., 2004; Ramachandran et al., 2011; Ni et al., 2012a).

Mitochondrial biogenesis (MB) is the growth and division of existing mitochondria, resulting in increased mitochondrial mass within cells. The primary purpose of MB is to maintain or restore energy homeostasis during energy deprivation or following a mitochondrial insult. Several signaling mediators control this process, but PPARγ co-

activator-1α (Pgc-1α) is the master regulator of MB. Although Pgc-1α itself does not bind to DNA, it interacts with other transcription factors in the nucleus to induce expression of genes that are important for MB. In particular, induction of nuclear respiratory factors (Nrf) 1 by Pgc-1α controls the coordinate expression of other genes involved in MB in the nucleus and the mitochondria, especially those encoding subunits of the electron transport chain (ETC) complexes (Baker et al., 2007; Scarpulla, 2008). Importantly, Pgc-1α is activated by the AMP-activated protein kinase (Ampk) and Sirtuin-1 (Sirt-1). It is also important to note that mitochondrial growth and mitochondrial fission and fusion are also carefully coordinated in order to ensure proper organization of the mitochondrial network (Chan, 2006 a,b). In some cases, MB may determine whether a cell survives or dies (Jornayvaz et al., 2010; Scarpulla, 2008), and impairment of MB contributes to several forms of tissue injury, and induction has been shown to be protective in these models (Finck and Kelly, 2007; Rasbach et al., 2007; St-Pierre et al., 2005; Funk et al., 2010; Rehman et al., 2013).

Surprisingly, although mitochondrial dysfunction is a key factor in APAP-induced liver injury and thus targeting MB may have high therapeutic potential, this has never been investigated in a model of APAP hepatotoxicity. Therefore, the major objective of the present study was to characterize the time course of MB following APAP overdose and to determine whether or not induction of MB could be beneficial during APAP hepatotoxicity.

5.3 Methods and Materials

Animals

Male C57Bl/6 mice (8-12 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME) and kept in an environmentally controlled room with a 12 h light/dark cycle and *ad libitum* access to food and water. Mice were i.p. treated with 200 or 300 mg/kg APAP (Sigma-Aldrich) dissolved in warm saline after overnight fasting, and euthanized at the indicated time points between 0 and 96 h after APAP injection for collection of blood and liver samples. SRT1720 (EMD Millipore) was dissolved in 10% DMSO plus 2% Tween 20 and was i.p. administered at 1.5h or 12h and 36h post-APAP. All vehicle control mice received the same volume of DMSO (1 mL/kg) and Tween 20 (0.2 mL/kg). Chloramphenicol (Sigma-Aldrich) dissolved in drinking water (2 mg/kg) was given starting 9h post-APAP, and three bonus doses (50 mg/kg) were i.p. administered at 9h, 24h and 36h post-APAP. Blood was drawn from the caudal vena cava using a heparinized syringe. The liver was divided into several pieces and aliquots were used for mitochondrial isolation (Du et al., 2015a), or fixed in OCT medium for immunofluorescent staining or in 10% phosphate-buffered formalin for histological analysis, and the remaining pieces were snap-frozen in liquid nitrogen and stored at −80°C for later experiments. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center and followed the criteria of the National Research Council for the care and use of laboratory animals.

Biochemical assays

Plasma ALT activity was determined using an ALT kit (Pointe Scientific, MI). Activity of mitochondrial complexes I and V were examined using blue native polyacrylamide gel electrophoresis (BN-PAGE) as previously described (Larosche et al., 2010; Schagger and von Jagow, 1991). GSH and GSSG levels were measured using a modified method of the Tietze assay as described (Jaeschke and Mitchell 1990; McGill and Jaeschke 2015).

mtDNA levels

mtDNA was measured as previously described (Cover et al., 2005). Briefly, total hepatic DNA was isolated with Genomic-tip 100/G columns (QIAGEN GmbH, Hilden, Germany) then blotted onto Hybond-N nylon membranes (GE Healthcare). Membranes were first hybridized with a 10.9-kilobase mtDNA probe (nucleotides 4964 –15,896) generated by long PCR and labeled by random priming, then stripped and hybridized with a mouse Cot-1 nDNA probe (Invitrogen, Cergy Pontoise, France). The levels were determined by densitometry analysis of autoradiographs and normalized to nuclear DNA levels.

Histology

Formalin-fixed tissue samples were embedded in paraffin and 5 µm thick sections were cut and transferred to glass slides. The slides were then stained with hematoxylin and eosin (H&E) for evaluation of tissue necrosis. Necrosis was quantified by a pathologist who was blinded to the sample identities. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed for assessment of DNA strand breaks with the In Situ Cell Death Detection Kit, AP (Roche Diagnostics, Indianapolis, IN) following manufacturer’s instructions. Sections were also stained for proliferating cell nuclear antigen (Pcna) using a rabbit polyclonal anti-Pcna antibody, according to the manufacturer's instructions (Santa Cruz Biotechnology, Dallas, TX). Pcna quantification

was performed using a custom macro for ImageJ that automatically thresholds images and counts the area of Pcna-positive cells based on staining darkness and size. Immunofluorescence staining was performed with OCT- embedded tissue. Cryosections were cut 6 µm thick and fixed with 5% paraformaldehyde for 10 mins. After washing with 1x PBS, tissues were blocked with 5% normal goat serum followed by overnight incubation with the rabbit anti-Tom20 antibody (1:250 dilution) (Santa Cruz, Dallas, TX) or anti-Pgc-1a antibody (1:250 dilution) (Pierce, Rockford, IL). The secondary antibody was Alexa Fluor 594-conjugated goat anti-rabbit antibody (Life Technologies, Eugene, OR). Nuclei were stained with DAPI containing mounting medium (Life Technologies, Eugene, OR) when placing coverslips, and images were obtained using a Zeiss Axiovert inverted fluorescence microscope (Carl Zeiss AG, Jena, Germany).

Western blotting

Western blotting was performed as previously described (Bajt et al., 2000). The primary antiobodies used in this study were Cyclin D1, Pcna and Nrf-1 from Santa Cruz Biotechnology (Dallas, TX); phosphorylated-Ampk, Drp-1 and beta-actin from Cell Signaling Technology (Danvers, MA); MitoProfile® Total OXPHOS Rodent WB Antibody Cocktail from Abcam (Cambridge, MA); COX IV subunit 2 & Pgc-1a antibodies were from Life Technologies (Carlsbad, CA) or Pierce (Rockford, IL) respectively. Horseradish peroxidase-coupled anti-mouse or anti-rabbit IgG was used as the secondary antibody. Proteins were visualized by enhanced chemiluminescence (Amersham Biosciences Inc., Piscataway, NJ).

Statistics

All data were expressed as mean ± SEM. For two groups with normally distributed data, the Student's t-test was used. For comparison of more than two groups, statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test for multiple comparisons. For non-normally distributed data, ANOVA was performed on ranks, followed by Dunn's multiple comparisons. $P < 0.05$ was considered significant.

5.4 Results

Induction of mitochondrial biogenesis during APAP hepatotoxicity.

To investigate the time course of mitochondrial biogenesis following APAP overdose, C57Bl/6 mice were treated with 200 or 300 mg/kg APAP and euthanized at multiple time points between 0 and 96 h post-APAP. APAP treatment caused dose-dependent acute liver injury that peaked at 12 h and then began to normalize, as indicated by plasma ALT and centrilobular necrosis in histology (Fig. 5.4.1A-C). Consistent with mitochondrial dysfunction, activities of the ETC complexes I and IV were also dose-dependently impaired, reduced by more than 50% at 300 mg/kg dose compared to baseline at 24 h post-APAP (Fig. 5.4.2A,B). mtDNA levels in the liver also decreased during this time (Fig. 5.4.2C,D). Interestingly, however, both ETC activity and mtDNA increased after 24 h, and were almost fully restored by 72 h (Fig. 5.4.2). These data suggest that APAP overdose caused dose-dependent MB in the mouse liver.

Mitochondrial biogenesis occurs selectively in hepatocytes surrounding necrotic areas.

To localize the area of MB in liver tissues after APAP overdose, mitochondria were stained with Tom 20, the central component of the TOM (translocase of outer membrane) receptor complex. Consistent with the time course of ETC activity and mtDNA content, Tom20 staining began to increase in a subset of hepatocytes a few cells away from the necrotic area boundary by 24h post-APAP (Fig. 5.4.3D, arrows). By 48 hours after APAP, intense Tom20 staining was evident exclusively in hepatocytes surrounding necrotic areas (Fig. 5.4.3E&F). Interestingly, we also noticed a less-stained zonal area sandwiched between the necrotic area and healthy hepatocytes at 12h (Fig. 5.4.3C), in which the hepatocytes might be in severe stress, with excessive mitophagy taking place to remove damaged mitochondrial, thus decreasing the amount of mitochondria in those hepatotcytes.

***Mitochondrial biogenesis signaling during acetaminophen hepatotoxicity*.**

To further investigate MB after APAP-induced liver injury, we performed immunoblotting for several major MB signaling mediators. Pgc-1α is the master regulator of MB and activates the transcription factors that coordinate the expression of nuclear and mitochondrial genes necessary for MB, particularly for expression of ETC subunits (Baker et al., 2007; Scarpulla, 2008). Pgc-1α is activated by Ampk. We observed phosphorylation of Ampk as early as 6 h after APAP treatment and this was sustained until at least 48 h (Fig. 5.4.4A). Though, hepatic Pgc-1α was diminished during early liver injury, protein levels were restored at 24h and appeared to increase at 48h (Fig. 5.4.4A). Nrf-1, which is important for Pgc-1α function and helps to coordinate the expression of MB genes from 6h post-APAP (Scarpulla, 2012), also showed increased expression (Fig. 5.4.4 A). Finally, although not all mitochondrial proteins that

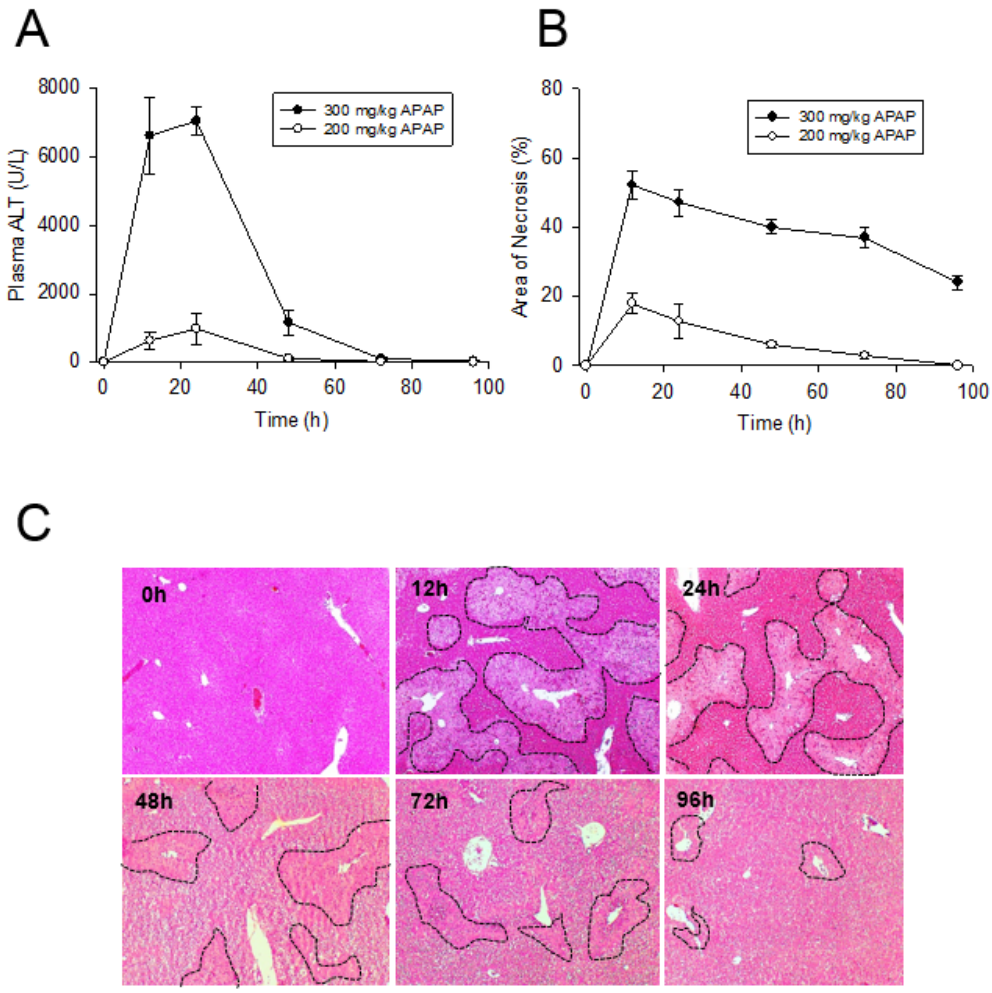


Figure 5.4.1 Time course and dose response of liver injury after acetaminophen treatment.

Mice were treated with 200 or 300 mg/kg acetaminophen (APAP) and sacrificed at various time points between 0 and 96 h. (A) Plasma alanine aminotransferase (ALT) activity. (B) Areas of necrosis (%) after 300 mg/kg APAP. (C) Representative H&E-stained liver sections after 300 mg/kg APAP with necrotic area outlined. Data are expressed as mean ± SEM for n = 4-6.

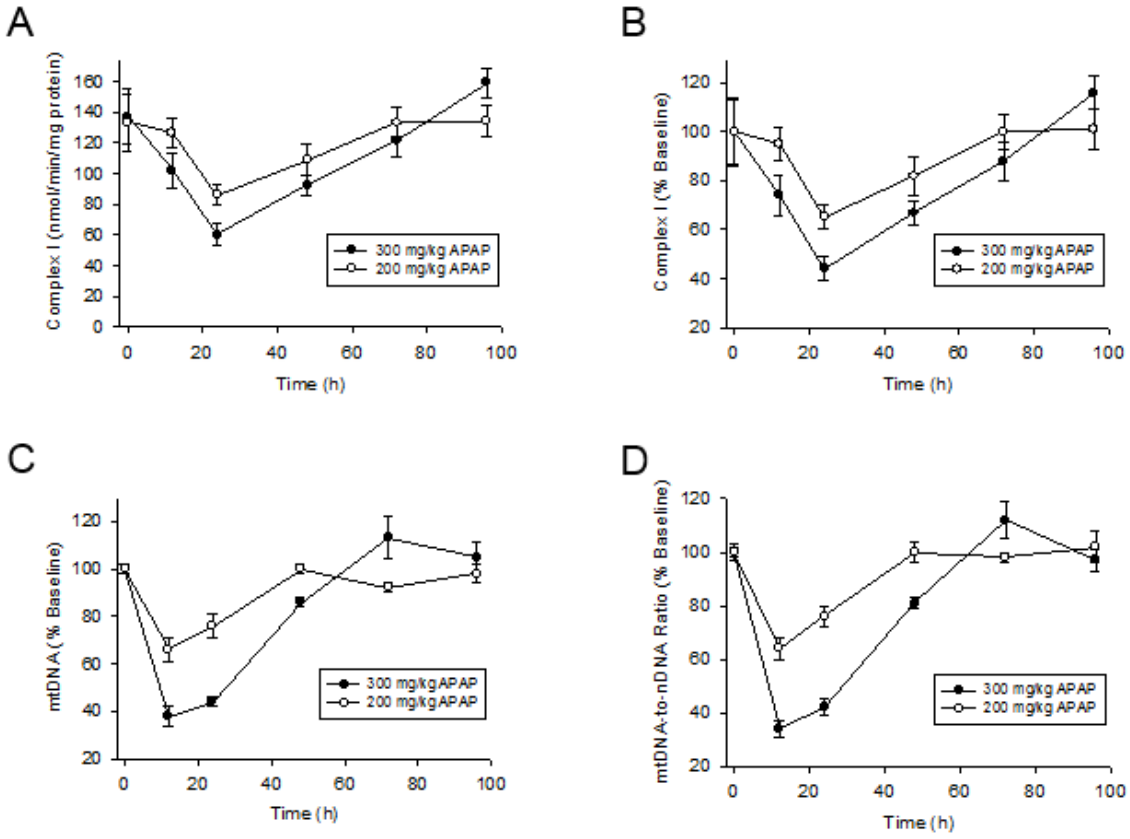


Figure 5.4.2 Electron transport chain activity and mitochondrial DNA levels in the liver after acetaminophen treatment.

Mice were treated with 200 or 300 mg/kg acetaminophen (APAP) and sacrificed at various time points between 0 and 96 h. (A) Electron transport chain (ETC) complex I activity over time. (B) ETC complex IV activity over time. (C) Mitochondrial DNA (mtDNA) levels in the liver over time expressed as absolute content. (D) mtDNA levels in the liver over time normalized to nuclear DNA. Data are expressed as mean ± SEM for n = 4-6.

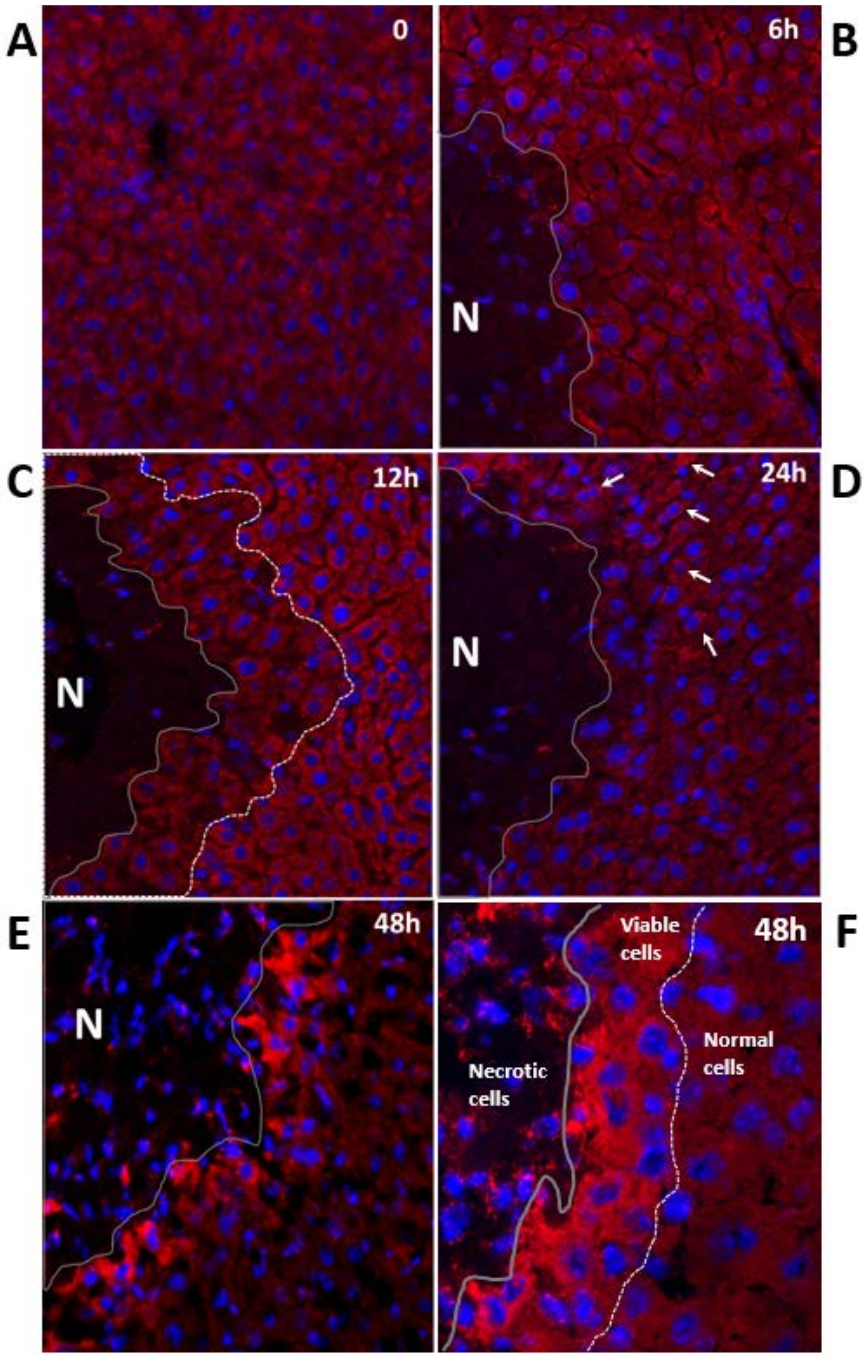


Figure 5.4.3 Immunofluorescent staining of Tom 20 (red) in the liver after APAP treatment with DAPI as a counterstain for nuclear (blue).

Mice were treated with 300 mg/kg acetaminophen (APAP) and sacrificed at various time points between 0 and 48 h. “N” indicated necrotic area and outlined with solid line and the arrows indicated the bright Tom 20 staining cells.

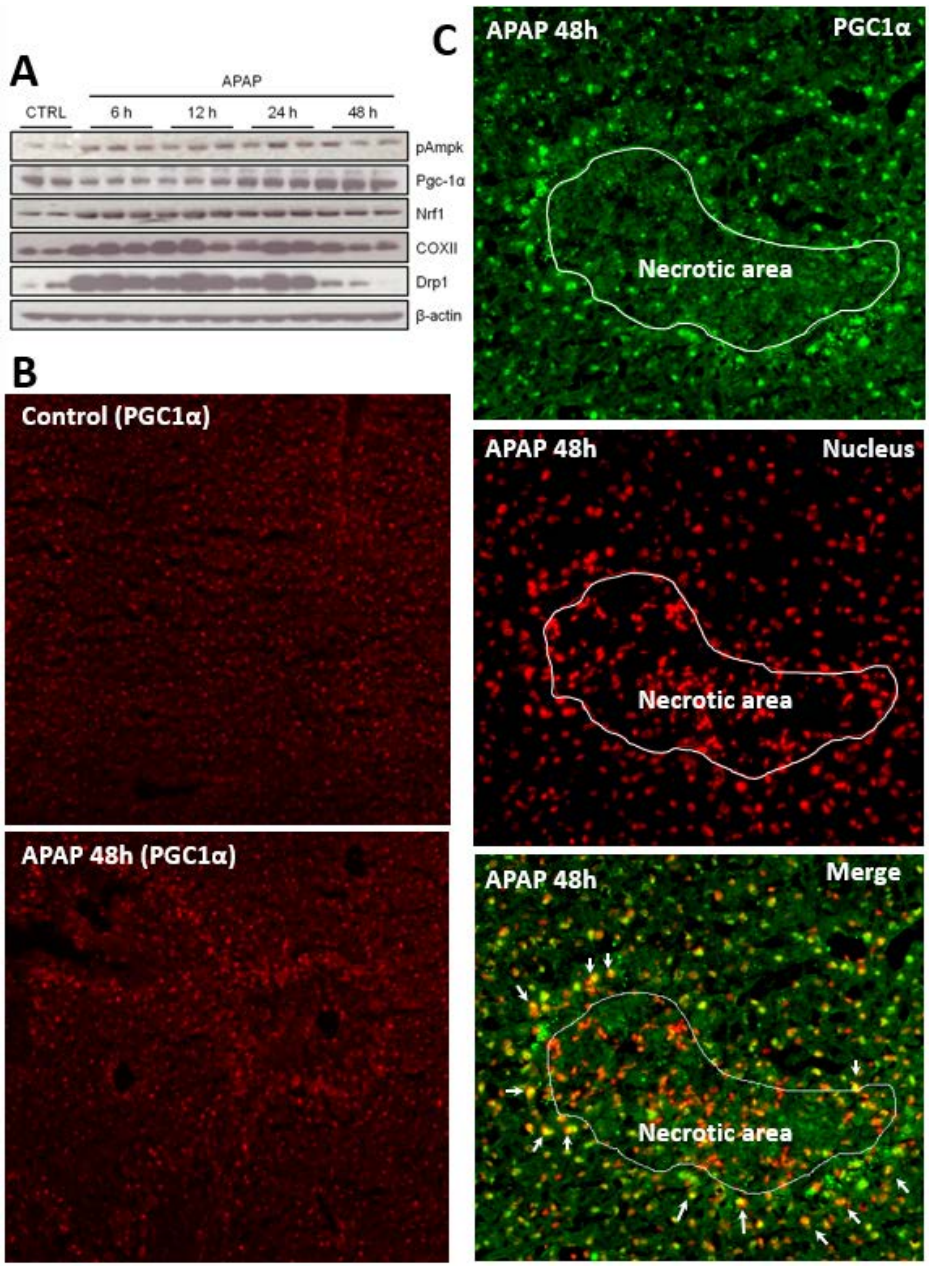


Figure 5.4.4 Activation or expression of mitochondrial biogenesis signaling mediators after acetaminophen treatment.

Mice were treated with 300 mg/kg acetaminophen (APAP) and sacrificed at various time points between 0 and 48 h. (A) Activation or expression of several major mitochondrial biogenesis (MB) markers was assessed by immunoblotting. Immunofluorescent staining of Pgc-1α (B) with DAPI as a counterstain for nuclear(C).

increased expression (Fig. 5.4.4 A). Finally, although not all mitochondrial proteins that we measured increased over time after APAP overdose, we did observe increased expression of ETC complex IV subunit 2 (Fig. 5.4.4 A), a protein encoded by mtDNA, suggesting that mitochondrial gene transcription is induced. Together with the late increase in mtDNA, these data suggest activation of the complete signaling axis leading to MB. Importantly, this was accompanied by dramatic induction of the mitochondrial fission protein Drp-1 (Fig. 5.4.4 A), suggesting not only growth of existing mitochondria but also division to form new mitochondria. To examine areas of induction of biogenesis signaling within the liver, sections were stained for the central regulator Pgc-1α. We found that Pgc-1α was evenly expressed in hepatocytes in all areas of the liver in control mice (Fig. 5.4.4 B). However, at 48h after APAP treatment, Pgc-1α expression was exclusively increased in hepatocytes surrounding necrotic areas (Fig. 5.4.4 B). In addition, Pgc-1α staining co-localized with the nucleus in these areas (Fig. 5.4.4 C). Together with the selective increase in staining of Tom 20 in Figure 3, these data suggested that Pgc-1α translocated to nuclei and activated MB in hepatocytes surrounding necrotic areas.

***Induction of mitochondrial biogenesis protects against APAP hepatotoxicity*.**

Stimulation of MB through PGC-1α signaling has been reported to rescue mitochondrial function and improve outcome in numerous pathologies, including cardiovascular diseases, nephrotoxicity, neurodegenerative diseases and also chronic liver injury (Finck et al., 2007; Degli et al., 2012; St-Pierre et al., 2005; Funk et al., 2010; Rehman et al., 2013). We hypothesized that induction of MB would protect against APAP-induced liver injury. To evaluate this, we treated mice with APAP, followed by SRT1720, an established inducer

of MB signaling (Milne et al., 2007). SRT1720 was given at 1.5h post-APAP to avoid its effect on metabolic activation of APAP. In support of the late induction of MB (after 12h post-APAP), SRT1720 did not affect the early injury at 6h but significantly decreased the late injury at 24h post-APAP, as indicated by the 38% reduction in plasma ALT, decrease in necrosis and TUNEL-positive cells (Fig. 5.4.5 A, B). This was also supported by the timely and advanced Tom 20 staining in hepatocytes surrounding necrotic areas in the SRT1720-treated mice, while it was still absent in the vehicle-treated mice (Fig. 5.4.5 C). In addition, total GSH levels were significantly increased while the GSSG/GSH ratio was decreased (Fig. 5.4.5 D, E), indicating a lower oxidant stress in SRT1720-treated mice. This may contribute to the protective effect of SRT1720.

***Induction of mitochondrial biogenesis promotes liver regeneration after APAP hepatotoxicity*.**

Promoting liver regeneration is a promising approach to the treatment of APAP-induced liver injury because of the relatively late presentation of most APAP-overdosed patients. MB has been reported to coincide with the tissue injury process, and its induction accelerates recovery and regeneration after injury in other organs (Wagatsuma et al., 2011; Yin et al., 2008; Rasbach et al., 2007; Tran et al., 2011). Since SRT1720 protected against late APAP induced injury, we hypothesized that MB may be important for liver regeneration after APAP hepatotoxicity. Consistent with our hypothesis, liver regeneration, as indicated by Pcna expression, began at 24 h post-APAP (Fig. 5.4.6 A), the same time point at which we first observed an increase in mtDNA. PCNA levels continued to increase until at least 48 h (Fig. 5.4.6 A), similar to the time course for MB. Interestingly, the regenerating cells, as indicated by the PCNA-positive cells, were also selectively around

the hepatocytes surrounding necrotic areas (Fig. 5.4.6 B). To further explore this idea, we treated mice with APAP followed by SRT1720 at 12 h and 36 h post-APAP. The time points were chosen to avoid any effect on the early liver injury which may give the appearance of improved recovery. Importantly, despite no difference in liver injury as shown by ALT between groups (APAP+veh: 185±61 U/L v.s. APAP+SRT1720: 142±43 U/L at 72h), we observed increased expression of Pgc-1α at 48h (Fig. 5.4.6 C) as well as both Pcna and cyclin D1 at both 48h and 72h (Fig. 5.4.6 D, E) in the livers from the APAP + SRT1720-treated animals compared with APAP + vehicle-treated mice, and this was further confirmed by densitometric analysis of the blots (Fig. 5.4.6 F, G). Quantification of Pcna-stained liver sections also showed a strong trend toward an increase in Pcna (134±12% of CTRL, $p = 0.078$), although it did not achieve significance. Overall, these data suggest that MB plays a role in liver regeneration and may be important for recovery after liver injury.

Inhibition of mitochondrial biogenesis by chloramphenicol prolonged liverinjury and impaired injury resolution after APAP hepatotoxicity.

Next we wanted to determine how MB inhibition affected APAP hepatotoxicity. Mice were treated with 300 mg/kg APAP, and chloramphenicol, an antibiotic that inhibits mitochondrial ribosome and protein synthesis, was given 9h later to avoid its effect on the early injury. As expected, chloramphenicol (CAP) specifically inhibited the expression of mtDNA encoded proteins such as complex 4 sub 2 but did not have a significant effect on those nuclear DNA encoded mitochondrial proteins (Fig. 5.4.7 A). In addition, CAP alone did not cause any liver injury but significantly prolonged APAP-induced liver injury, as indicated by the increased ALT at 48h (Fig. 5.4.7 B) and greater necrosis areas at both

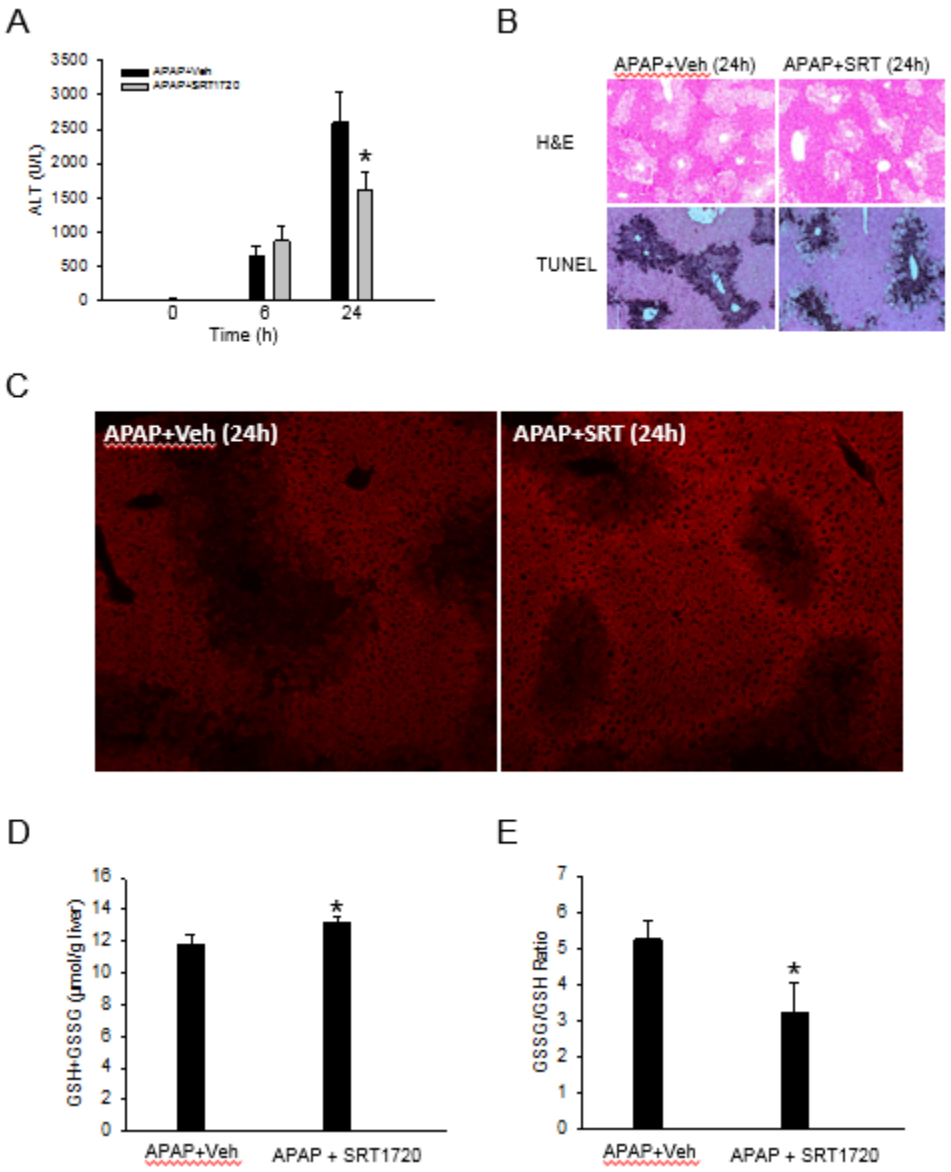
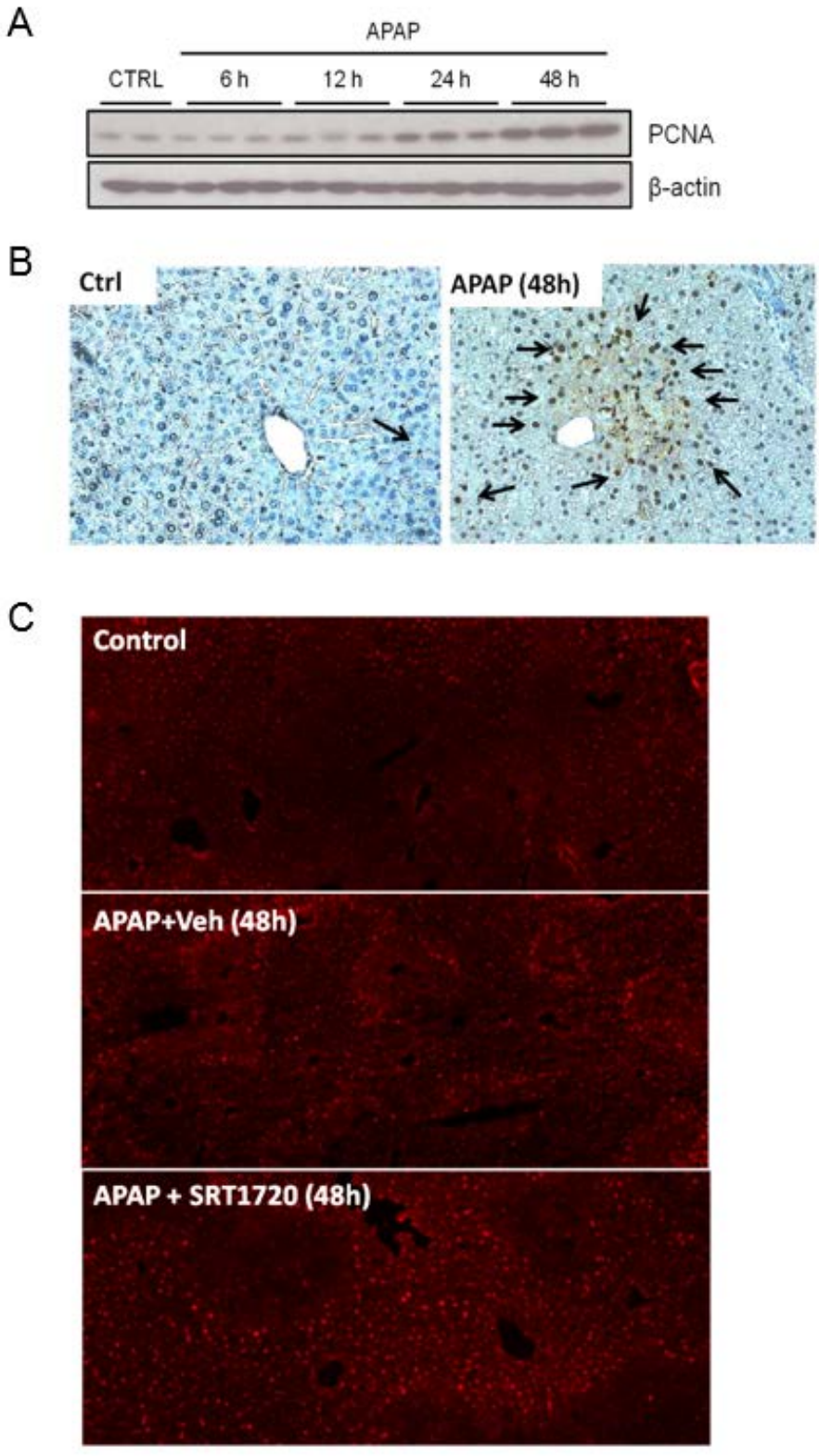


Figure 5.4.5 Induction of mitochondrial biogenesis by SRT1720 protected against acetaminophen hepatotoxicity.

Mice were treated with 300 mg/kg acetaminophen (APAP) followed by 10 mg/kg SRT1720 or its vehicle at 1.5h later, and sacrificed at 6h or 24h post-APAP. (A) Plasma alanine aminotransferase (ALT) activity. (B) H&E- or TUNEL-stained liver sections at 24h. (C) Immunofluorescent staining of Tom 20 in liver tissue at 24h. (D) Total GSH levels and (E) GSSG/GSH ratio at 24h. Data are expressed as mean ± SEM for n = 4-6. *$p < 0.05$ vs. APAP+Veh.


A
APAP
CTRL
6 h
12 h
24 h
48 h
PCNA
β-actin
B
Ctrl
APAP (48h)
C
Control
APAP+Veh (48h)
APAP + SRT1720 (48h)

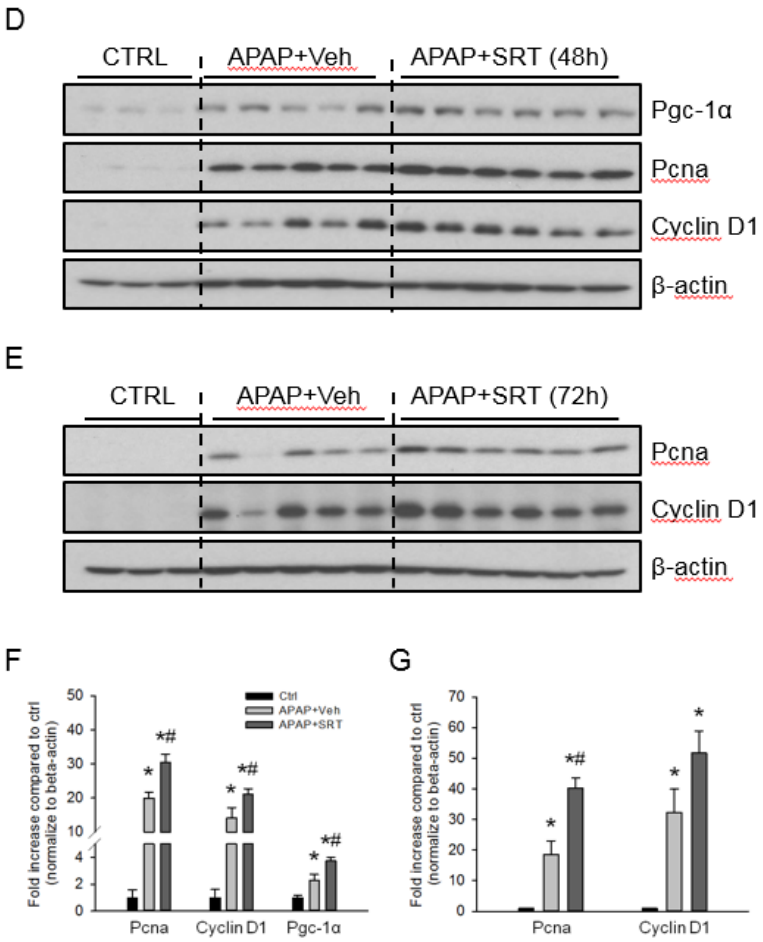


Figure 5.4.6 Induction of mitochondrial biogenesis by SRT1720 promoted liver regeneration after acetaminophen treatment.

Mice were treated with 300 mg/kg acetaminophen (APAP). Some animals were treated with either SRT1720 (SRT) or its vehicle (Veh.) 12 h and 36h post-APAP and sacrificed at 48h or 72h post-APAP. (A) Proliferating cell nuclear antigen (Pcna) expression over time after APAP treatment. (B) Representative staining of Pcna in the liver tissue at 48h post-APAP. (C) Immunofluorescent staining of Pgc-1α. (D) Pgc-1α, Pcna and Cyclin D1 expression in livers from mice treated with SRT1720 (SRT) or its vehicle (Veh.) at 48h and 72h (E). (F) Densitometry for 48 h samples. (G) Densitometry for 72 h samples. Data are expressed as mean ± SEM for n = 3-5. *p < 0.05 vs. Ctrl. #p < 0.05 vs. APAP + Veh.

48h and 72h (Fig. 5.4.7 C). Mitochondrial staining with Tom20 further confirmed these results (Fig. 5.4.7 D). Intense Tom20 staining were exclusively seen in hepatocytes surrounding necrotic areas at 48h, and it spreads to the entire damaged area by 72h (Fig. 5.4.7 D). However, the positive staining was significantly less and the necrotic area was significantly larger in the mice with chloramphenicol treatment at both time points (Fig. 5.4.7 D).

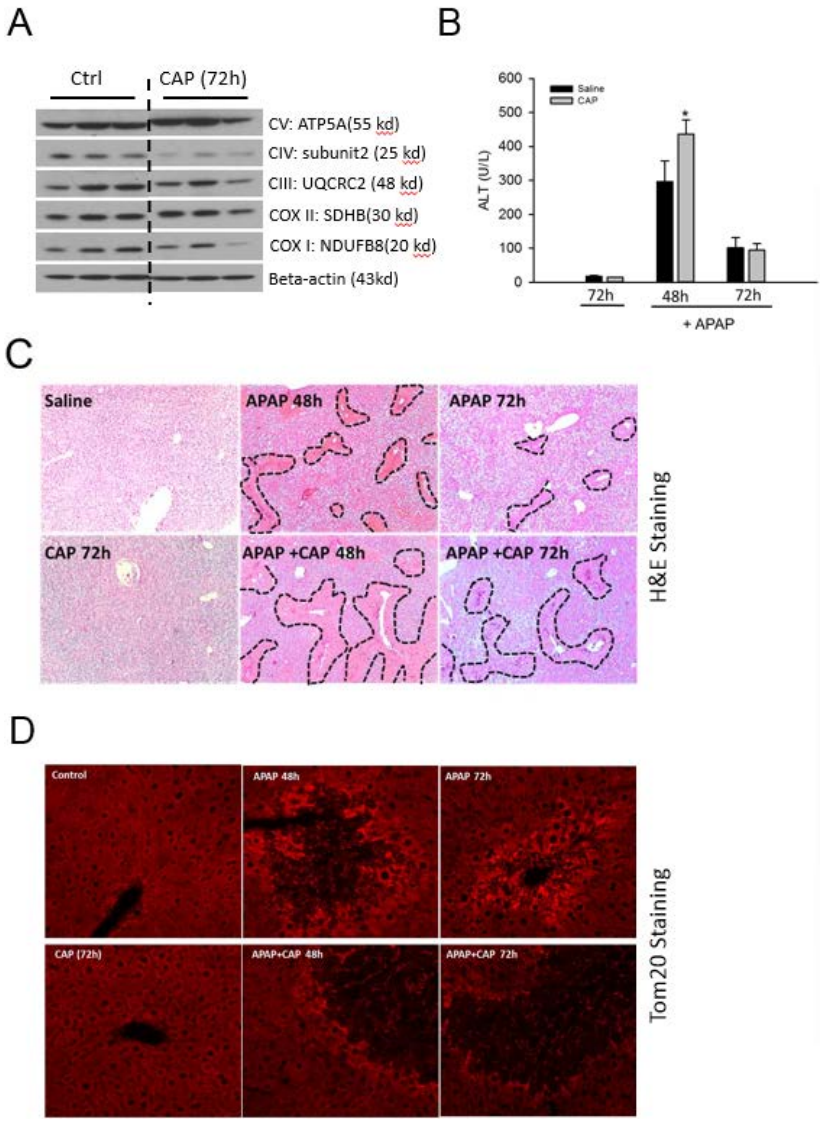


Figure 5.4.7 Inhibition of mitochondrial biogenesis by chloramphenicol prolonged the injury and impaired the injury resolution

Mice were treated with 300 mg/kg acetaminophen (APAP). Some animals were treated with 2 mg/kg chloramphenicol (CAP) in the drinking water beginning 9h post-APAP, and 50 mg/kg bonus doses were i.p. administered at 9h, 24h and 36h post-APAP. Mice were sacrificed at 48h or 72h post-APAP. (A) Protein expression of ETC subunits in control and CAP-treated mice. (B) Plasma alanine aminotransferase (ALT) activity. (C) Representative H&E-stained liver sections with necrotic area outlined. (D) Representative Tom 20-stained liver sections. Data are expressed as mean ± SEM for n = 3-6. *$p < 0.05$ vs. APAP + Saline.

5.5 Discussion

There is considerable evidence that mitochondrial damage plays a major role in the various mechanisms of APAP-induced liver injury in both mice and humans (Placke et al., 1987; Meyers et al., 1988; Jaeschke, 1990; Kon et al., 2004; Reid et al., 2005; Ramachandran et al., 2011; McGill et al., 2012a; 2014). However, little is known about the mechanisms of recovery of mitochondrial mass and function or the role of MB in liver regeneration after injury resolution. Our data suggest that MB signaling begins early after APAP overdose, but that mitochondrial mass and function are not fully restored until late time points. In addition, MB occurs selectively in hepatocytes surrounding necrotic areas. Furthermore, induction of MB with a known MB-inducer protects against APAP hepatotoxicity and promotes liver regeneration, while its inhibition prolonged the injury and impaired the injury resolution after APAP hepatotoxicity.

Pgc-1α is central mediator of MB signaling (Komen and Thorburn, 2014). Activation of Pgc-1α signaling can occur through both increased expression of the protein and post-translational modification, including phosphorylation by active Ampk and deacetylation by Sirt1 (Komen and Thorburn, 2014). However, activation of Pgc-1α is not enough to induce MB. As a co-activator, Pgc-1α must interact with other transcription factors to transactivate expression of MB genes. One such transcription factor is Nrf1 (Scarpulla, 2012). Upon activation, Pgc-1α and Nrf1 induce expression of nuclear genes that are important for MB, including mitochondrial transcription factor A (Tfam). Tfam is a key transcription factor for expression of genes encoded in mtDNA and is also involved in mtDNA replication (Scarpulla, 2012). The fact that we observed phosphorylation of Ampk and increased expression of Nrf1, Pgc-1α and an mtDNA-encoded subunit of complex IV

in our samples suggests that all of the key signaling steps required for MB are activated during APAP hepatotoxicity. The reason for the delayed Pgc-1α induction in our experiments is not clear; however, it is important to remember that induced expression is only one of the ways in which Pgc-1α activity can be increased. It is possible that post-translational modifications of Pgc-1α could occur at earlier time points prior to activation of protein expression. Additional studies are needed to test that possibility.

We have previously suggested that APAP overdose induces zonated histologial changes in the centrilobular areas of the mouse liver; ranging from inner to outer areas includes necrosis (zone 1), mitochondrial spheroid formation (zone 2), autophagy (zone 3) and mitochondrial biogenesis (zone 4) (Ni et al., 2013). By staining mitochondria with mitochondrial protein Tom 20, we noticed a less-stained zonal area sandwiched between the necrotic area and healthy hepatocytes at 12h post APAP (Fig. 3C). Although these cells were still surviving, their mitochondrial function may have been severely impaired due to the close proximity to the centrilobular areas, and excessive mitophagy could be taking place to remove damaged mitochondria, thus decreasing mitochondrial staining in those hepatotcytes. Interestingly, we also observed that the Tom20 staining started to increase from 24h post-APAP in a subset of cells, and the increased Tom20 staining was exclusively in hepatocytes surrounding necrotic areas by 48 hours (Fig. 3D, E, F). The fact that we also observed a selective increase of Pgc-1α at 48h in these areas (Fig. 4B) further demonstrates that activation of Pgc-1α signaling may promote MB in these hepatocytes. Coincidently, most of the cells surrounding necrotic areas at 48h were positive for the hepatocyte proliferation marker PCNA (Fig. 6B). This suggested that induction of MB may predispose these hepatocytes to enter the cell cycle towards cell regeneration.

SRT1720 is a derivative of the natural product resveratrol that is a potent activator of Sirt1 and Pgc-1α (Milne et al., 2007). However, it is not known if SRT1720 directly or indirectly promotes MB (Pacholec et al., 2010; Huber et al., 2010; Komen and Thorburne, 2014). In fact, the effect of resveratrol and its derivatives also seems to involve activation of Ampk (Komen and Thorburne, 2014). In any case, it is widely accepted as a useful inducer of Pgc-1α signaling and MB. Our data provide preliminary evidence that post-treatment with SRT1720 to induce Pgc-1α signaling protects against APAP-induced late liver injury and enhances liver regeneration after APAP overdose (Fig. 5, 6). It has previously been shown that knockdown of Pgc-1α in mice exacerbates APAP-induced liver injury (Ye et al., 2014). Although the authors of this study concluded that the protective effects of Pgc-1α are due to upregulation of antioxidant genes, it is possible that impairment of MB also played a role. In addition, we also demonstrated that inhibition of MB by chloramphenicol, a commonly used antibiotic that specifically inhibits mitochondrial ribosome and protein synthesis and has been shown to S (Wagatsuma et al., 2011; Kao et al., 2012), prolonged the injury and impaired the injury resolution. Together, the data suggest that MB is important for liver repair after APAP treatment.

Although MB had not previously been studied in APAP hepatotoxicity, it does occur in other forms of tissue injury. In the liver, there is some evidence that the stress caused by chronic ethanol feeding induces MB (Han et al., 2012), however it is not yet clear what role this plays in alcohol-induced liver injury. In extrahepatic tissues, kidney cells treated with pro-oxidants showed increased MB after the initial stress, and overexpressing Pgc-1α enhanced recovery of mitochondrial function (Rasbach et al., 2007). Importantly, several studies from the same group and others have shown that pharmacological induction of MB

enhances regeneration and recovery in various rodent models of acute kidney injury (Rehman et al., 2013; Whitaker et al., 2013; Funk and Schnellmann, 2013; Jesinkey et al., 2014; Garrett et al., 2014; Khader et al., 2014). Our data support the idea that the same may be true after APAP-mediated hepatotoxicity.

In summary, we demonstrated that MB occurs selectively in hepatocytes surrounding necrotic areas induced by APAP hepatotoxicity and that its induction protects against liver injury and promotes liver regeneration. We found that, after an initial reduction due to mitochondrial damage and possibly mitophagy, mitochondrial mass and function increase and return to normal levels after APAP-induced liver injury. We also observed the activation of MB signaling in hepatocytes surrounding necrotic areas. Furthermore, post-treatment with the Sirt1 activator protects against APAP-induced liver injury and induced Pcna and Cyclin D1 expression in the liver while its inhibition by CAP prolonged the injury and impaired the injury resolution, suggesting that MB is important in both injury and liver regeneration after APAP toxicity. Induction of MB may be a promising therapeutic approach for clinical APAP overdose in the future.

Chapter 6. Discussion and Future Directions

Part of this section is adapted from Du et.al (2015), "Pathophysiological significance of c-jun N-terminal kinase in acetaminophen hepatotoxicity", Expert Opinion on Drug Metabolism & Toxicology, 11(11), 1769-1779; Du et.al (2016), "Oxidative stress during acetaminophen hepatotoxicity: source, pathophysiological role and therapeutic potential", Redox Biology, 10, 148-156, with permission from the publishers

N-acetylcysteine (NAC) was introduced as a clinical antidote against APAP poisoning in the 1970's (Prescott et al., 1977). Until today, it is the only pharmacological treatment option for APAP overdose patients. The extensive mechanistic insights from preclinical models have proposed multiple protective mechanisms for NAC treatment (Fig. 6). If it is given shortly after APAP overdose, i.e. within the metabolism phase of APAP, NAC supplements the GSH pool as it is a synthetic precursor and thus protects by scavenging the reactive metabolite NAPQI (Corcoran and Wong, 1986; Corcoran et al, 1985). During the injury progression phase, the newly synthesized GSH is transported into mitochondria, where it detoxifies ROS and peroxynitrite (Cover et al., 2005; Knight et al., 2002). Interestingly, surplus NAC in the circulation can be degraded and turned into Krebs cycle intermediates, which supports mitochondrial energy metabolism and recovery of mitochondrial function (Saito et al., 2010b). These protective mechanisms are supported by the varied efficacy of NAC in the overdose patients. It is obvious that the patients who benefit most are those receiving NAC within 8 h after APAP overdose, in which the replenished GSH can detoxify NAPQI in the metabolism phase and prevent the initiation of the injury (Smilkstein et al., 1988; Whyte et al., 2010). Although the effectiveness of NAC declines at later times, the delayed administration is still beneficial in the clinic (Larson, 2007; Xie et al., 2015a), probably by detoxifying ROS and supporting mitochondrial energy metabolism. Unfortunately, in the clinic most APAP overdose patients only seek medical attention during or after the peak of injury (Larson, 2007; Xie et al., 2015a), at which time the effectiveness of NAC has already significantly decreased (Smilkstein et al., 1988; Whyte et al., 2010). Although liver transplant is a lifesaving procedure when the liver injury has progressed to irreversible liver failure,

only a very small portion of patients receive a transplant due to the severe organ shortage in clinic practice (Larson et al., 2005). In addition, the transplantation is a highly expensive procedure and carries significant risk of mortality and morbidity, and those patients receiving the transplant still suffer from the potential complications including acute or chronic donor organ rejection, life-long immunosuppression, infections, biliary complications and recurrence of disease (Yang et al., 2014). Therefore, a pharmaceutical intervention that still works during this late period would surely have therapeutic potential and greatly help those late-presenting patients.

6.1 Therapeutic potential of targeting mitochondrial oxidative stress

Acetaminophen hepatotoxicity is characterized by extensive oxidative stress. However, its source, pathophysiological role and possible therapeutic potential if targeted, have not been well clarified. Current experimental evidence, including data from us, demonstrates that neither oxidative stress induced by drug metabolism nor extracellular oxidant stress from inflammatory cells are relevant sources of ROS in APAP hepatotoxicity (Du et al., 2016a). In contrast, P450-mediated APAP metabolism generates excessive reactive metabolite NAPQI, which targets mitochondrial proteins and especially the complex subunits in ECT. This impairs ETC respiration and results in ROS formation, leading to an overwhelming mitochondrial oxidant stress and mitochondrial dysfunction (Du et al., 2016a).

The protective effect of NAC by detoxifying ROS encouraged the use of antioxidants for late treatment of APAP poisoning. Indeed, numerous interventions that can act as antioxidants are protective in preclinical models of APAP toxicity. For example, delayed treatment with exogenous GSH or the natural product resveratrol after APAP metabolism

significantly decreases oxidative stress and protected against injury (Du et al. 2015b; Knight et al. 2002). In addition, pharmacological inhibition of the JNK signaling pathway, which amplifies the mitochondrial oxidant stress in APAP hepatotoxicity, reduces oxidant stress and subsequent injury (reviewed in Du et al., 2015c; Han et al., 2013). However, despite massive evidence in preclinical studies supporting a beneficial potential of antioxidants, no drugs that specifically target ROS are available clinically for APAP overdose patients. There are several reasons for this issue. First, due to the high efficacy of the current standard-of-care antidote NAC, any promising antioxidant must be compared with NAC, and only those that can provide additive protection to NAC possess therapeutic potential. Interestingly in our recent studies, the mitochondrial targeted antioxidant Mito-Tempo, either as a late treatment alone or together with NAC offered better protection than NAC alone, which supports its use as a therapeutic option for late treatment of APAP overdose in patients (Fig. 6) (Du et al., 2017). Comparison of its efficacy to its analog Tempo highlights not only the importance of the mitochondrial oxidant stress in the development of APAP toxicity but also the therapeutic potential of other mitochondrial targeted antioxidants (Du et al., 2017). It would be interesting to test the efficacy of other antioxidants, e.g. the mitochondria-targeting peptide Elamipretide (Sabbah et al., 2016), which is currently in multiple clinical trials with FDA approval pending for primary mitochondrial myopathy. Second, due to the high costs of de novo drug development and the limited number of patients, it is unlikely that a pharmaceutical company will develop a drug specifically for APAP poisoning. Therefore, a better strategy for getting a new antidote against APAP overdose would be by re-purposing an existing drug. For example, an earlier study identified benzyl alcohol, a marketed drug

for treatment of head lice, as a promising intervention in APAP hepatotoxicity (Cai et al., 2014), though our follow-up study revealed that the protection of benzyl alcohol is mainly caused by inhibition of APAP metabolism and thus is unlikely a realistic therapeutic option for treatment of APAP overdose in patients (Du et al., 2015a). Methylene blue, a clinical used drug for diseases including methemoglobinemia and psychiatric disorders, targets to mitochondria and reduce mitochondrial oxidant stress (Lee et al., 2015). It also restores the compromised ETC function and thus protects against APAP hepatotoxicity (Lee et al., 2015). Recently, we reported that metformin, a first line drug for type 2 diabetes, attenuates the mitochondrial oxidant stress and protects against APAP hepatotoxicity even as a late treatment (Fig. 6) (Du et al., 2016b). In addition, its effectiveness is reproducible in human HepaRG cells, a clinically relevant model for APAP overdose (Du et al., 2016b), further supporting metformin as a therapeutic option for patients. Nevertheless, more studies are still needed to establish its effective therapeutic dose in humans and assess potential side effects of high doses of metformin before its application in APAP overdose patients.

Research into natural products isolated from plants has revealed a large number of potential drug candidates that may act as antioxidants. Unfortunately, the frequent use of inappropriate preclinical models in testing the efficacy of antioxidants or other interventions has been a significant prohibitive factor in recent years, and experimental data from such studies can be confusing, leading to controversial conclusions and thus hampering the progress in translational research in testing antioxidants. For example, the rat is widely regarded as a poor model since even high overdoses of APAP do not cause oxidative stress and liver injury in these animals (McGill et al., 2012b). However, large

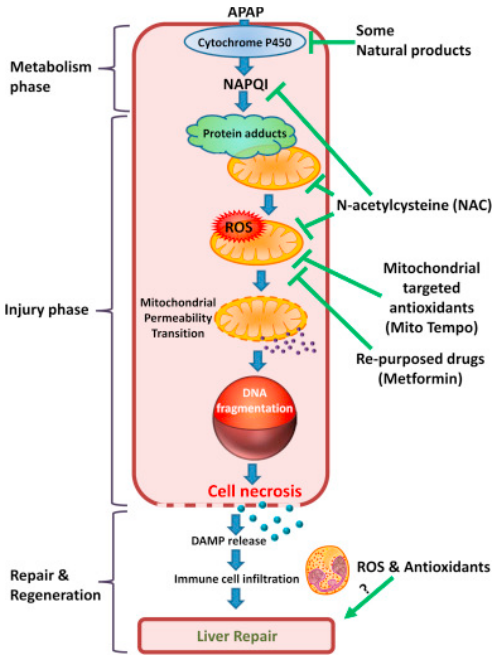


Fig. 6.1 The effect of ROS and antioxidants on APAP hepatotoxicity

Liver toxicity of APAP is initiated after a metabolism phase, where the drug is metabolized by cytochrome P450 to generate the reactive metabolite NAPQI. Excessive formation of NAPQI results in formation of protein adducts, especially on mitochondrial proteins, which initiates the injury phase. Mitochondrial protein adduct formation results in generation of reactive oxygen species (ROS) within the organelle, which ultimately leads to activation of the mitochondrial permeability transition and release of mitochondrial proteins such as apoptosis inducing factor and endonuclease G and translocation to the nucleus. This then causes nuclear DNA fragmentation and subsequently cell necrosis. Necrotic cells release damage associated molecular patterns (DAMPs), which initiate the regeneration phase, with infiltration of immune cells, and ultimately liver repair. Interventions which inhibit APAP metabolism, as seen with a number of natural products, would consequently prevent NAPQI generation and thus prevent hepatotoxicity. However, these types of therapeutics would not be clinically beneficial, since most patients present much later, i.e., at which time the injury phase has already been initiated. N-acetylcysteine (NAC), which is the current standard of care, protects by replenishing glutathione stores and scavenging NAPQI, as well as supporting mitochondrial recovery. However, since it is effective only early during the injury phase, it may not be as beneficial for patients who present late after APAP consumption. Mitochondrial targeted antioxidants such as Mito Tempo, or repurposed drugs such as Metformin, robustly prevent mitochondrial oxidative stress, which attenuates downstream signaling and cell necrosis, and could be a future therapeutic option. The effect of ROS and antioxidants on the repair phase remains unclear and deserves further investigation.

numbers of studies testing phytotherapeutics or other antioxidant interventions are still being conducted in this model (Jaeschke et al., 2014) (Fig. 6.1). Several human hepatoma cell lines (e.g. HepG2, Hep3B, Huh7) are frequently used as *in vitro* models in APAP studies. However, their human pathophysiological relevance is highly questionable because of the lack of P450 enzymes for the metabolic activation of APAP in these cells, which initiates the toxicity in rodents and humans. Even if an intervention, including an antioxidant, shows protection in these cell lines, it may not be therapeutically relevant for APAP overdose patients. In contrast, the mouse model closely resembles the human pathophysiology and so far has served as one of the most physiologically relevant models. The most successfully applied *in vitro* model is primary mouse hepatocytes. Although it has some limitations, such as the lack of non-parenchymal cells and loss of P450 enzyme activity over time in culture, for an acute model such as APAP-induced cell death, it generally reproduces most aspects of the *in vivo* pathophysiology (Jaeschke et al., 2012; 2014). The HepaRG cell model closely resembles the human pathophysiology for APAP overdose (except the requirement for JNK) (McGill et al., 2011; Xie et al., 2015a). Freshly isolated primary human hepatocytes (PHH) are the gold standard for drug toxicity studies and our recent investigation has documented their relevance for the human pathophysiology in APAP hepatotoxicity (Xie et al., 2014). However, due to their limited availability and high cost if commercially acquired, the use of primary human hepatocytes is still very limited in APAP studies. Cryopreserved PHH were introduced to overcome these disadvantages, and currently more advanced PHH culture systems like 3D culture or sandwich culture systems are being developed to more closely recapitulate the normal liver tissue architecture (Godoy

et al., 2013). We encourage the use of pathophysiologically relevant models of human cells (e.g. primary mouse hepatocytes; HepaRG cells; PHH and *in vivo* mouse model) in testing the efficacy of antioxidants or any other therapeutic interventions in the future investigations.

6.2 Therapeutic potential of targeting JNK signaling pathway

JNK signaling is critical in liver injury caused by numerous etiologies, including I/R injury, fibrosis, HCC, and NASH, and inhibition of JNK is emerging as an attractive therapeutic strategy for these pathologies (Seki et al., 2012). Intriguingly, various JNK inhibitors have been discovered and some of them, such as SP600125 and D-JNKI1, have demonstrated effectiveness in preclinical studies. One inhibitor, CC-930, has even gone into clinical trials (Wagner and Nebreda, 2009; Hui et al., 2008; Plantevin et al., 2012; Stebbins et al., 2008).

Similar to these findings, JNK has also emerged as a promising therapeutic target for APAP overdose in recent years. Early studies suggested that JNK signaling plays a detrimental role in the toxicity by serving as a second hit to amplify the cellular stress (Hanawa et al., 2008; Kaplowitz et al., 2008; Saito et al., 2010a). It is believed that the first hit caused by GSH depletion, protein binding, and oxidative stress moderately impairs mitochondrial respiration, but it is insufficient to cause cell death. However, the initial oxidative stress sensitizes mitochondria to JNK signaling and when JNK is activated, it translocates to mitochondria and provides a second hit. The second hit further amplifies the oxidant stress and leads to MPT pore formation, complete loss of the

mitochondrial membrane potential, and subsequently cell necrosis. This hypothesis has been supported by numerous studies using genetic or pharmacological intervention strategies. And more intriguingly, JNK activation also occurs in primary human hepatocytes and human liver tissues after APAP overdose (Henderson et al., 2007; Xie et al., 2014), supporting the relevance of JNK activation to human pathophysiology.

However, this concept has also been challenged by several findings, especially those from recent studies. While a few initial studies failed to reproduce the protection of JNK deficiency in APAP toxicity, concerns over off-target effects of JNK inhibitors and even in knock-out mice are increasing now. For example, the role of JNK2 in APAP hepatotoxicity has been controversial due to the earlier compelling findings in JNK2-deficient mice (Gunawan et al., 2006; Henderson et al., 2007 and Saito et al., 2010; Bourdi et al., 2008), although follow-up studies demonstrated that this controversy may be due to the mispairing of C57BL/6 sub-strains between WT mice and deficient mice (Bourdi et al., 2011; Duan et al., 2016). More recently, Cubero et al. showed that JNK inhibitor SP600125 even protected against APAP hepatotoxicity in mice with the hepatocyte-specific knockout of JNK1 and JNK2 genes, raising the possibility that the protection by SP600125 comes from its off-target effects (Cubero et al., 2016). Therefore, the development of more specific, water soluble JNK inhibitors would certainly be helpful in clarifying these problems. In addition, many details of the JNK signaling pathway are still unknown. It was suggested that an initial oxidant stress after APAP overdose causes JNK activation in cytoplasm and consequent mitochondrial translocation. However, most of the oxidant stress in APAP hepatotoxicity develops in mitochondria, and the oxidant stress in cytoplasm is minimal (Jaeschke, 1990; Du et al.,

2016a). Therefore, the source of the initial oxidant stress and how it activates JNK in the cytosol is not yet known. The connection between mitochondrial P-JNK and the MPT is also not fully understood. The effect of JNK activation and translocation to the mitochondria appears to dependent on the presence of mitochondrial Sab, which is the binding target of JNK but not RIP1 (Dara et al., 2015; Win et al., 2011). Because sustained JNK activation is necessary for the development of toxicity (Hanawa et al., 2008; Mobasher et al., 2014; Hu et al., 2016), while transient JNK activation is normally beneficial for cells (Czaja, 2007; Lamb et al., 2003), we also need to explore how the balance between transient activation and sustained activation is regulated in APAP toxicity.

Another critical issue for JNK would be its pathophysiological relevance in APAP overdose patients. Since liver biopsies are rarely taken from APAP overdose patients in the clinic, some basic questions such as the time course of JNK activation and mitochondrial translocation remain unknown in humans. Primary human hepatocytes are emerging as a clinically relevant model for study of APAP hepatotoxicity that can help us to answer some of these questions (Xie et al., 2014). Although JNK activation was maintained up to 24 h post-APAP in human hepatocytes, inhibition of JNK 3 h post-APAP was only partially effective, while the clinical antidote NAC displayed complete protection against the toxicity when administered as late as 6 h (Xie et al., 2014). This direct comparison of their effectiveness indeed does not support the use of SP600125 in clinical trials. However, the low efficacy might be due to the fact that the dose of the JNK inhibitor SP600125 used in this study was limited by its solubility (Xie et al., 2014). Therefore, other direct or indirect interventions in JNK signaling may still be worth

trying in this model. JNK is a potential therapeutic target for APAP poisoning, while future studies are warranted for more in-depth testing of specific inhibitors in well-defined preclinical models and human hepatocytes.

6.3 Mitochondrial dynamics in APAP hepatotoxicity.

Mitochondria are dynamic organelles that undergo constant morphological changes in response to environmental stimuli and pathophysiological conditions. Besides serving as the “power house” of the cell, mitochondria also fulfil various vital cellular function, including cell metabolism, cell death, cell differentiation and proliferation. Multiple mechanisms evolved maintain to the homeostasis of mitochondria to ensure normal cellular function. For example, mitochondria undergo constant fission and fusion process to allow exchange of matrix contents or separation of unhealthy segments under cellular stress (Twig et al., 2008). Damaged mitochondria can fuse with lysosomes and undergo a degradation process termed mitophagy as a cell survival mechanism (Ding and Yin, 2012). Interestingly, due to their endosymbiotic origin, mitochondria have their own genome, which allows them to proliferate to meet the bio enegetic needs and be distributed during their division (Clapier et al., 2008).

Mitochondrial fusion in mammals is mediated by the fusion proteins mitofusin 1 (Mfn1) and Mfn2 for the outer mitochondrial membrane and optic atrophy 1 (OPA1) for the inner membrane (Cipolat et al., 2006). Mitochondrial fission is mainly mediated by dynamin related protein 1 (Drp1). Drp1 is recruited to the mitochondrial outer membrane through its interaction with the mitochondrial receptor proteins (e.g. fission 1 (Fis1),

mitochondria fission factor (Mff), MID49 and MID51) that constricts mitochondria resulting in mitochondrial division (Losón et al., 2013). Mitophagy is typically mediated through the Pink1–Parkin pathway, but recently a Parkin-independent pathway has also been proposed (Ni et al., 2013; Williams et al 2015). Mitochondrial biogenesis in mammals is mediated by AMPK/Sirt – PGC1a – Nrf2 – TFAM pathway, as mentioned in the introduction chapter.

Recent studies have just begun to explore mitochondrial dynamics (fission, fusion, mitophagy and biogenesis) during APAP hepatotoxicity (Ni et al., 2012a; b; 2013; Ramachandran et al., 2013). Interestingly, electron and confocal microscopic analysis of mouse liver tissues revealed that APAP overdose causes distinct zonated morphological changes in the centrilobular areas, where the areas with increasing distance from the perivenous region are necrotic area (Zone 1), spheroid area (Zone 2), mitophagy area (Zone 3) and mitochondrial biogenesis area (Zone 4) (Ni et al., 2013; 2015). This is most likely due to the distribution gradient of P450 enzymes and GSH, which are responsible for metabolic activation of APAP and scavenging of NAPQI and ROS, respectively. Therefore, it is not unexpected that the centrilobular hepatocytes in Zone 1 that have high P450 enzymes but low GSH form high levels of protein adducts that induce overwhelming ROS and peroxynitrite formation, leading to severe mitochondrial damage and finally necrotic cell death in this area. In Zone 2 and Zone 3 areas where NAPQI formation is decreasing but GSH level is increasing, mitochondrial damage may be more moderate and thus mitochondrial spheroid and mitophagy take places to remove damaged mitochondria, which ensures the cell survival and restricts further growth of necrotic areas. With increasing distance from the perivenous areas, the hepatocytes in zone 4 have

only limited mitochondrial dysfunction, and therefore may be able to undergo mitochondrial biogenesis, which can generate more ATP for cell metabolism and get prepared for ensuing cell regeneration and repair (Ni et al., 2013; 2015; Du et al., unpublished data).

Studies from our lab and others have also offered other experimental insights into the pathophysiological roles of mitochondrial dynamics in APAP hepatotoxicity. For example, APAP induces autophagy/mitophagy in mouse liver to remove protein adducts and damaged mitochondria (Ni et al., 2012a; 2016). Intriguingly, pharmacological inhibition of autophagy exacerbated APAP-induced hepatotoxicity. In contrast, its induction attenuated the toxicity, supporting a beneficial role of mitophagy after APAP overdose (Ni et al., 2012a; 2016). In addition, we also demonstrated that APAP induces translocation of Drp-1 to mitochondria in mouse livers and appears to cause mitochondrial fission in primary mouse hepatocytes (Ramachandran et al., 2013; Du et al., unpublished data). Drp-1 activation is mediated by receptor-interacting protein (Rip) kinase signaling, as indicated by the fact that knockdown of Rip3 expression in the liver prevents Drp-1 translocation and reduces the liver injury (Ramachandran et al., 2013). Other studies have also shown protection against APAP by inhibition or deficiency of Rip1 (Zhang et al., 2014; Takemoto et al., 2014; Dara et al., 2015). Inhibition of Drp-1 is protective against APAP *in vitro* and *in vivo* (Ramachandran et al., 2013; Du et al., unpublished data). Although these data suggest that mitochondrial fission is detrimental during APAP hepatotoxicity, it is known that mitochondrial fission is necessary for MB. Thus, while excessive fission is harmful at early time points during APAP hepatotoxicity, it is likely beneficial at late time points. The latter idea is supported by our data that MB

occurs selectively in hepatocytes surrounding necrotic areas induced by APAP and its inhibition prolonged the injury and delayed the injury resolution (Ni et al., 2013; Du et al., unpublished data). In addition, further induction of MB with a pharmacological agent SRT1720 protects against APAP-induced liver injury and enhances liver regeneration. By contrast, inhibiting MB using chloramphenicol, prolongs the injury and impairs the injury resolution. Together, these data suggest that induction of MB may be a promising therapeutic approach for clinical APAP overdose in the future (Du et al., unpublished data). In contrast to the dramatic induction of Drp-1 in APAP-treated mouse livers (Du et al., unpublished data), no obvious changes in Mfn1 and Mfn2 levels were found (Ni et al., 2015) and thus the role of fusion is still largely unknown. Future studies are needed for further clarification of the pathophysiological role of mitochondrial dynamics in APAP hepatotoxicity. A better understanding of the underlying signal pathways may allow us to identify potential therapeutic targets for treatment of APAP overdose or acute liver failure in patients.

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