ACETAMINOPHEN HEPATOTOXICITY IN PRIMARY HUMAN HEPATOCYTES AND MICE

BY

YUCHAO XIE

Submitted to the graduate degree program in Toxicology and the Graduate Faculty of the University of Kansas Medical Center in partial fulfillment of the requirements for the degree of Doctor of Philosophy

_____

Committee Chair, Hartmut Jaeschke, PhD

_____

Wen-Xing Ding, PhD

_____

Partha Kasturi, PhD

_____

Kenneth E McCarson, PhD

_____

John Wood, PhD

Date defended: May 9, 2016

The Dissertation Committee for Yuchao Xie

certifies that this is the approved version of the following dissertation:

ACETAMINOPHEN HEPATOTOXICITY IN PRIMARY HUMAN HEPATOCYTES AND MICE

_____

Committee Chair, Hartmut Jaeschke, PhD

Date approved: May 9, 2016

Abstract

Acetaminophen is the most prevalent cause of acute liver failure (ALF) and drug-induced liver injury (DILI) in western countries. Extensive studies have revealed important intracellular events during the pathogenesis after APAP *in vivo* and *in vitro*. However, no detailed mechanistic research has been conducted in freshly isolated primary human hepatocytes (PHH), which are the gold standard to test drug-induced toxicity. To that end, the detailed injury time course, dose-response curves and signaling events were characterized. The overall time course and sequence of events mirror the clinical situation in APAP overdose patients, but occur significantly more slowly than in APAP-treated rodents, emphasizing cautious data extrapolation across experimental models. In addition, c-Jun N-terminal kinase (JNK) inhibitor moderately attenuated cell death after APAP, suggesting a detrimental role of JNK in injury progression.

Although APAP-induced hepatotoxicity is reproducible in the murine model, it is not the case for AMAP, a regioisomer of APAP. AMAP was considered for long to be non-hepatotoxic in mice, primary mouse hepatocytes (PMH), hamsters and hepatoma cell lines. The lack of toxicity was largely due to the significantly less mitochondrial protein adduct formation after AMAP compared with APAP. In PHH, significant cell death was observed after AMAP, accompanied by a loss of mitochondrial membrane potential and the absence of JNK activation or P-JNK translocation to mitochondria. Further investigation indicated that AMAP toxicity was readily explained by mitochondria protein adducts formation in primary human but not mouse hepatocytes, highlighting the critical role of mitochondrial protein arylation in determining APAP or AMAP hepatotoxicity. Additional studies were performed to investigate the toxicity of ATP *in*

vitro. ATP released from necrotic hepatocytes is considered a damage-associated molecular pattern (DAMP) molecule which could elicit innate immune responses, and therefore contributes to cell death. A recently published paper also suggested a direct toxicity of ATP. However, experiments in four different hepatocyte types including PHHs demonstrated an absence of toxicity directly by ATP. The fourth study focuses on characterization of APAP metabolites and adducts formation in APAP overdose patients, suggesting the importance of profiling both metabolites and protein adduct formation in the clinical diagnosis of APAP overdose.

Given the importance of c-Jun N-terminal kinase (JNK) in APAP-induced liver injury, two pharmacological inhibitors of apoptosis signal-regulating kinase 1 (ASK1), an upstream kinase of JNK, were tested in mice. The ASK1 inhibitor attenuated liver injury both as a pre-treatment and as a 1.5h post-treatment by blocking JNK activation and P-JNK translocation to mitochondria. Importantly, inhibiting ASK1 activity did not affect liver regeneration.

Acknowledgment

First, I would like to thank my mentor Dr. Hartmut Jaeschke for the opportunity to work in the lab and his mentoring over the years. I have learned so much from his scientific integrity and dedication, critical thinking, continuous research inspirations and humor. He teaches me how to be a better scientist in word and deed. It has been a wonderful time studying and working under his guidance.

I would like to acknowledge my committee members, Dr. Wen-Xing Ding, Dr. Ken E McCarson, Dr. Partha Kasturi and Dr. John Wood for all the valuable help, suggestions and encouragement. I would also like to thank all the faculty members especially Dr. McCarson, Dr. Weir and Dr. Apte for their unselfish help for my career development. Besides, I would like to thank the cell isolation core and analytical core for their continuous support for my research, and also the administrative staff for the wonderful coordination.

I express my appreciation to all my previous and current Jaeschke lab members, Huimin, Min, Anup, Mary Lynn, Dave, Mitch, Margitta, Ben, Kuo, James and Luqi. It is always so much fun to be in the lab with you all and share about science, life and others. I feel lucky to be surrounded by a group of nice, fun and smart people. I treasure all these moments and laughter we had together.

I extend my thanks to my peer students in the department. Going through the departmental courses and comprehensive exam are not the most enjoyable experiences, and thank you for all the mutual support, encouragement and company. Also I want to

thank previous colleagues and alumni who are now all over the country for their valuable advice and help.

I would like to thank my fiancé Rupert. Maintaining a long-distance relationship for 5 years is not easy. Thank you for supporting my decision to choose KU 5 years ago and keeping flying back and forth across the country over the years. I still remember all the overnight stays at various airports when you were trying to catch the flights early in the morning. All the difficulties we have overcome together nourish the relationship and make us stronger in every single way. Last but not least, I would like to thank my parents for their unconditional love. They are happy and proud of every of my steps forward and are always calming and encouraging when things do not work out. The love runs deep through all my ups and downs.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1. Introduction

1.1 Background of APAP

Acetaminophen (APAP, N-Acetyl-Para-Aminophenol) is a worldwide popular analgesic and antipyretic. It is the top-selling generic drug, with around 120 million prescriptions, 20 billion doses and 6 billion in sales each year (Lee, 2004; IMS, 2008; IMS, 2014). However, APAP is also the leading cause of either acute liver failure (ALF) or drug-induced liver injury (DILI) in industrialized nations, such as the US and UK (Lee, 2012).

APAP is different from most drugs on the market in many ways. First, there are no widely accepted scientific explanations for its pharmacological effects, while most of the drugs nowadays have very specific targets and mechanisms of actions to support their approval. Proposed mechanisms for APAP include increasing pain thresholds, modulating the endogenous cannabinoid system, and inhibiting cyclooxygenases. However, none of them have received widespread recognition (Ferreira et al., 1997; Botting 2000; Bertolini et al., 2006; Hinz et al., 2008). Second, current drugs are strictly regulated in terms of toxic responses. For the liver, drugs with 1/10000 incidence of liver dysfunction could face the risks of disapproval, market withdrawal or black box warning according to FDA guidance. In this regard, APAP would probably not be approved as a drug.

The third difference between APAP and current drugs on the market is the discovery process. In contrast to current methodical drug development as practiced today, the discovery and application of APAP started with serendipity. As the last survivor of the aniline-derived analgesics, the popularity of APAP was preceded by the ups and downs of other aniline derivatives. Acetanilide was the first one discovered in this family,

simply due to a mistake by the local pharmacy as they accidentally replaced naphthalene with acetanilide, which lead to alleviated fever symptoms in patients. Commercialization of acetanilide followed immediately, accompanied by the discovery of side effects including methemoglobinemia which resulted in bluish skin (Prescott, 1996). The adverse effects of acetanilide sparked a search for safer alternatives, leading to the discovery of phenacetin. Phenacetin was widely used in the 1950s to reduce work-related headache and exhaustion, especially for factory workers. In the same group of people, nephropathy was observed and its relation to phenacetin intake was later pointed out (Dubach et al., 1991). These undesirable effects of phenacetin and reports of Reye's syndrome after aspirin paved the way for the popularity of APAP. However, APAP toxicity was later realized.

Currently, APAP is the major cause of acute liver failure in western countries. Featuring coagulopathy and encephalopathy, acute liver failure is the result of hepatocyte dysfunctions. Without liver transplantation, the mortality of ALF exceeds 90% due to multi-organ failure, infection, cerebral edema and hemorrhage (Bernal et al., 2010). In the 1990s, although APAP was the most prevalent cause for ALF, the incidence was around 20~28% (Schiodt et al., 1999; Shakil et al., 2000). The number increased considerably to 39% between1998-2001, and even more to 51% in 2004 and 46% in 2012 (Lee, 2004; Bernal et al., 2010; Lee, 2012). Several interesting facts about APAP deserve attention. First, mortality rates are higher in accidental APAP overdose patients than the suicidal patients using APAP, as the former are soon discovered and treated with NAC while the latter usually ingest APAP over days without knowing the harm (Schiodt et al., 1997). Second, concomitant use of alcohol or drugs could on one hand clouds the

judgement and delays the hospitalization of patients, while on the other hand causes drug-drug interactions which enhance inflammation and exacerbate liver injury (Thummel et al., 2000).

1.2 Intracellular mechanisms of liver injury after APAP

1.2.1 APAP metabolism

Acetaminophen is safe at therapeutic doses, whereas an overdose could trigger hepatotoxicity, liver dysfunction and even liver failure. After being ingested and absorbed, APAP reaches the liver through the portal vein. At therapeutic doses, around 50% of APAP is conjugated with glucuronide and 30% is conjugated with sulfate. These reactions are catalyzed by UDP-glucuronosyltransferase (UGT) and sulfotransferase (SULT), especially UGT1A and SULT1 (de Morais et al., 1992; Court et al., 2001; Lindsay et al., 2008; Navarro et al., 2011). The resultant conjugates are removed from the hepatocytes into either bile or plasma through canalicular and basolateral transporters including multidrug resistant protein-2 (mrp2) and 3 (mrp3) as well as breast cancer resistant protein 1 (Bcrp 1). Studies using the plasma samples of APAP overdose patients indicated that, as the sulfation pathway was easily saturated, the distribution of APAP conjugation could be skewed to glucuronidation as much as 90% (Clements et al., 1984; Xie et al., 2015a). With the purpose of increasing water solubility of APAP metabolites and preparing them for excretion, these phase II conjugating reactions contribute significantly to the detoxification of APAP. Indeed, Gunn rats with impaired glucuronidation were more susceptible to APAP compared with other rat strains (de Morais and Wells 1989). In humans, polymorphisms of UGT1A allowed higher glucuronidation activities, and resultantly lower incidence of liver failure after toxic doses of APAP (Court et al., 2013). Therefore, deficiency in conjugating pathways presents a risk of increased bioactivation and aggravated hepatotoxicity in individuals.

In addition to the two phase II conjugation pathways, phase I metabolisms mediated by Cytochrome P450 enzymes account for the conversion of the remaining 5-10% APAP to the toxic metabolite N-acetyl- p- benzoquinone imine (NAPQI) (Dahlin et al., 1984). Note that, phase I and phase II metabolisms are not sequential but happen simultaneously, as subtoxic doses of APAP also elicit APAP protein adduct formation where phase II metabolisms are clearly not overwhelmed. Therefore, the saturation of phase II pathways is not a prerequisite for NAPQI formation (McGill et al., 2013).

Among all CYP450s, CYP2E1 is the most important one for APAP bioactivation while CYP3A4, CYP1A2 and CYP2D6 are also involved. Evidence supporting the critical role of CYP2E1 comes from the observation that APAP-induced liver injury was exacerbated by isoniazid and alcohol which are inducers of CYP2E1 (Sato and Lieber, 1981; Zand et al., 1993; Thummel et al., 2000). Besides, genetic deletion of cyp2e1 desensitized mice to APAP hepatotoxicity, while transgenic expression of human cyp2e1 in cyp2e1-null mice restored the metabolic activities and hepatotoxicity after APAP (Lee et al., 1996; Cheung et al., 2005). A spectrum of chemicals, natural products and drugs can block CYP450 activities and effectively prevent APAP toxicity. However, in studies testing the therapeutic interventions of APAP hepatotoxicity, suppression of APAP metabolism can lead to misinterpretations of the protective mechanisms by theses interventions. As inhibiting the upstream metabolic activation automatically shuts down the downstream events after APAP, further tests on the roles of downstream events are precluded and any related conclusions are unsupported (Jaeschke et al., 2011; Jaeschke et al., 2013). Cases in point are, an important and common solvent dimethyl sulfoxide (DMSO), ethanol, purinergic receptor P2X7 antagonist A438079, gap junction inhibitor 2-APB, benzyl

alcohol and a series of natural products (Jaeschke et al., 2006; Jaeschke et al., 2011; Du et al., 2013; Jaeschke et al., 2013; Xie et al., 2013). In those scenarios, inhibition of CYP450 enzyme activities invalidated any claims on alternative protective mechanisms like inhibiting sterile inflammation or enhancing antioxidant defense. To circumvent this issue, evaluations of metabolic activities and protein adduct formation at the initial phase after APAP are recommended, and a >1.5h posttreatment regime to avoid any interference on APAP metabolism is also helpful to confirm the protective mechanisms.

1.2.2 Protein adducts

As the principle toxic metabolite of APAP, NAPQI is an electrophile scavenged by conjugation with glutathione (GSH). This reaction could happen spontaneously or is catalyzed by glutathione-S-transferase (GST). Once GSH is depleted, the excessive NAPQI reacts with alternative targets, especially the sulfhydryl groups of cysteine residues on cellular proteins to form protein adducts (Streeter et al., 1984; Hoffmann et al., 1985). That being said, GSH depletion is not a prerequisite of protein adducts formation, as APAP-adducts are detectable after nontoxic dose of APAP when GSH is not exhausted (McGill et al., 2013).

Arylation of cellular proteins represents a hallmark of APAP toxicity. However, follow-up studies revealed that total protein adducts formation was necessary but not sufficient for liver injury. First, it is mitochondrial protein binding rather than total protein biding which correlates with injury. In mice, comparison of APAP with N-acetyl-meta-aminophenol (AMAP), a nontoxic regioisomer of APAP, indicated that the disparity of their toxicities was due to the significantly different mitochondria protein adduct levels

even though the total protein binding were similar (Tirmenstein and Nelson 1989; Myers et al., 1995). This suggests that total protein binding does not necessarily lead to toxicity. Consistently, mitochondrial protein binding was significantly lower in rats which are resistant to APAP compared with mice (McGill et al., 2012b). Besides, in primary human hepatocyte where AMAP is toxic, mitochondria adduct level was significantly higher than in primary mouse hepatocytes which were resistant to AMAP (Xie et al., 2015b). Given these studies, it is generally believed that mitochondrial protein adduct levels rather than total protein adduct levels mirror APAP toxicity (Cohen et al., 1997; Qiu et al., 2001; McGill et al., 2012b; Xie et al., 2015b). Second, liver protein adducts were detectable at subtoxic doses like 75mg/kg, and therefore liver injury is not mandatory in the presence of total protein binding (Heard et al., 2011; McGill et al., 2013). Third, subsequent amplification processes involving apoptosis-signaling kinase (ASK1), c-jun N-terminal kinase (JNK), mixed-lineage kinase 3(MLK3), glycogen synthase kinase 3-β, receptor-interacting protein 1&3 (RIP1&3) are necessary for the execution of ultimate hepatocyte necrosis and liver injury, and these players will be further discussed below. Therefore, total protein binding is only an initiating event and does not necessarily lead to liver injury, while further amplification processes are necessary for ultimate cell death.

As APAP-adducts could be detected in the serum of patients and also possess a longer half-life compared with APAP (Pumford et al., 1989; Muldrew et al., 2002), serum adduct measurement emerged as a diagnostic tool for APAP overdose in patients (Pumford et al., 1989; Muldrew et al., 2002; Davern et al., 2006; James et al., 2008). Although it holds promise for diagnosis, a translational study in serum of APAP overdose patients suggests that early-presenting patients may have deceivingly low adduct levels,

which could affect clinical diagnosis and timely treatment (Muldrew et al., 2002). To that end, a threshold of >1.1 nmol/ml APAP-cys together with ALT> 1000U/L was suggested as relevant for toxicity. In addition, evaluation of APAP metabolites could also assist in the diagnosis of APAP overdose (James et al., 2009; Heard et al., 2011; Xie et al., 2015a).

1.2.3 Mitochondria

The importance of mitochondria in APAP-induced hepatotoxicity was first revealed by a series of comparison studies between APAP and the regioisomer of AMAP in mice mainly conducted by Dr. Sidney Nelson's group. The lack of hepatotoxicity after AMAP at doses which are toxic with APAP was confirmed by several studies in various species (Tirmenstein and Nelson, 1989; Roberts et al., 1990; Holme et al., 1991; Pierce et al., 2002). It was also indicated that there were less cytosolic and mitochondrial GSH depletion, reduced protein nitration and an absence of JNK activation after AMAP compared with APAP (Tirmenstein and Nelson, 1989; Myers et al., 1995; Abdelmegeed et al., 2013). Most importantly, AMAP and APAP resulted in comparable total cellular protein adducts formation while there were significantly more mitochondrial protein adducts formation after APAP (Tirmenstein and Nelson, 1989; Myers et al., 1995; Qiu et al., 2001). Another important piece of evidence supporting the critical role of mitochondria is about the source of oxidant stress. Study from our lab reported an increase of both hepatic and mitochondrial GSSG but no changes in biliary GSSG levels (Jaeschke, 1990). As cytosolic GSSG is partially excreted into bile at physiology states, the absence of biliary GSSG excretion suggests that the elevated GSSG in the hepatocytes are restricted to organelles that do not release GSSG into the cytosol, e.g. mitochondria (Jaeschke, 1990). In fact, the increased GSSG content in mitochondria

accounted for the increase in the total GSSG content in the liver (Jaeschke, 1990). Of note, this also suggests that the oxidant stress does not come from the process of APAP metabolism. Although CYP450s catalyzes electron transfers during the reaction and is known to induce oxidative stress. However, under *in vivo* conditions, there is no direct evidence for oxidant stress during APAP metabolism (Smith and Jaeschke, 1989; Bajt et al., 2004). Further support for mitochondrial oxidant stress came from the observation that peroxynitrite was selectively generated in the mitochondria and acted as a critical mediator of APAP toxicity (Knight et al., 2002; Cover et al., 2005). Besides, loss of SOD2 in mitochondria in mice enhanced APAP-induced liver injury, and mitochondrial translocation of iron exacerbated the injury (Kon et al., 2010).

The result of mitochondrial adducts formation after APAP is multifaceted. On one hand, binding of NAPQI to mitochondrial proteins like ATP synthase could directly lead to cessation of ATP production (Jaeschke, 1990). On the other hand, attack of mitochondrial proteins by NAPQI could impair mitochondrial respiration and initiate oxidative stress (Meyers et al., 1988). Specifically, the presumably damaged electron transfer chains (ETC) donate free electrons directly to O_2, generating superoxide O_2^- and other reactive oxygen and nitrogen species as discussed above. One critical downstream result of the initial oxidant stress is the activation of several signaling pathways especially JNK (Gunawan et al., 2006; Hanawa et al., 2008) through ASK1 (Nakagawa et al., 2008; Xie et al., 2015c), MLK3 (Sharma et al., 2012), GSK3β (Shinohara et al., 2010) and also RIP1/3(Ramachandran et al., 2013; Zhang et al., 2014). The activated/phosphorylated JNK translocates to the mitochondria and amplifies the early oxidant stress (Saito et al., 2010). As a result, mitochondria permeability transition (MPT) pores open at the inner

mitochondrial membrane to elicit collapse of mitochondrial membrane potential and loss of the proton gradient. With that, the mitochondria are unable to maintain the osmolality and undergo oncotic swelling (Placke et al., 1987; Kon et al., 2004). Several strategies that prevent the MPT have shown protective effects. Using cyclosporine A or NIM811which are pharmacological inhibitors of the MPT pore regulator cyclophilin D, APAP hepatoxicity was significantly reduced *in vitro* and *in vivo* (Kon et al., 2004; Masubuchi et al., 2005). Besides, APAP toxicity was significantly attenuated in cyclophilin D knockout mice (Ramachandran et al., 2011a). However, debio 025, an analog of cyclosporine A failed to provide protection (LoGuidice and Boelsterli, 2011). Although the accurate composition of MPT pores is still in dispute, its indispensable role in APAP hepatotoxicity has been confirmed.

In the later phase of cell death after APAP, the opening of MPT pore and mitochondrial swelling cause release of mitochondrial intermembrane proteins, in particular apoptosis-inducing factor (AIF) and endonuclease G (endoG), which are responsible for nuclear DNA fragmentation after APAP (Cover et al., 2005; Bajt et al., 2006; Bajt et al., 2011). Previous data demonstrated a lack of caspase contribution to DNA fragmentation after APAP as there was no caspase activation and a pan-caspase inhibitor Z-VAD-fmk did not protect against the injury (Lawson et al., 1999; Jaeschke et al., 2006). Although MPT results in AIF and endoG translocation in the later phase, in the early phase, bax translocation to the mitochondrion is responsible for the release of AIF, endo G and second mitochondria-derived activator of caspases (smac) (Bajt et al., 2008). Therefore, it is not surprising to see an early but not lasting protection in $Bax^{-/-}$ mice after APAP (Bajt

et al., 2008). It looks like although the cells activate some of the apoptotic mechanisms, the ultimate injury pattern is still necrosis (Gujral et al., 2002).

1.3 Signaling pathways in APAP-induced hepatotoxicity

The study of intracellular signaling pathways of APAP-induced hepatoxicity has sparked a continuous wave of publications in the recent decade and has filled in multiple knowledge gaps in this model. It started with the findings that both genetic deletion of c-Jun N-terminal kinase (JNK) 2 and pharmacological inhibition by the JNK inhibitor SP600125 protected mice against APAP toxicity (Gunawan et al., 2006; Henderson et al., 2007; Cubero et al., 2015). Although this was soon challenged by a similar study using a different mice strain (Bourdi et al., 2008), subsequent observations that apoptosis-signaling kinase 1 (ASK1) knockout mice were protected against APAP supports the conclusion that JNK is critical, as ASK1 is an upstream kinase of JNK (Nakagawa et al., 2008). This protection by inhibiting ASK1 was later confirmed using an ASK1 specific inhibitor GS-459769 from Gilead Sciences, Inc. (Xie et al., 2015c). Furthermore, two other JNK inhibitors, leflunomide (LEF) which is an anti-rheumatic drug and D-JNK1 which is a peptide inhibitor both alleviated APAP-induced liver toxicity in mice (Latchoumycandane et al., 2006; Henderson et al., 2007). Also, mice deficient in Mkp-1which is a phosphatase of P-JNK displayed worse phenotype (Wancket et al., 2012). Although conflicting observations exist, the preponderance of data favors a deleterious effect by JNK (Cubero et al., 2015; Du et al., 2015).

These initial studies stimulated the investigations of JNK in APAP-induced liver injury, and revealed possibilities of alleviating the injury by modulating signaling pathways in addition to the traditional antidote NAC (Saito et al., 2010). It was soon discovered that JNK is only part of the signaling network, as upstream players like glycogen synthase kinase 3 b (GSK3β), mixed lineage kinase 3 (MLK3) and apoptosis signal-regulating

kinase 1(ASK1) were subsequently identified (Nakagawa et al., 2008; Shinohara et al., 2010; Sharma et al., 2012) . As demonstrated by gene knockout or knockdown studies in mice, GSK3β and MLK3 are considered early regulators of JNK activation while ASK1is a late regulator maintaining the activation. Genetic deletion of any of the three genes significantly protected mice from APAP-induced liver injury (Nakagawa et al., 2008; Shinohara et al., 2010; Sharma et al., 2012). As both ASK1 and MLK3 are mitogen-activated protein kinases (MAP3K), it is not surprising to see the attenuated activation of the MAPK namely JNK and alleviated injury after the blockade of ASK1 or MLK3 (Ichijo et al., 1997; Lotharius et al., 2005). However, what is important is that ASK1, MLK3 and GSK3β are all redox-sensitive enzymes, and therefore the link between JNK and the upstream mitochondrial oxidant stress is established (Saitoh et al., 1998; Grimes and Jope 2001; Noguchi et al., 2005; Hong and Kim 2007; Lee et al., 2014). A player downstream of JNK was also identified, which is Sab (SH3 domain-binding protein that preferentially associates with Btk) (Win et al., 2011). Sab is a scaffold protein on the outer membrane of mitochondria. Immunoprecipitation indicated that JNK and Sab physically interacted with each other (Chambers et al., 2011; Win et al., 2011). Silencing or loss of Sab abrogated sustained JNK activation and translocation, and also significantly reduced hepatic injury (Win et al., 2011; Win et al., 2016). Recent study suggested that the interaction between JNK and Sab lead to SHP1 (also named PTPN6: protein tyrosine phosphatase, non-receptor type 6) dissociation from Sab, and subsequently SHP1 and DOK4 (docking protein 4)-dependent inactivation of Src protein. Src inactivation then triggered inhibition of mitochondrial electron transport chain and ROS increase to sustain JNK activation (Win et al., 2016). With that, a complete ROS

amplification loop beginning with and ending in JNK activation was suggested. Collectively, ASK1, MLK3 and GSK3β mediate JNK activation and phospho-JNK translocation to mitochondria in response to initial mitochondrial oxidant stress, while Sab interacts with JNK to mediate Src inactivation and ultimately mitochondrial ROS production.

Despite a clearer picture of the signaling network, the specific role of JNK remains yet unanswered. Several hypotheses have been proposed. First, JNK may induce Bax translocation to mitochondria. To test that, Bax-/- mice were exposed to APAP. At 6h, the injury was significantly attenuated as indicated by ALT. However, the protection was lost later on at 12h (Bajt et al., 2008). Therefore, the temporary protection by Bax deficiency does not fully explain the lasting protection in JNK knockout mice (Bajt et al., 2008; Saito et al., 2010). Second, JNK may activate iNOS leading to increased peroxynitrite formation (Gunawan et al., 2006; Latchoumycandane et al., 2007; Hanawa et al., 2008). However, the JNK inhibitor SP600125 failed to affect NO formation and iNOS inhibitor did not reduce the NO level after APAP (Saito et al., 2010). Third, the initial oxidative stress could elicit JNK activation and translocation to mitochondria which may be responsible for mitochondrial membrane permeability transition (MPT). Indeed, the JNK inhibitor SP600125 lowered the GSSG level after APAP, indicating the importance of JNK in promoting oxidative stress (Saito et al., 2010). Current thinking is that both GSH depletion and oxidant stress are necessary for the activation of JNK but not sufficient for the final injury, while either replenishing GSH repository or scavenging ROS is enough to inhibit JNK activation and the resultant injury (Saito et al., 2010).

Although the weight of evidence generally favors a pathological role of JNK in APAP-induced liver injury, studies with alternative results challenge the hypothesis and increased the complexity (Du et al., 2015). Among those, studies from multiple labs using JNK1 or 2 $^{-/-}$ mice have demonstrated that these knockout mice are not protected against APAP toxicity (Henderson et al., 2007; Bourdi et al., 2008; Hanawa et al., 2008; Saito et al., 2010). Part of the reason for the disparities in toxicity is the inconsistent genetic background of the mice used across studies (Bourdi et al., 2011). On the other hand, JNK1 and JNK2 most likely compensate for each other and therefore selective elimination of either one was not completely protective (Hanawa et al., 2008; Du et al., 2015).

Even though JNK is now generally considered essential in the progression of injury, it is still counterintuitive that one single molecule acts as an on/off switch for the whole injury process. In agreement with that, recent data suggested a protein kinase C (PKC)-mediated while JNK-independent pathway contributing to the pathogenesis of APAP toxicity (Saberi et al., 2014). In addition to the PKC pathway, receptor-interacting protein kinase 1 and 3 (RIP1 or RIP3) also profoundly contribute to the injury. Both genetic deletion and knockdown of RIP3 protected the mice at early but not late time points, suggesting that RIP3 is an early injury mediator after APAP. Besides, pharmacological interventions using RIP1 inhibitor necrostatin-1 or RIP3 inhibitor dabrafenib significantly alleviated injury (Sharma et al., 2012; Li et al., 2014). Although conflicting data exist, JNK is not the sole player in APAP-induced liver injury.

Although currently necrosis is a widely-accepted mode of cell death after APAP, it was under debate for long as to whether apoptosis contributes to the injury. A few robust

arguments suggest a minor role of apoptosis. First, histological evaluations demonstrated extensive cell swelling and massive cell content release after APAP, with very few apoptotic cells (Gujral et al., 2002). Additionally, in fast mice there was no significant caspase activation after APAP and caspase inhibitors did not protect against the injury (Lawson et al., 1999; Gujral et al., 2002; Williams et al., 2010). Although transient caspase activation was observed in fed Swiss Webster mice treated with APAP, the overall cell death was still apoptotic and did not fundamentally change (Williams et al., 2011b). Consistently, as DNA fragmentation is induced by Endo G and AIF rather than caspases, APAP-induced hepatic injury was not affected by caspase inhibitor treatment (Lawson et al., 1999).

1.4 Treatment strategies for APAP-induced liver injury

Investigations of APAP-induced hepatotoxicity over the past decades expanded our understanding of the injury mechanisms, and more importantly provided new treatment strategies. Although in the clinic N-acetylcysteine (NAC) remains the most convenient and economic antidote for APAP overdose nowadays, with APAP toxicity being not only a single OTC drug toxicity but also a classical model of DILI and ALF, the exploration of novel therapeutic options is especially enlightening.

1.4.1 Enhance antioxidant defenses

One major protective mechanism of NAC is to enhance the antioxidant defenses. The popularity of NAC dates back to four decades ago largely due to the excellent efficacy and easy administration given the commercially available dosage forms (Prescott et al., 1977; Vale et al., 1981; Polson and Lee 2005). Furthermore, the Rumack-Matthew nomogram delineates the relationship between serum concentrations of APAP and hours after ingestion to assists better decision of NAC treatment in clinic (Rumack and Matthew, 1975; Rumack et al., 1981).

Mechanistically, NAC serves as a reservoir of cysteine residues and provides this rate-limiting substrate to stimulate the biosynthesis of the tri-peptide glutathione (Lauterburg et al., 1983). *In vivo*, N-acetyl-L-cysteine but not the isomer N-acetyl-D-cysteine stimulated GSH synthesis, and therefore only the former was able to alleviate the injury after APAP (Corcoran and Wong, 1986). With NAC replenishing the GSH pool after APAP, NAPQI could be detoxified through conjugation with GSH. Therefore, cellular proteins are spared from NAPQI attack and less protein adducts are formed. *In vivo*, NAC

treatment regimens which reduce covalent binding were most effective in attenuating liver injury after APAP (Corcoran et al., 1985). However, NAC needs to be administered in patients early after APAP overdose.

By restoring the GSH level, NAC also scavenges the reactive oxygen and nitrogen species after APAP (Knight et al., 2002). These interferences on early cellular events after APAP are sufficient to explain the efficacy of NAC when given during or soon after APAP metabolism. However, in some cases, late treatment of NAC still improves the patient outcome, indicating that additional protective mechanisms are operative (Harrison et al., 1990; Saito et al., 2010). For that matter, a third mechanism was proposed, suggesting that excess NAC was able to improve the energy supply by providing substrates to the Krebs cycle (Saito et al., 2010). By comparing GSH and NAC treatment after APAP, it was recognized that the most effective strategy to attenuate the injury is to supply excessive cysteines for GSH and simultaneously amino acids for energy production in the Krebs cycle (Saito et al., 2010). In summary, NAC replenishes GSH pool to scavenge NAPQI formation, prevents insults to proteins and also reduces ROS and RNS formation. In addition, NAC provides energy substrates for the Krebs cycle.

Methionine can be converted to cysteine and therefore promotes GSH biosynthesis similar to NAC. Evidence indicates that methionine is comparable in efficacy as NAC, although the latter is more commonly prescribed (Brok et al., 2006). Other antidotes with similar mechanisms include cysteamine and dimercaprol, which are less preferable based on patient outcome (Crome et al., 1976; Hughes et al., 1977; Vale et al., 1981; Brok et al., 2006). Actually acetaminophen/methionine formulation did exist under the name

Paradote in UK. However, it was discontinued to the unpleasant odor from the sulfur and higher costs.

In addition to the effort of promoting GSH resynthesis, other antioxidants have also been tested and have shown protection. Metallothionein is a low molecular weight protein rich in cysteines (Coyle et al., 2002). Zn-induced metallothionein was able to significantly alleviate APAP toxicity in mice without interfering with the metabolism, as metallothionein scavenged NAPQI when GSH was depleted (Saito et al., 2010). Also, resveratrol, a polyphenol small molecule antioxidant, protected against APAP-induced liver injury even as a posttreatment possibly through directly scavenging peroxynitrite and preventing DNA fragmentation (Fremont 2000; Du et al., 2015). On the other hand, attempts to manipulate lipid peroxidation by extra supply of vitamin E failed to achieve any protective effect, suggesting that LPO is not a relevant mechanism of injury (Knight et al., 2003).

1.4.2 Target signaling pathways

Like current cancer therapies which focus on several important receptor tyrosine kinases, targeting signaling pathways also emerges as potential treatment strategies for APAP overdose. The majority of the effort was devoted to the MAPK pathway, and several pharmacological inhibitors have been tried in this model. Specifically, inhibitors of apoptosis signaling kinase 1(ASK1), c-Jun N-terminal kinase (JNK), receptor interacting protein (RIP) 1 or 3 are protective after APAP in mice as discussed in 1.2.4. Importantly, data have shown the lack of interference on liver regeneration by inhibiting ASK1 (Xie et al., 2015c). The key question remains as to whether the efficacy is superior to NAC or

not. Comparison of the JNK inhibitor SP600125 with NAC in primary human hepatocytes indicates that, the JNK inhibitor SP600125 is partially protective against APAP as a 3h-posttreatment while NAC is completely protective at 6h after APAP, suggesting a superior efficacy of NAC (Xie et al., 2014). Similarly, in mice the protection of ASK1 inhibitor but not NAC is lost at 3h post APAP (Xie et al., 2015c). Thus, targeting events upstream of JNK activation including scavenging mitochondrial ROS is necessary and could potentially provide better protection than modulating MAPK pathway (Du et al., unpublished data).

1.4.3 Stimulate autophagy

Autophagy is a conservative catabolic process which degrades cellular contents and recycles them for synthesis of macromolecules or ATP (Mizushima and Komatsu 2011). Previous findings demonstrated that an autophagy inducer rapamycin prevented, while autophagy inhibitors 3-methyladenine or choloroquine exacerbated APAP-induced toxicity *in vitro* and *in vivo*, suggesting a protective role of autophagy (Ni et al., 2012; Ni et al., 2012). The reason for the protection is that autophagy selectively removes damaged mitochondria and therefore attenuates the toxicity (Ni et al., 2012a &b & 2013). Specifically, targeting PINK1-Parkin-mediated autophagy could be a therapeutic option for APAP overdose (Williams 2015 a&b). Also, broad spectrum protein kinase C (PKC) inhibitors protected against APAP in mice by upregulating AMPK-mediated autophagy (Saberi et al., 2014; Du et al., 2015). Together, enhancing autophagy represents a potential therapeutic strategy which promotes the clearance of impaired mitochondria and limits the progression of cell death.

1.4.4 Promote liver regeneration

As liver is a regenerative organ, stimulation of liver regeneration has always been a potential treatment approach for liver diseases. Previous studies have demonstrated the importance of liver regeneration after APAP administration in mice, and several mediators including VEGF, IL-6, TNF-α and beta-catenin activation are shown to promote liver regeneration (Chiu et al., 2003; James et al., 2003; Donahower et al., 2006; Apte et al., 2009; Bhushan et al., 2014). Also, the increase of serum alpha-fetoprotein, which is indicative of liver regeneration, correlated with favorable outcomes in patients (Schmidt and Dalhoff, 2005). Importantly, exogenous administration of stem cell factors (SCF) and human recombinant VEGF, which stimulates liver regeneration, managed to attenuate the injury after APAP (Hu and Colletti et al., 2008; Donahower et al., 2010). With that, enhancing liver regeneration holds promise as a therapeutic option for APAP overdose in the clinic.

1.4.5 Others

In additional to the discussed therapeutic strategies, pharmacological interventions on mitochondria membrane permeability transition (MPT) have shown some effect. Cyclosporine A and NIM 811are able to attenuate APAP hepatotoxicity, which is consistent with the reduced injury in cyclophilin D knockout mice (Kon et al., 2004; Masubuchi et al., 2005; Ramachandran et al., 2011a). Also, modulating mitochondrial fission by inhibiting DRP1 (Dynamin-related protein 1) prevents APAP toxicity *in vitro*, suggesting the relationship between mitochondria dynamics and hepatocyte cell death (Ramachandran et al., 2013).

1.5 Current experimental models of APAP-induced hepatotoxicity

With the popularity of acetaminophen as a model of DILI, the mechanisms of injury have been extensively explored in a spectrum of *in vitro* and *in vivo* models. Overall, disparities of human relevancy exist among different models. However, there is no perfect model for APAP hepatotoxicity, and each model illuminates cellular events to a certain degree. A summary of advantages and disadvantages of these experimental models can be found in table 7.2.1.

1.5.1 Animal models

In the clinic, acetaminophen-induced liver injury is a fulminant, dose-dependent and predictable type of DILI (Jaeschke, 2015). The reproducibility of these characteristics in the mouse readily explains its prevalence as an experimental model for APAP studies. Consequently, the majority of the understanding about APAP-induced liver injury in the past few decades was developed in mice, and most of the understanding was confirmed in humans by measuring mechanistic biomarkers in plasma samples from APAP overdose patients and in primary human hepatocytes (McGill et al., 2012a; McGill et al., 2014c; Xie et al., 2014). Despite these advantages of the mouse model, a few limitations still exist. First, there are gender differences in mice which are not obvious in human. Studies revealed the resistance of female mice to APAP and this was partially explained by a faster GSH recovery (Franc et al., 1997; Du et al., 2014). Second, the progression of injury is accelerated in mice compared with humans. In mice, significant liver injury as indicated by ALT release is observed starting from 2h after APAP overdose with peak injury between 6~12h (Gujral et al., 2002; Bhushan et al., 2013). Compared with that, the

onset of injury in human is delayed and the injury culminates between 24-48 h after APAP exposure (Singer et al., 1995; Larson 2007; McGill et al., 2012a). Different administration routes in human and mice are partially responsible for the disparities in injury progression, as mice are administered with dissolved APAP saline while patients ingest APAP tablets which take extra time for absorption.

Although the rat is a common species in toxicology studies as required by regulatory agencies such as the FDA and EPA, it is not a relevant model for APAP-induced liver toxicity. Consistently, rats are resistant to APAP at even 2g/kg. Also, rats have significantly less mitochondrial protein adduct formation and oxidative stress, and there is no JNK activation and P-JNK translocation to mitochondria (McGill et al., 2012b). However, the usage of rats to test the hepatoprotection of herbal therapeutics is commonly seen. Most of the time, the ALT release in APAP-treated rats are close to control rats, making it difficult to test any reduction of ALT by the herbal products (Jaeschke et al., 2011). Thus, the rat is not a relevant model for APAP toxicity studies.

Studies of APAP-induced hepatotoxicity are also conducted in other animal species, including hamster, zebrafish, cats, dogs and others (Tee et al., 1986; Allen, 2003). However, the relevancy of the injury pattern for human has not been established and may be questionable.

1.5.2 Primary human and mouse hepatocytes

In vivo models are most ideal for systemic toxicity testing. However, *in vitro* models are also informative when the mechanisms of actions are due to hepatocytes but not other liver cell types.

Among all *in vitro* models, primary human hepatocytes (PHHs) remain the gold standard of toxicity testing which allow experimental interventions (Godoy et al., 2013). Needless to say, PHH has a full repertoire of drug metabolizing enzymes and transporters, and it is an *in vitro* model closest to human livers (Wilkening et al., 2003). Generally, PHHs are isolated from liver tissues of individual patient donor, which renders PHHs susceptible to interindividual variability in drug responses. The first hepatocyte isolation method was established more than 40 years ago and the current protocol follows a similar two-step collagenase digestion method (Bojar et al., 1976; Seglen, 1976; Strom et al., 1982).

Despite the advantages of PHH, several limitations exist, and continuous efforts have been made to improve the availability, convenience and human relevance of PHHs. The first limitation is that PHHs are available on an unpredictable basis depending on the liver donors, and therefore the duration of study is considerably prolonged (Xie et al., 2014). Cryopreservation of isolated PHHs was introduced for this reason, and the frozen PHHs are ready to use after thawing. However, the viability of cryopreserved PHHs depends on the PHH quality before processing as well as the freezing plus storage procedures, and the viability is generally low.

Another disadvantage of PHH is the progressive cell dedifferentiation, mainly reflected by the loss of cell polarity and alterations of drug metabolizing enzymes or transporters. As these features are critical for hepatocyte functionality, several approaches are adapted to overcome these issues. First, for short-term toxicity studies, it is recommended to perform the experiment soon after proper attachment of PHHs to avoid the gradual dedifferentiation process especially the loss of Cytochrome P450 enzyme expression (Xie et al., 2014). Second, cells can be plated in sandwich culture or 3D culture, which have

been shown to prevent the dedifferentiation to some degree (Godoy et al., 2013; Schyschka et al., 2013). A more widespread application of cryopreserved PHHs relies on the improvement of all these determinants.

Similar to PHHs, precision–cut liver slices which are liver sections from human liver donors, have also been used for APAP toxicity studies. The intact tissue architecture, expression of drug metabolizing enzymes and transporters, as well as the presence of non-parenchymal cells could provide new insights in addition to PHHs (Hadi et al., 2013).

As an *in vitro* counterpart of mice, primary mouse hepatocytes (PMHs) are also frequently used. The PMH is not only a model to confirm cellular events *in vivo*, but also a tool to investigate parenchymal cell responses or non-inflammatory activities to APAP. As the role of sterile inflammation after APAP has been a highly controversial topic in recent years, primary hepatocytes serve as a reference model to test any potential artifacts (Jaeschke et al., 2012). If sterile inflammation does play a role during the injury progression after APAP, any blockade on sterile inflammation or non-parenchymal cells should not affect APAP-induced cell death in PMHs. On the other hand, if the same protection against APAP *in vivo* is reproducible in PMHs, then most likely the protection is not due to modulating sterile inflammation (Xie et al., 2013).

1.5.3 Hepatoma cell lines

As PMH isolation requires mouse liver perfusion, more accessible alternatives are hepatoma cell lines, including HepaRG, HepG2, Hep3B and Huh7 (Jaeschke et al., 2011; McGill et al., 2011; Jaeschke et al., 2013). HepaRG cells are bipotent hepatoma cells derived from a liver tumor infected with HCV, and display the phenotypes of both

hepatocytes and biliary epithelial cells (Parent et al., 2004; Cerec et al., 2007; Guillouzo et al., 2007). Most importantly, the HepaRG cell expresses sufficient amount of CYP450s for drug metabolism and therefore is a suitable model for drug toxicity testing (Guillouzo et al., 2007; Kanebratt and Andersson, 2008; McGill et al., 2011). The key cellular events and time courses after APAP in HepaRG cells are similar to human (McGill et al., 2011; Xie et al., 2015a). Although APAP does not activate JNK in HepaRG cells and the cells are not responsive to JNK inhibitor SP600125, the cell line provides an experimentally convenient and relevant model for APAP hepatotoxicity studies (McGill et al., 2011; Xie et al., 2014). In contrast, a number of hepatoma cells including HepG2, Hep3B, Huh7 lack CYP450 enzyme expression and have limited biotransformation properties. Therefore, these cell lines are unsuitable to study APAP-induced liver cell death (Jaeschke et al., 2011; Jaeschke et al., 2011).

1.5.4 Clinical samples of APAP overdose patients

Although APAP overdose is the most frequent cause of acute liver failure in the US, liver biopsies are not recommended due the risk of bleeding in patients (Lee, 2012). With that, blood samples from consented APAP overdose patients are being used. Although no experimental interventions are permitted, studies using patient blood samples have allowed significant process especially on mechanistic biomarker identification (McGill and Jaeschke 2014). A spectrum of APAP biomarkers including HMGB1 (Antonie et al., 2009& 2012), keratin-18 (Antonie et al., 2009& 2012), micro RNAs (Ward et al., 2014; Vliegenthart et al., 2015), APAP-protein adduct (Daven et al., 2006; McGill et al., 2013), mtDNA, nuclear DNA (McGill et al., 2012a &2014c), glutamate dehydrogenase (GDH) (McGill et al., 2012a), argininosuccinate synthetase (McGill et al., 2014a), acylcarnitines

(McGill et al., 2014b), glycodeoxycholic acid (Woolbright et al., 2014) and others have all demonstrated a correlation with the injury and with the clinical outcome in most cases. The continuous search for superior biomarkers especially progenitor biomarkers comes from the fact that traditional clinical endpoints of liver injury like ALT do not predict who survives or recovers and who dies. Future exploration of potential biomarkers still relies on a better understanding of pathophysiology after APAP.

1.6 Specific aims of the dissertation

Specific Aim 1 - Investigate the time course and dose-response of APAP toxicity in primary human hepatocytes

The time course (0-48h) and dose response (1-20 mM) of APAP-induced cell death will be studied in primary human hepatocytes. In these studies, it will be investigated if key events observed in mouse hepatocytes and HepaRG cells, i.e. GSH depletion, protein adducts formation, mitochondrial dysfunction and oxidant stress are critical for cell necrosis in PHH.

Specific Aim 2 – Investigate the involvement of ASK1 and JNK in the pathophysiology of APAP-induced cell death

The importance of JNK-ASK1 pathway has been documented in the mouse model. Thus, the objective is to assess if this pathway is also critical in human hepatocytes. The activation of this pathway will be evaluated using western blotting for P-JNK. In addition, mitochondrial translocation of P-JNK will be assessed. The pathophysiological relevance will be tested using pharmacological inhibitors of ASK1 and JNK and by gene knockdown experiments.

Specific Aim 3 – Investigate the effect of cell culture oxygen tension on APAP-induced cell death in primary human hepatocytes.

Our lab has demonstrated that the artificially high oxygen concentration in incubators using room air (21% oxygen) leads to enhanced oxidant stress and accelerated cell death in mouse hepatocytes. To assess if human hepatocytes respond similarly, APAP-induced

cell oxidant stress and cell death will be compared at 21%, 10% and 5% oxygen concentrations.

Chapter 2. Acetaminophen-induced Hepatotoxicity in Primary Human Hepatocytes

This section is adapted from Xie et.al (2014), "Mechanisms of acetaminophen-induced cell death in primary human hepatocytes", Toxicology and Applied Pharmacology, 279(3): 266-274, with permission from the publisher

2.1 Abstract

Acetaminophen (APAP) overdose is the most prevalent cause of drug-induced liver injury in western countries. Numerous studies have been conducted to investigate the mechanisms of injury after APAP overdose in various animal models; however, the importance of these mechanisms for humans remains unclear. Here we investigated APAP hepatotoxicity using freshly isolated primary human hepatocytes (PHH) from either donor livers or liver resections. PHH were exposed to 5 mM, 10 mM or 20 mM APAP over a period of 48 h and multiple parameters were assessed. APAP dose-dependently induced significant hepatocyte necrosis starting from 24 h, which correlated with the clinical onset of human liver injury after APAP overdose. Interestingly, cellular glutathione was depleted rapidly during the first 3 h. APAP also resulted in early formation of APAP-protein adducts (measured in whole cell lysate and in mitochondria) and mitochondrial dysfunction, indicated by the loss of mitochondrial membrane potential after 12 h. Furthermore, APAP time-dependently triggered c-Jun N-terminal kinase (JNK) activation in the cytosol and translocation of phospho-JNK to the mitochondria. Both co-treatment and post-treatment (3 h) with the JNK inhibitor SP600125 reduced JNK activation and significantly attenuated cell death at 24 h and 48 h after APAP. The clinical antidote N-acetylcysteine offered almost complete protection even if administered 6 h after APAP and a partial protection when given at 15 h. Conclusion: These data highlight important mechanistic events in APAP toxicity in PHH and indicate a critical role of JNK in the progression of injury after APAP in humans. The JNK pathway may represent a therapeutic target in the clinic.

2.2 Introduction

Acetaminophen (APAP; paracetamol) is widely used as an analgesic and antipyretic drug. At therapeutic doses, it is considered effective and safe but an overdose can cause severe liver injury, liver failure and even death (Larson, 2007). Currently, APAP is the leading cause of drug-induced liver failure in the US and UK (Lee, 2012). Considerable progress has been made in the understanding of the mechanisms of injury after APAP overdose in animal models (Jaeschke et al., 2012). Although most of the drug is conjugated with glucuronic acid or sulfate and excreted, a small fraction is metabolized by cytochrome P450 enzymes to a reactive metabolite, presumably *N*-acetyl-*p*-benzoquinone imine (NAPQI). NAPQI can be scavenged through conjugation with glutathione (GSH) and can react with protein sulfhydryl groups to form adducts (Mitchell et al., 1973). It is thought that protein adducts, especially in mitochondria, cause mitochondrial dysfunction and an early oxidant stress, which initiates the activation of various MAP kinases (Jaeschke et al., 2012). Ultimately, the activities of different kinases converge to phosphorylate c-jun-N-terminal kinase (P-JNK), which then translocates to the mitochondria and amplifies the oxidant stress and peroxynitrite formation (Hanawa et al., 2008 and Saito et al., 2010a). This mitochondrial oxidant stress leads to the opening of the membrane permeability transition (MPT) pore with collapse of the membrane potential and cessation of ATP synthesis. In addition, the MPT causes mitochondrial matrix swelling, with rupture of the outer membrane and the release of intermembrane proteins, such as endonuclease G and apoptosis-inducing factor, which translocate to the nucleus and induce DNA fragmentation (Jaeschke et al., 2012). Together, these events result in cell necrosis (Gujral et al., 2002).

After the early mechanistic insight into APAP hepatotoxicity in mice leading to the development of the first clinical antidote N-acetylcysteine (NAC) (Prescott et al., 1977), no further therapeutic intervention has been developed during the last 35 years. One of the main reasons is limited understanding of the human pathophysiology, which makes it difficult to directly extrapolate potential therapeutic targets in mice to humans. However, some progress has been made recently. In the metabolically competent human hepatoma cell line HepaRG, APAP-induced protein adducts formation, mitochondrial dysfunction and oxidant stress preceded cell necrosis at 24–48 h (McGill et al., 2011). In patients with APAP overdose, high levels of protein adducts have been detected in serum (Davern et al., 2006 and Muldrew et al., 2002). Furthermore, evidence for mitochondrial damage, i.e. the leakage into serum of the mitochondrial matrix enzyme glutamate dehydrogenase (GDH) and mitochondrial DNA, were measured in patients with liver injury (McGill et al., 2012a). The rise of serum acylcarnitines reflected mitochondrial dysfunction in mice; however, this effect was not observed in humans because of the suppression by the standard of care NAC treatment (McGill et al., 2014b). In addition, cell death markers and damage associated molecular patterns found in mice, such as high mobility group box 1 protein, microRNA-122, cytokeratin-18, nuclear DNA fragments, and argininosuccinate synthetase, were also observed in serum of patients (Antoine et al., 2012, McGill et al., 2012a, McGill et al., 2014a and Starkey Lewis et al., 2011). Despite these similarities in cellular responses to APAP overdose in mice and in humans, detailed signaling mechanisms of APAP-induced cell death in human hepatocytes remain unclear. A better understanding of these events is critical for development of any additional therapeutic interventions. Therefore, the aim of the present study was to investigate the

mechanisms of hepatotoxicity of APAP in freshly isolated primary human hepatocytes (PHH). Specifically, we wanted to determine critical but as yet unexplored events in PHH such as mitochondrial protein binding, mitochondrial dysfunction and mitochondrial translocation of P-JNK. Our data show similarities but also essential differences to mouse hepatocytes, some of which may explain the delayed toxicity in humans and PHH.

2.3 Materials and methods

Isolation of primary human hepatocytes

All human tissues were obtained with informed consent from each patient, according to ethical and institutional guidelines. The study was approved by the University of Kansas Medical Center Institutional Review Board. A summary of medical information of donors is provided in Table 1. No donor tissue was obtained from executed prisoners or other institutionalized persons. All liver specimens were obtained in accordance with a Human Subject Committee approved protocol from patients undergoing a hepatic resection procedure or from donor organs. Liver specimens were obtained from the hospital operating room and placed in a sterile container that contains either cold sterile saline or cold University of Wisconsin's (UW) solution. The liver specimens were then placed in a secondary container with ice and transported back to the cell isolation lab. Once in the lab, all procedures were then performed in a biological safety cabinet using aseptic technique. A pan of ice was placed in the biological safety cabinet and covered by a plastic bag and then a sterile field was placed over the bag. The liver specimen was then placed on top of the sterile field. Catheters were inserted into the major portal and/or hepatic vessels and

the tissue was perfused with cold EMEM (Mediatech cat # 15-010-CM) containing 25 mM HEPES buffer (Mediatech cat # 25-060-CI) to determine which vessels provide the most uniform perfusion of the tissue. The catheters were then secured into the vessels either by sutures or surgical grade glue. All remaining major vessels on the cut surfaces were closed with sutures and/or surgical grade glue. The liver tissue was then placed in a sterile plastic bag and connected to a peristaltic pump with the flow rate dependent on the number of catheters and size of tissue. The bag containing the tissue was placed in a water bath at 37 ℃ and the tissue was perfused with calcium, magnesium and phenol red free HBSS (Hyclone cat # SH30588.02) containing EGTA (1.0 mM) without recirculation for 10–15 min. The EGTA chelates calcium which leads to the separation of cell junctions and helps to remove any residual blood. The liver specimen was then perfused for 8–10 min using HBSS without EGTA to flush residual EGTA from the tissue. Finally, the liver specimen was perfused with EMEM containing 0.1 mg/ml of collagenase (VitaCyte cat# 001-2030) and 0.02 mg/ml of protease (VitaCyte cat# 003-1000) which was recirculated as long as needed (18–30 min.) to complete the digestion. All perfusion solutions were maintained at 37 ℃ via a water bath. Perfusion of the specimen was stopped when the liver tissue began to show fissures and separation from the liver capsule. The liver tissue was then removed from the plastic bag and placed in a sterile plastic beaker that contains ice-cold EMEM + 25 mM HEPES. The specimen was then gently chopped with sterile scissors to release hepatocytes. The cell suspension was filtered through sterile gauze-covered funnels to remove cellular debris and clumps of undigested tissue. Hepatocytes were isolated from the other cell types in the suspension by low speed centrifugation at 80 *g* for 5 min at 4 ℃. The supernatant was decanted and

the centrifugation steps were repeated 2 more times using cold EMEM + 25 mM HEPES to re-suspend the hepatocytes after each spin. After three washes in EMEM + 25 mM HEPES, the hepatocytes were re-suspended in cold Williams' Medium E (Life Technologies cat # A12176-01 supplemented with l-Glutamine (2 mM) (Life Technologies cat # 35050-061), HEPES (10 mM), insulin (10–7 M) dexamethasone (10–7 M), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml). Cell viability was assessed by trypan blue exclusion and cell number was determined by using a hemocytometer viewed through an inverted microscope. Viabilities were expressed as a percentage of the total cell number. The hepatocytes were brought to a concentration of 0.5×10^6 cells/ml in Williams' Medium E, as described above, plus 5% bovine calf serum. The hepatocytes were then seeded on collagen coated plates and allowed to attach in a humidified 37 ℃, 5% CO_2incubator for 2–12 h and then the medium was changed to serum free Williams' Medium E supplemented as described.

Primary human hepatocytes experiments

Approximately 3 h after being seeded on collagen-coated dishes, cells were washed with sterile phosphate-buffered saline (PBS) and treated with APAP dissolved in Hepatocyte Maintenance Medium (Lonza, Walkersvill, MD) supplemented with insulin, dexamethasone, and gentamicin/amphotericin-B. Due to the limited number of hepatocytes obtained from each isolation procedure it is not possible to perform a complete set of assays on each batch of hepatocytes. We did not specifically assign patients to certain experiments. However, for every batch of cells, we measured ALT release of hepatocytes 24 and 48 h after 10 mM APAP to make sure the cells are responding properly. For JNK inhibitor treatment, SP600125 (Calbiochem, Darmstadt,

Germany) dissolved in dimethyl sulfoxide (DMSO) was added to the cell culture medium to achieve a final concentration of 10 μM, either at the same time as APAP treatment, or 3 h afterwards. For caspase inhibition experiments, cells were co-treated with 20 μM of Z-VD-fmk (generous gift from Dr. S.X. Cai, Epicept, San Diego, CA) and APAP. For N-acetylcysteine (NAC) treatment, NAC was dissolved in H_2O and then added to the culture medium at a series of time points after APAP to achieve the final concentration of 20 mM.

APAP-protein adducts measurement

APAP-protein adducts were measured in total cell homogenate and in mitochondria. Briefly, hepatocytes or isolated mitochondria were lysed in 10 mM sodium acetate buffer, pH 6.5, by brief sonication or several cycles of freeze–thaw, respectively, and the lysates were filtered through a size exclusion column (Biospin-6 columns, Bio-Rad Laboratories, Hercules, CA) to remove low molecular weight compounds. The purified proteins were then digested overnight with proteases. Proteases were precipitated with an acetone/methanol mixture and pelleted, and the supernatants evaporated to a residue. The residue was then dissolved in 50 μL sodium acetate buffer and subjected to high pressure liquid chromatography with electrochemical detection (HPLC-ECD) of protein-derived APAP-cysteine (McGill et al., 2013 and Muldrew et al., 2002). Protein in the lysates was measured using the bicinchoninic acid assay (BCA).

Biochemical assays

ALT levels were determined using an ALT reagent kit (Pointe Scientific, MI). GDH activity was evaluated as described (McGill et al., 2012a). Using Ac-DEVD-AMC as the

substrate, caspase-3 activity was measured as previously described in detail (Jaeschke et al., 1998). A modified method of the Tietze assay was carried out to measure cellular glutathione levels as described (Jaeschke and Mitchell 1990). To detect the mitochondrial membrane potential, JC-1 Mitochondrial Membrane Potential Kit (Cell Technology, Mountain View, CA) was used as described in detail (Bajt et al., 2004)

PI staining

PHH were allowed to adhere on glass bottom dishes for 3 h, followed by 10 mM APAP. The medium was removed after 24 h and cells were stained with 500 nM of propidium iodide (Invitrogen) for 5 min. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and images were recorded with a fluorescence microscope.

Isolation of subcellular fractions and Western blotting

Mitochondria and cytosolic fractions were isolated from cell lysates using differential centrifugation as described (Cover et al., 2005). Standard Western blotting was performed using a rabbit anti-JNK antibody and a rabbit anti-phospho-JNK antibody (Cell Signaling Technology, Danvers, MA).

HepaRG cells

HepaRG cells were differentiated and treated as described (McGill et al., 2011).

Statistics

All results were expressed as mean ± standard error (SE). Comparisons between multiple groups were performed with one-way ANOVA followed by post hoc Student–Newman–Keuls test. If the data were not normally distributed, the Kruskal–Wallis Test was used

(nonparametric ANOVA) followed by Dunn's Multiple Comparisons Test. $P < 0.05$ was considered significant.

2.4 Results

APAP induces necrotic cell death in PHH

We evaluated the toxicity of APAP in freshly isolated PHH by performing a time course study with 5 mM and 10 mM APAP. APAP resulted in significant cellular ALT release into the culture medium starting from 24 h with a progressive further increase up to 48 h (Fig. 2.4.1A). The injury was dose-dependent (Fig. 2.4.1B). PI staining of the nuclei confirmed the necrotic cell death (Fig. 2.4.1C). Furthermore, no increase in caspase-3 activity was detectable in APAP-treated PHH cells at 24 h (data not shown).

The total number of cell isolations for this manuscript was 44 with the donor patient information listed in Table 2.4.1. For each batch, cell injury was determined at 24 and 48 h after APAP exposure and compared to cell death in controls. Fig. 2.4.2A demonstrates the variability in the cell death response between different batches of cells. In addition, the groups were separated into cells from male and female donors (Fig. 2.4.2B). Our results do not indicate a statistically significant gender difference in toxicity in response to APAP exposure. Furthermore, GSH depletion of cells from 7 individual donors is shown in Fig. 2.4.2C. Together, these data demonstrate a limited inter-individual variability in APAP-induced toxicity between cells from different donors.

APAP triggers GSH depletion, mitochondria dysfunction and protein adducts formation in PHH

APAP is metabolized to the electrophile NAPQI, which can react with the sulfhydryl groups of cysteine in GSH or cellular proteins. Exposure to 10 mM APAP reduced cellular GSH levels by > 70% within 3 h, followed by further depletion over the next 20 h (Fig. 2.4.3A). Measurement of the mitochondrial membrane potential with the JC-1 assay revealed significant mitochondrial dysfunction and loss of mitochondrial membrane potential between 6 and 15 h after APAP (Fig. 2.4.3B). Protein adducts in the cells were formed mainly during the first 6 h, with no further increase up to 15 h (Fig. 2.4.3C). Interestingly, protein adducts in mitochondria did not increase during the first 3 h, i.e. before GSH was depleted by 70%. However, mitochondrial adduct levels dramatically increased between 3 and 6 h (Fig. 2.4.3D). These time course experiments revealed a sequence of events in PHH after APAP exposure that includes rapid GSH depletion and protein adduct formation, with mitochondrial protein adduct formation and mitochondrial dysfunction occurring later, only after extensive GSH depletion.

APAP causes JNK activation and mitochondrial translocation in PHH

JNK activation and the mitochondrial translocation of phospho-JNK (P-JNK) have been well established as a second hit in mouse hepatocytes to amplify the initial oxidative stress (Hanawa et al., 2008 and Saito et al., 2010a). To explore the role of JNK in PHH,

Table 2.4.1 Liver donors medical information

Criteria	Number (total = 44)
Source of hepatocytes	
Resected livers	17 (39%)
Donor livers	27 (61%)
Age	
Range	23–79
Average	50.8
Gender	
Male	21 (48%)
Female	23 (52%)
Ethnicity	
Caucasian	37 (84%)
Asian	2 (5%)
Black	4 (9%)
N/A	1 (2%)
Body mass index (BMI)	
Range	21.2–49.5
Average	29.6
Smoke/Alcohol use	
Both	11 (25%)
Smoke only	6 (14%)
Alcohol only	1 (2%)
Neither	7 (16%)
N/A	19 (43%)
Diagnosis	
Metastatic colon cancer	16 (36%)
Head injury	7 (16%)
ICH/Stroke	5 (11%)
Drug overdose	4 (9%)
Cardiac arrest	4 (9%)
Asthma attack	2 (5%)
Asphyxiation/hanging	2 (5%)
Metastatic rectal cancer	2 (5%)
CVA/Stroke	1 (2%)
SIGSW	1 (2%)

ICH; intracerebral hemorrhage.
CVA; cerebrovascular accident.
SIGSW; self-inflicted gunshot wounds.
N/A; not available.

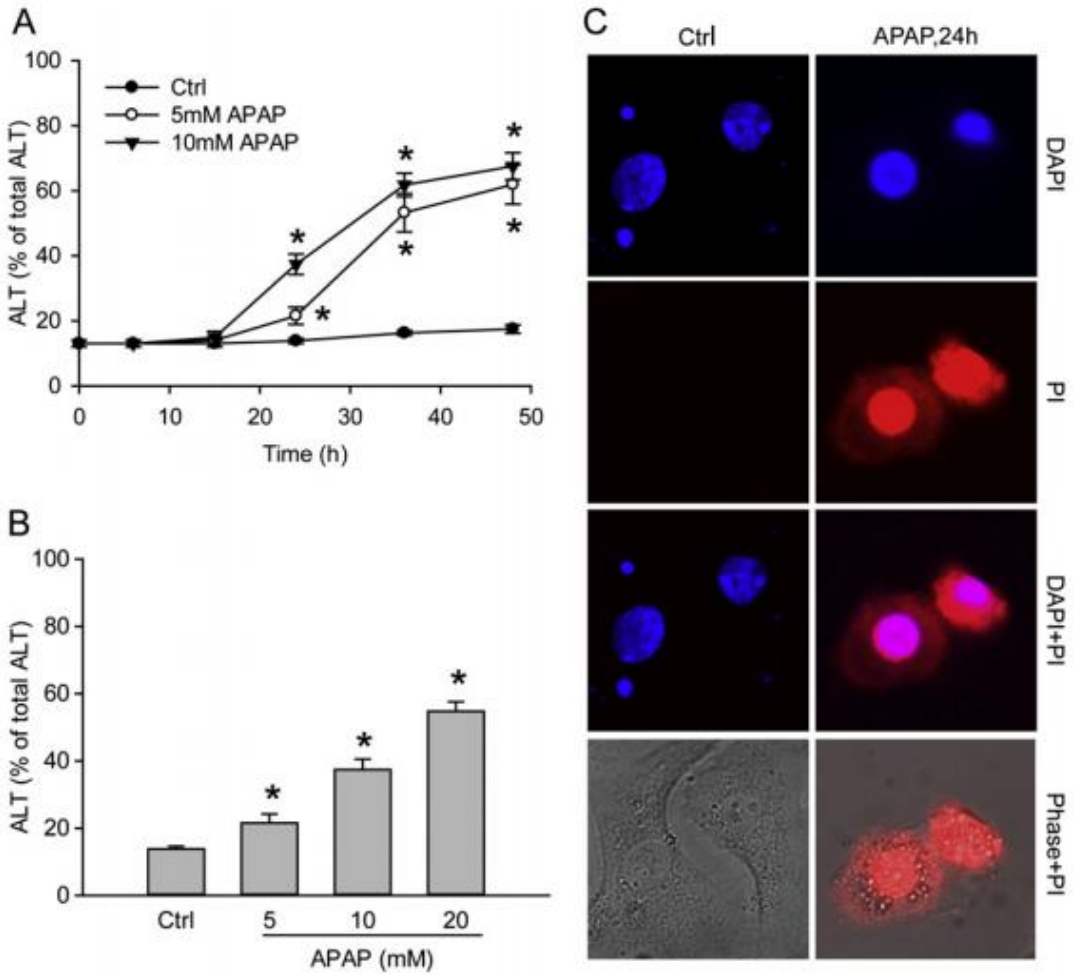


Figure 2.4.1 APAP Induces Cell Death In Primary Human Hepatocytes (PHH).

Cells were treated with 5 mM or 10 mM APAP over a period of 48 h. (A) Time course of toxicity over 48 h, as indicated by percentage of alanine aminotransferase (ALT) activity found in the culture medium, (B) dose–response of APAP toxicity at 24 h after treatment. (C) Necrotic cell death as indicated by nuclear PI staining after 24 h after 10 mM APAP exposure. The nucleus was stained with DAPI. Data in A and B represent mean ± SE of 20 independent cell isolations. *$P < 0.05$ (compared with time 0 or control).

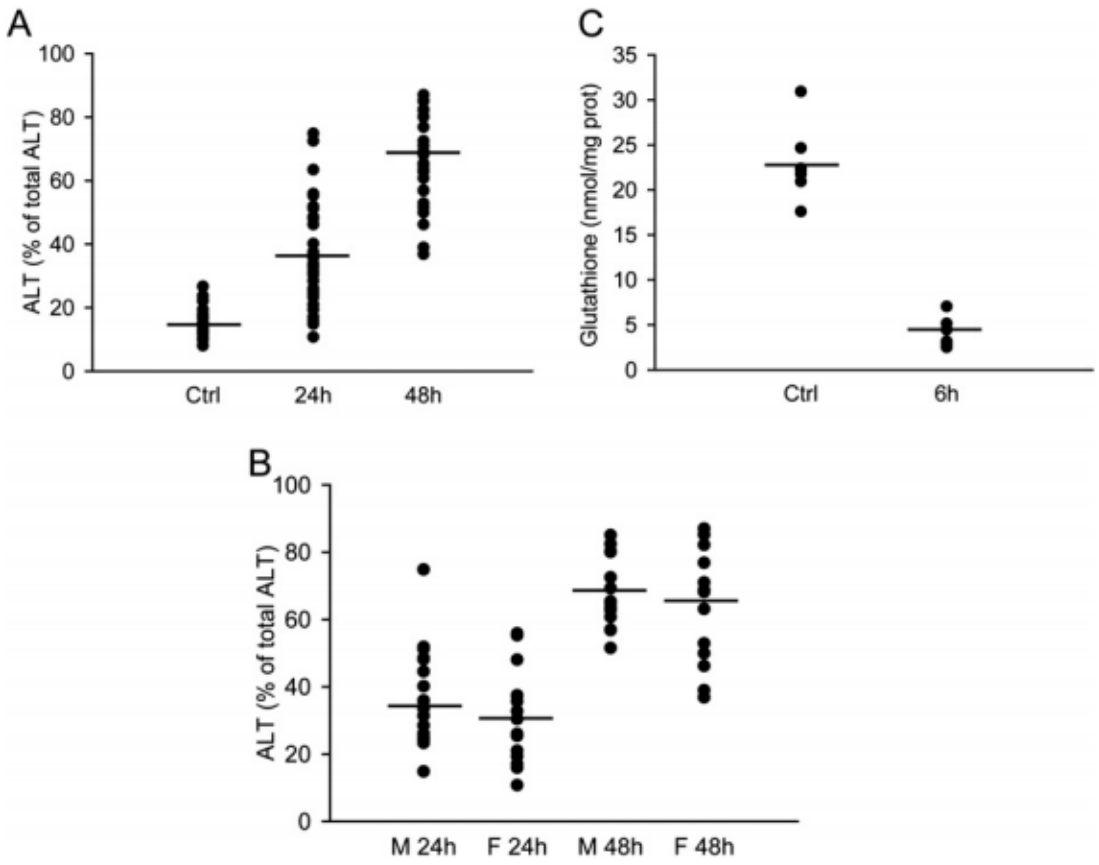


Figure 2.4.2 Inter-individual variation of ALT and GSH levels in primary human hepatocytes.

(A) ALT release from hepatocytes isolated from 44 patients. Each batch of cells (individual patient) were either untreated (0), or exposed to 10 mM APAP for 24 and 48 h. (B) ALT release from hepatocytes isolated from male and female patients 24 and 48 h after 10 mM APAP. (C) GSH levels of hepatocytes from each individual (n = 7) at 0 and 6 h after 10 mM APAP. Horizontal lines indicate average values.

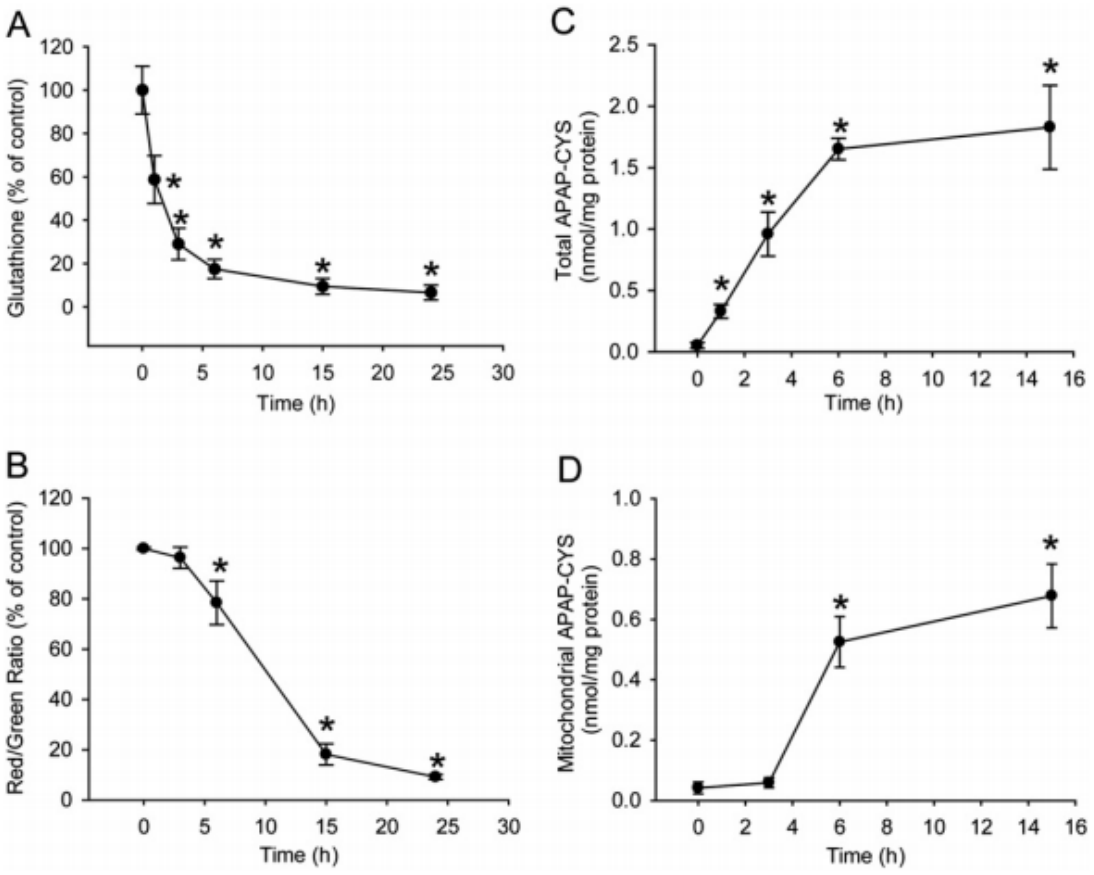


Figure. 2.4.3 APAP triggers GSH depletion, mitochondria dysfunction and protein adduct formation in PHH.

(A) Time course of cellular glutathione depletion after 10 mM APAP. (B) Loss of mitochondria membrane potential over 24 h after 10 mM APAP, as indicated by decrease of red/green fluorescence ratio using the JC-1 assay. APAP-protein adduct formation (C) in the whole cell and (D) in the mitochondria fraction over 15 h after 10 mM APAP. Data represent mean ± SE from experiments using cells from 3 to 8 donors. * $P < 0.05$ (compared with time 0).

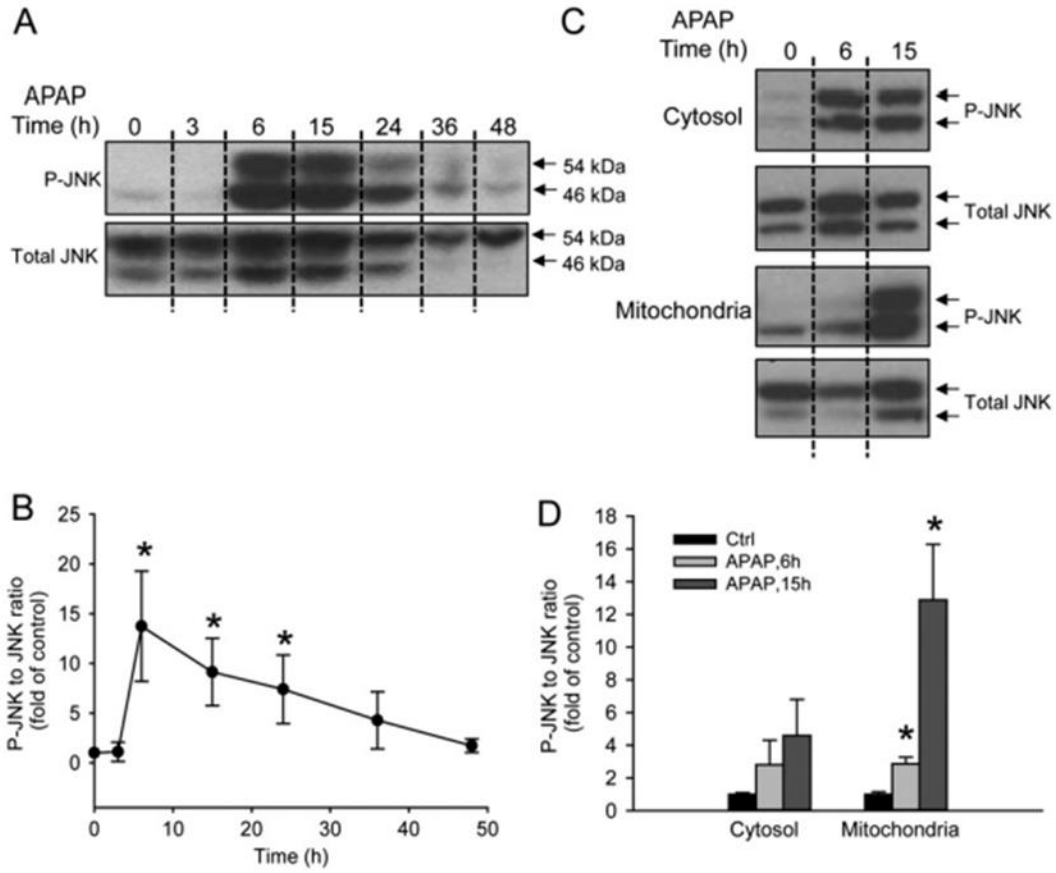


Figure. 2.4.4 APAP leads to cellular JNK activation in PHH and phospho-JNK translocation to the mitochondria.

(A) Time course of JNK activation in whole cell homogenate after 10 mM APAP and (B) Densitometry. (C) JNK activation in the cytosol and phospho-JNK translocation to the mitochondria and (D) Densitometry. Data represent mean ± SE of independent Western blotting using PHH samples from 3 to 4 donors. **P* < 0.05 (compared with controls).

APAP-treated cells were harvested at several time points and the lysates were probed for JNK and P-JNK by Western blotting. APAP resulted in time-dependent JNK activation which was first detectable at 6 h, with progressive decrease subsequently (Fig. 2.4.4A). This result was confirmed by densitometry and the calculation of the P-JNK-to-JNK ratio (Fig. 2.4.4B). To determine whether activated JNK translocated into mitochondria as it does in mouse hepatocytes, cytosolic and mitochondrial fractions were isolated from PHH and JNK activation was evaluated at 6 and 15 h after APAP treatment. Similar to what was observed in total cell lysate, APAP exposure caused JNK activation in the cytosol at 6 and 15 h (Fig. 2.4.4C). Some JNK (mainly 54 kDa) was observed in mitochondria from controls, with a modest but significant increase in P-JNK 6 h after APAP exposure and massive P-JNK at 15 h (Fig. 2.4.4C). These results were confirmed by densitometry and the calculation of the P-JNK-to-JNK ratio (Fig. 2.4.4D).

JNK inhibitor SP600125 partially protects against APAP-induced cell death in PHH

Given the beneficial effect of the JNK inhibitor SP600125 in animals, we sought to determine whether it protects against APAP in PHH. Cells were treated with SP600125 either simultaneously with APAP or 3 h after APAP, and cell death was evaluated at 24 and 48 h. As SP600125 was dissolved in DMSO, a well-known inhibitor of cytochrome P450 enzymes, the concentration of SP600125 was limited to 10 μM to reduce interference with APAP metabolism. Despite this low concentration, SP600125 consistently reduced cell death at both time points and with both treatment paradigms (Figs. 5A, B). To verify the ability of SP600125 to inhibit JNK activation in human hepatocytes, Western blotting was conducted. Treatment with this low concentration of SP600125 moderately lowered the activation of JNK in both co-treated and post-treated

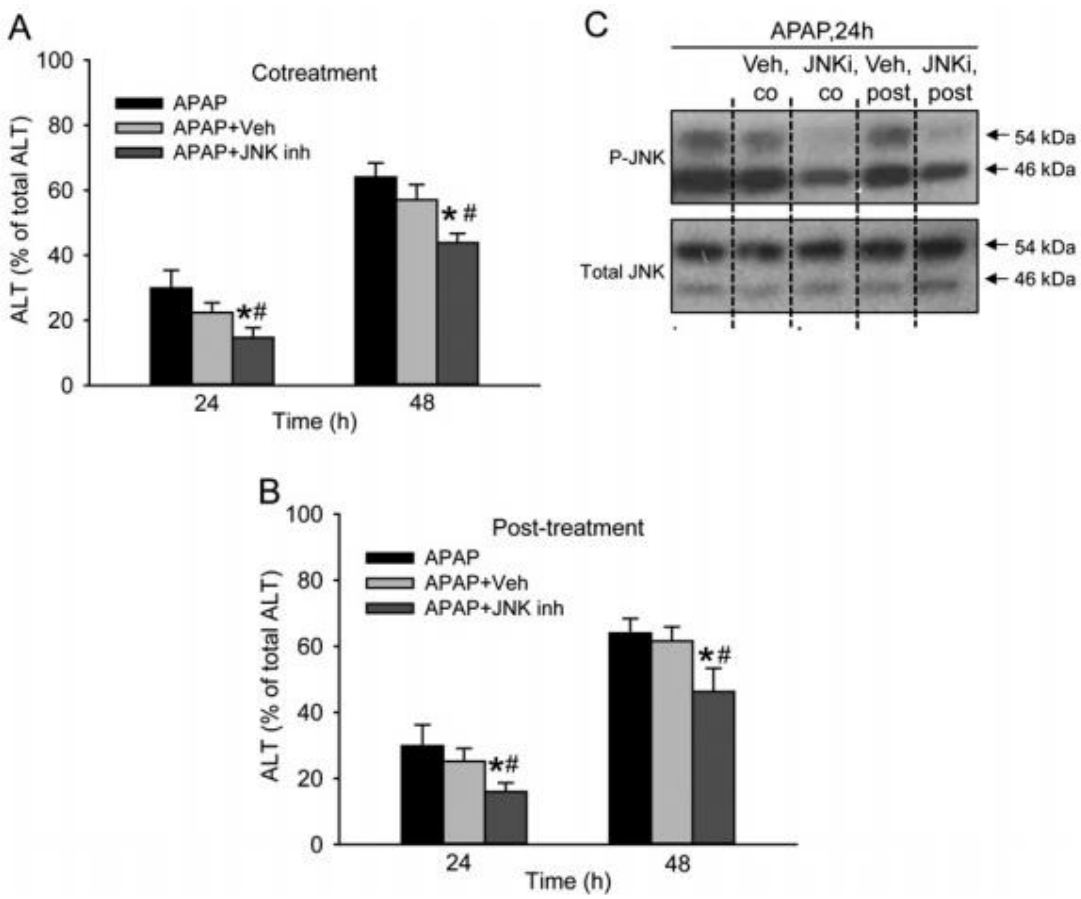


Figure 2.4.5 JNK inhibitor SP600125 partially protects against APAP-induced cell death in PHH.

JNK inhibitor SP600125 using 0.2% DMSO as the vehicle was administered either as (A) a cotreatment with APAP or (B) a 3-hour post-treatment after APAP. (C) Western blotting was performed to verify the efficacy of SP600125. Data represent mean ± SE from experiments using cells from 6 donors. # $P < 0.05$ (compared with APAP + Veh group).

cells without altering total JNK levels (Fig. 2.4.5C). Thus, JNK is involved in the progression of cell death after APAP in PHH.

JNK is not relevant for APAP-induced cell death in HepaRG cells

Previous data have demonstrated that HepaRG cells, a bipotent human hepatoma cell line, are a useful model to study APAP hepatotoxicity (McGill et al., 2011). For comparison, JNK activation in HepaRG cells was evaluated. Over a period of 24 h, JNK activation was barely detectable after APAP treatment, and total JNK levels remained almost constant (Fig. 2.4.6A). Surprisingly, co-treatment of SP600125 with APAP aggravated the injury rather than preventing it (Fig. 2.4.6B). Based on these data, JNK activation does not appear to be relevant for cell death after APAP in HepaRG cells.

Protection against APAP-induced cell death in PHH by the clinical antidote N-acetylcysteine

To test if NAC is able to reduce APAP toxicity in PHH, cells were treated with 10 mM APAP and 20 mM NAC at the same time or 3-24 h after APAP. APAP alone caused 29% and 68% cell death at 24 and 48 h, respectively (Fig. 2.4.7A). NAC treatment completely prevented cell death at 24 h when given up to 6 h after APAP and significantly attenuated ALT release by 77% at 48 h (Fig. 2.4.7A). There was still a trend of reduced ALT release at 48 h when the cells were treated with NAC at 15 h. However, the protection by NAC was completely lost in the 24 h post-treatment group (Fig. 2.4.7A). When GDH as indicator for mitochondrial dysfunction was measured, NAC given either at the same time or 6 h after APAP totally eliminated the release of GDH at 24 or 48 h after APAP

exposure (Fig. 2.4.7B). Furthermore, NAC treatment eliminated JNK activation at 24 h (Figs. 2.4.7C, D).

2.5 Discussion

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.

GSH depletion and protein adducts

The electrophilic metabolite NAPQI reacts rapidly with GSH, causing extensive GSH depletion in mouse livers *in vivo* (> 80% within 30 min) (McGill et al., 2013) and in cultured mouse hepatocytes (> 80% within 3 h) (Bajt et al., 2004). Compared to the much more delayed GSH depletion in the metabolically competent human hepatoma cell line HepaRG (McGill et al., 2011), PHH responded very similar to mouse hepatocytes with a rapid GSH depletion of > 70% within 3 h. In addition, extensive protein adduct formation was observed in PHH with peak levels at 6 h. APAP protein adducts in mice and mouse hepatocytes peak around 2 h and 3 h, respectively (McGill et al., 2012b and McGill et al., 2013). More importantly, protein adducts in mitochondria, which are thought to initiate mitochondrial dysfunction, also peak at the same time in mouse cells (McGill et al., 2013 and Xie et al., 2013). However, mitochondrial protein formation was delayed in

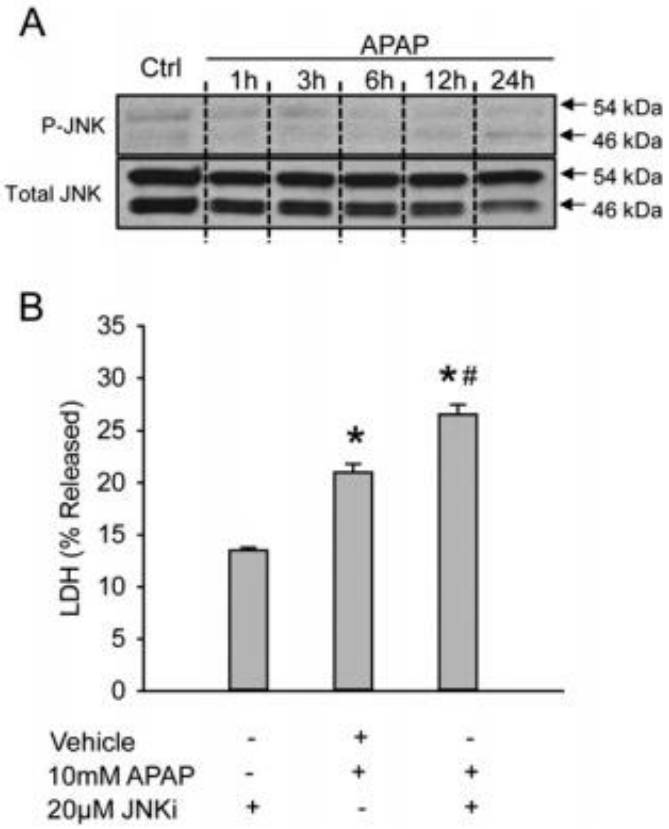


Figure. 2.4.6 No JNK activation in HepaRG cells after APAP and no protection using JNK inhibitor SP600125.

(A) Time course of JNK activation over 24 h in HepaRG cells. (B) Cell death as suggested by percentage of lactate dehydrogenase (LDH) released into the culture media at 24 h after APAP with or without JNK inhibitor SP600125 as a co-treatment. Data represent mean ± SE of n = 4. *$P < 0.05$ (compared with control), # $P < 0.05$ (compared with APAP group).

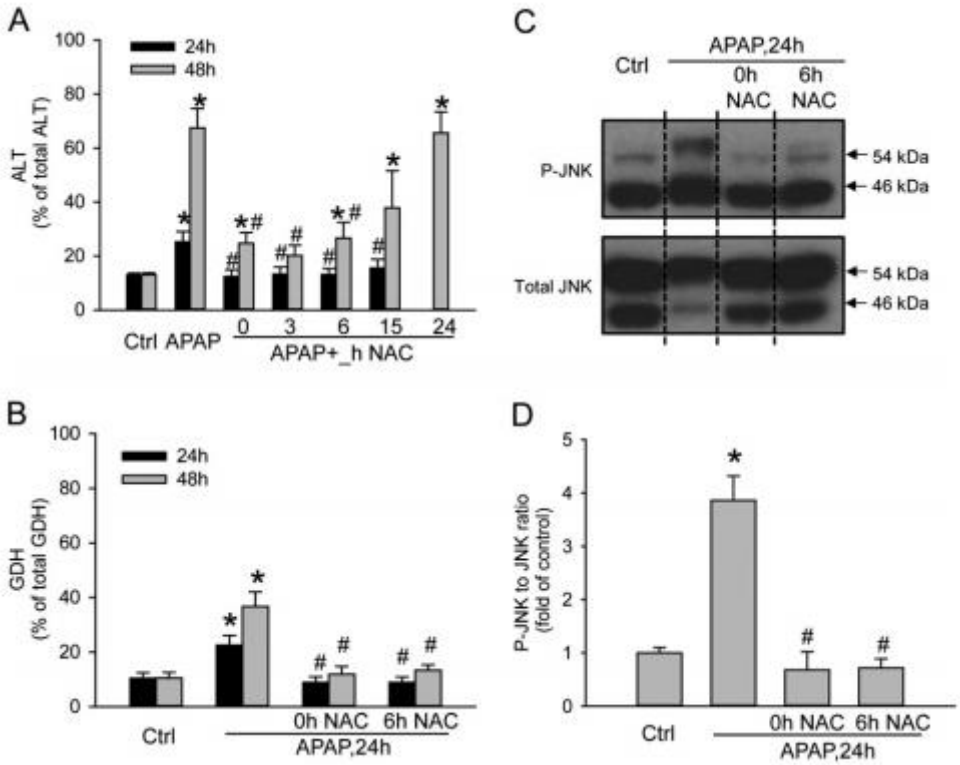


Figure 2.4.7 Protection by N-acetylcysteine (NAC) against APAP-induced hepatotoxicity in PHH.

20 mM NAC was applied at various time points after APAP and (A) ALT activity, (B) glutamate dehydrogenase (GDH) activity and (C–D) JNK activation was evaluated at 24 h or 48 h after APAP. (D) Densitometry. Data represent mean ± SE from experiments using cells from 3 to 7 donors. *P < 0.05 (compared with control), # P < 0.05 (compared with APAP group).

PHH compared to mouse hepatocytes and dramatically increased mainly after GSH depletion. Interestingly, on the basis of mg protein, APAP-Cys adducts levels were 1.5-to-3-times higher in PHH than in mouse hepatocytes or livers *in vivo*. Thus, while GSH depletion and total protein binding in response to APAP is only minimally delayed compared to mouse hepatocytes, two fundamental differences between PHH and mice are the delayed mitochondrial protein adduct formation and the overall higher protein binding in human cells. A potential reason for the different time line in mitochondrial adduct formation could be an involvement of mitochondrial cyp2e1 in the process (Knockaert et al., 2011). However, further studies are required to investigate this hypothesis in detail.

Mitochondrial dysfunction and JNK activation

It is generally accepted that the early protein adduct formation in mouse hepatocytes leads to mitochondrial oxidant stress, which triggers the activation of several MAP kinases, including apoptosis signal-regulating kinase 1 (Nakagawa et al., 2008), mixed lineage kinase 3 (Sharma et al., 2012), receptor interacting protein kinases 1 and 3 (Ramachandran et al., 2013 and Zhang et al., 2014), and glycogen synthase kinase-3 beta (Shinohara et al., 2010), all of which are thought to eventually trigger the downstream phosphorylation of JNK. P-JNK then translocates into mitochondria (Hanawa et al., 2008) and amplifies the mitochondrial oxidant stress (Saito et al., 2010a) to a level that can trigger MPT pore opening and cell necrosis (Kon et al., 2004). In PHH, JNK activation in the cytosol was delayed for several hours compared to mice, consistent with the delayed mitochondrial protein adduct formation. In addition, the mitochondrial translocation of P-JNK, which can be observed within 2–3 h after APAP exposure in mice (Hanawa et al.,

2008 and McGill et al., 2012b), was even more delayed in PHH. P-JNK translocation to mitochondria correlated with the collapse of the mitochondrial membrane potential followed by the onset of cell necrosis. Thus, many events observed in mouse hepatocytes in response to APAP also occur in PHH, but most of these events are substantially delayed and this may explain the delayed onset of necrosis in humans.

The critical role of JNK activation as an amplification system in the pathophysiology of APAP hepatotoxicity has been demonstrated by the protection provided by the JNK inhibitor SP600125 or knock-down of JNK genes *in vivo* (Gunawan et al., 2006 and Henderson et al., 2007). In contrast, the JNK inhibitor was only partially effective in cultured mouse hepatocytes, and only in the presence of the GSH synthesis inhibitor BSO (Gunawan et al., 2006). We have confirmed that the inhibitor was ineffective in protecting against APAP-induced cell death in cultured mouse hepatocytes (Y Xie and H Jaeschke, unpublished). Thus, the partial protection in PHH by SP600125 suggests that JNK activation is an important step in the pathophysiology in humans. However, the concentration of the inhibitor used in our experiments was sub-optimal. A limitation of SP600125 is the need to use DMSO as solvent, which can prevent APAP-induced hepatotoxicity due to inhibition of P450 enzymes even at very low concentrations and thus protect through off-target effects (Jaeschke et al., 2006). Interestingly, APAP did not cause JNK activation and SP600125 did not protect against APAP toxicity in HepaRG cells. Although we cannot rule out the possibility that hepatoma cells have altered signaling mechanisms, another explanation could be that other signaling pathways besides JNK are involved in the progression of injury. Recent data suggest that protein kinase C (PKC) inhibitors alleviated APAP-induced liver injury

both *in vivo* and *in vitro* (Saberi et al., 2014). However, a classical PKC inhibitor and knocking down of PKC-α protected through inhibition of JNK while broad-spectrum PKC inhibitors protected through a JNK-independent but AMPK-dependent pathway (Saberi et al., 2014). Preventing phosphorylation of AMPK by these PKC inhibitors led to activation of survival pathways such as autophagy (Saberi et al., 2014), which removes damaged mitochondria and protects against APAP-induced hepatotoxicity (Ni et al., 2012a-b). Thus, the JNK pathway is an important amplification mechanism that aggravates the oxidant stress and promotes APAP-induced cell death in murine and human hepatocytes but PKC-mediated signaling independent of JNK may also contribute to the pathophysiology.

Protection by the clinical antidote N-acetylcysteine in PHH

For more than 35 years, NAC has been the only clinically approved antidote against APAP poisoning (Polson and Lee, 2005). NAC is most effective when given within 8 h of APAP overdose, but has been shown to be partially beneficial even when administered up to 24 h after APAP exposure (Smilkstein et al., 1988). The mechanism of protection during the early, metabolism phase primarily involves improved scavenging of the reactive metabolite NAPQI due to accelerated GSH synthesis (Corcoran et al., 1985), while the later effects include scavenging of mitochondrial oxidant stress and support of mitochondrial energy metabolism (Saito et al., 2010b). The experiments with delayed NAC treatment of PHH demonstrated complete protection up to 6 h after APAP, and partial protection when the cells were treated at 15 h. These results closely resemble the clinical effectiveness of NAC. In contrast, delayed NAC treatment in mice or mouse hepatocytes is not effective beyond 2 h after APAP exposure, underscoring the much

more accelerated pathophysiology in the murine systems. Thus, despite the usefulness of mice and mouse hepatocytes to study APAP-induced cell death, our results demonstrate that freshly isolated PHH are clearly superior to animal models and human cell lines.

In summary, our results demonstrated rapid GSH depletion, formation of cellular and mitochondrial protein adducts, JNK activation and P-JNK translocation to mitochondria, loss of the mitochondrial membrane potential and cell necrosis in PHH after APAP exposure. Although the overall sequence of events was similar to mouse hepatocytes, there was clearly a substantial delay in formation of mitochondrial protein adducts, which occurred mainly after extensive GSH depletion. Similarly, JNK activation and translocation to the mitochondria were delayed. These events triggered the later loss of the mitochondrial membrane potential and cell necrosis. In addition, JNK inhibition was partially effective in PHH. Furthermore, NAC post-treatment was completely protective in PHH when administered as late as 6 h, and partially effective up to 15 h, after APAP. Overall, this timeline of APAP-induced cell death in PHH is very close to events observed in APAP overdose patients. Therefore, we can conclude that freshly isolated PHH are the best available model to study mechanisms of APAP-induced cell death and are the most relevant model for the human pathophysiology. Mouse hepatocytes or mice *in vivo* share many similarities with PHH, but differences in the time line of cell death and potentially in the details of signaling events are emerging. This means that it will be important to use PHH to test new therapeutic targets discovered in murine models before contemplating translating these findings into the clinic.

Chapter 3. N-acetyl-m-aminophenol (AMAP)-induced Hepatotoxicity in Primary Human Hepatocytes

This section is adapted from Xie et.al (2015), "Mitochondrial protein adducts formation and mitochondrial dysfunction during N-acetyl-m-aminophenol (AMAP)-induced hepatotoxicity in primary human hepatocytes", Toxicology and Applied Pharmacology, 289(2): 213–222, with permission from the publisher

3.1 Abstract

3′-Hydroxyacetanilide or N-acetyl-*meta*-aminophenol (AMAP) is generally regarded as a non-hepatotoxic analog of acetaminophen (APAP). Previous studies demonstrated the absence of toxicity after AMAP in mice, hamsters, primary mouse hepatocytes and several cell lines. In contrast, experiments with liver slices suggested that it may be toxic to human hepatocytes; however, the mechanism of toxicity is unclear. To explore this, we treated primary human hepatocytes (PHH) with AMAP or APAP for up to 48 h and measured several parameters to assess metabolism and injury. Although less toxic than APAP, AMAP dose-dependently triggered cell death in PHH as indicated by alanine aminotransferase (ALT) release and propidium iodide (PI) staining. Similar to APAP, AMAP also significantly depleted glutathione (GSH) in PHH and caused mitochondrial damage as indicated by glutamate dehydrogenase (GDH) release and the JC-1 assay. However, unlike APAP, AMAP treatment did not cause relevant c-jun-N-terminal kinase (JNK) activation in the cytosol or phospho-JNK translocation to mitochondria. To compare, AMAP toxicity was assessed in primary mouse hepatocytes (PMH). No cytotoxicity was observed as indicated by the lack of lactate dehydrogenase release and no PI staining. Furthermore, there was no GSH depletion or mitochondrial dysfunction after AMAP treatment in PMH. Immunoblotting for arylated proteins suggested that AMAP treatment caused extensive mitochondrial protein adduct formation in PHH but not in PMH. In conclusion, AMAP is hepatotoxic in PHH and the mechanism involves the formation of mitochondrial protein adducts and mitochondrial dysfunction.

3.2 Introduction

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′-Hydroxyacetanilide or N-acetyl-meta-aminophenol (AMAP) is a regioisomer of APAP and also possesses analgesic and antipyretic properties (Baker et al., 1963). However, unlike APAP, studies in mice (Tirmenstein and Nelson, 1989), hamsters (Roberts et al., 1990), primary mouse hepatocytes (Holme et al., 1991) and TAMH cells (Pierce et al., 2002) have suggested that AMAP is not hepatotoxic. Several groups have proposed possible mechanisms to explain the discrepancy in toxicity, and most of the evidence suggests a critical role for mitochondria. It was demonstrated that, although AMAP and APAP caused similar total cellular protein binding, there was significantly less mitochondrial protein binding after AMAP (Tirmenstein and Nelson, 1989 and Myers et al., 1995). Similarly, it was shown that the reactive metabolite of APAP extensively bound to mitochondrial proteins, while metabolites of AMAP preferentially bound to microsomal and cytosolic proteins (Tirmenstein and Nelson, 1989, Streeter et al., 1984, Matthews et al., 1997 and Qiu et al., 2001). APAP also triggered robust nitrotyrosine protein adduct formation in mitochondria (Cover et al., 2005), while there was no appreciable mitochondrial protein nitration after AMAP (Abdelmegeed et al., 2012). Consistent with those data, there is less cytosolic and mitochondrial GSH depletion after AMAP compared to APAP (Nelson, 1980, Tirmenstein and Nelson, 1989, Rashed et al., 1990 and Hanawa et al., 2008). Furthermore, the lack of JNK activation and translocation to mitochondria in mice after AMAP suggested that AMAP did not particularly target mitochondria (Hanawa et al., 2008). Together these data indicated that mitochondrial dysfunction and mitochondrial protein binding are critical in determining toxicity. A widely accepted hypothesis to explain the difference in mitochondrial effects is that the reactive metabolite(s) of AMAP is more active and not stable enough to escape

the site of formation in the endoplasmic reticulum (ER), leading to greater microsomal protein binding, while NAPQI can escape the ER and attack other organelles, including mitochondria (Rashed et al., 1990).

A recent study revisited AMAP toxicity in precision-cut liver slices and found that AMAP was as toxic as APAP based on ATP depletion in rat and human liver slices (Hadi et al., 2013). However, the mechanism of AMAP toxicity in human liver cells was not investigated. These findings prompted us to evaluate the differences in the mechanisms of action between AMAP and APAP in PHH, and to compare across species. A better understanding of AMAP toxicity in PHH could provide new insights into APAP-induced hepatotoxicity.

3.3 Materials and methods

Isolation and treatment of primary human hepatocytes (PHH)

All human tissues were acquired with informed consent from each patient according to ethical and institutional guidelines. The study was approved by the University of Kansas Medical Center Institutional Review Board. Table 3.4.1 provides a summary of medical information of donors. No donor tissue was obtained from executed prisoners or other institutionalized persons. All liver specimens were acquired in accordance with a Human Subject Committee approved protocol from patients undergoing a hepatic resection procedure or from donor livers. Hepatocytes were isolated as described in detail (Xie et al., 2014). Around 3 h after being seeded, cells were washed with sterile phosphate-buffered saline (PBS) and treated with AMAP or APAP. Cells were harvested at different time points according to assays.

Isolation and treatment of primary mouse hepatocytes (PMH)

Primary hepatocytes were isolated as described in detail (Bajt et al., 2004). Cell viability of each isolation was generally more than 90%, and hepatocyte purity was > 95%. Following isolation, primary hepatocytes were plated with a concentration of 6×105 cells per well and allowed 3 h to attach. The cells were then washed with PBS and exposed to AMAP or APAP. Cells were harvested at the indicated time points.

Biochemical assays

ALT activities were measured using an ALT reagent kit (Pointe Scientific, MI). LDH and GDH levels were determined as described (Xie et al., 2013 and Du et al., 2013). A

modified Tietze assay was used to measure glutathione levels as described (Jaeschke and Mitchell, 1990). The JC-1 Mitochondrial Membrane Potential Kit (Cell Technology, Mountain View, CA) was used to measure the mitochondrial membrane potential as described in detail (Bajt et al., 2004).

Propidium iodide (PI) staining

PHH and PMH were treated with AMAP or APAP 3 h after seeding. The medium was removed after 48 h (PHH) or 15 h (PMH) and cells were stained with 500 nM of PI (Invitrogen) for 5 min. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and images were obtained with a fluorescence microscope.

Isolation of subcellular fractions and Western blotting

Mitochondria and cytosolic fractions were isolated using differential centrifugation as described (Xie et al., 2014). Western blotting was performed as described (Bajt et al., 2000). A rabbit anti-JNK antibody and a rabbit anti-phospho-JNK antibody (Cell Signaling Technology, Danvers, MA) were used for JNK and phospho-JNK detection. A rabbit primary antibody was used to detect APAP or AMAP protein adducts. The antibody was raised against acetamidobenzoic acid and has been demonstrated to detect both APAP and AMAP protein adducts (Matthews et al., 1997 and Salminen et al., 1998).

Statistics

All results are expressed as mean ± standard error (SE). Comparisons between multiple groups were performed with one-way ANOVA followed by post hoc Student–Newman–

Keul's test. If the data were not normally distributed, the Kruskal–Wallis Test was used (nonparametric ANOVA) followed by Dunn's Multiple Comparisons Test. $P < 0.05$ was considered significant.

Table 3.4.1 Liver donor medical information

Donor	Source	Age	Gender	Diagnosis
1	Resection	41	Female	Metastatic colon cancer
2	Donor	25	Female	Head trauma
3	Donor	64	Female	Anoxia
4	Donor	38	Male	Head trauma
5	Donor	29	Female	CVA/stroke
6	Donor	25	Male	Cardiac arrest
7	Donor	13	Male	Asthma attack
8	Donor	50	Male	Head trauma
9	Donor	N/A	Male	Anoxia
10	Donor	19	Male	Head trauma
11	Donor	24	Female	Cardiac arrest
12	Donor	16	Male	Head trauma

CVA; cerebrovascular accident; N/A; not available.

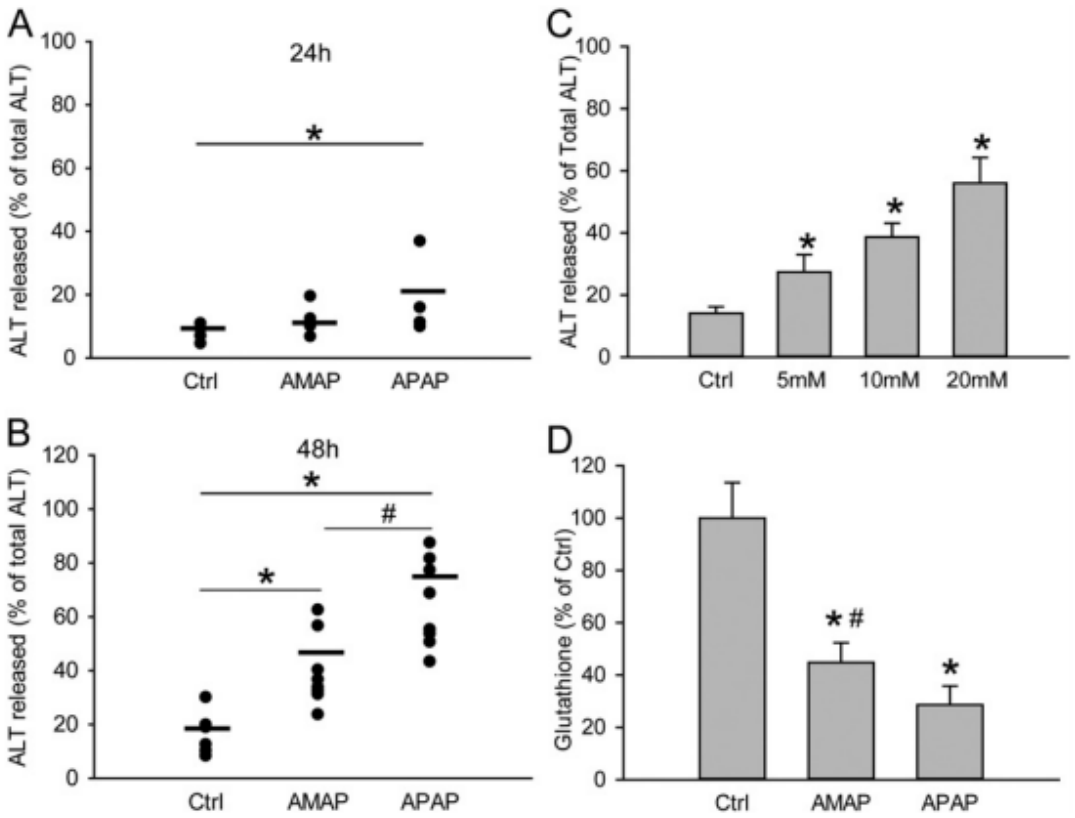


Figure 3.4.1 AMAP induces cell death and GSH depletion in human hepatocytes.

Primary human hepatocytes (PHH) were treated with 10 mM of AMAP or APAP over a period of 48 h, and the percentage of alanine aminotransferase (ALT) release was measured at 24 h (A) and 48 h (B) to evaluate toxicity. (C) Dose–response of AMAP toxicity at 48 h after treatment. (D) Glutathione depletion after 10 mM AMAP or APAP exposure for 24 h. Data represent mean ± SE from experiments using cells from 3 to 10 donors. *P < 0.05 (compared with controls); # P < 0.05 (compared with APAP group).

3. 4 Results

AMAP induces cell death and GSH depletion in primary human hepatocytes

Freshly isolated primary human hepatocytes (PHH) were treated with 10 mM AMAP or APAP for 48 h. APAP triggered significant cell death as indicated by the percentage of alanine aminot after treatment (Fig. 3.4.1A,B). AMAP did not cause significant cytotoxicity until 48 h (Fig. 3.4.1B). Consistent with that, toxicities of both AMAP and APAP at 48 h were confirmed by the cellular uptake of red fluorescent PI stain (Fig. 3.4.2). PI cannot enter live cells but upon membrane permeabilization during necrosis, the stain enters the cells and binds to nuclear DNA, which enhances the red fluorescence. An overlay of PI with the nuclear stain DAPI or the phase contrast images confirms the nuclear localization of the PI staining indicative of necrotic cell death in PHH after exposure to APAP and AMAP (Fig. 3.4.2). At both 24 h and 48 h, AMAP was less toxic than APAP (Fig. 3.4.1A, B). The hepatotoxicity of AMAP was dose-dependent (Fig. 3.4.1C). Exposure to 10 mM AMAP triggered substantial GSH depletion at 24 h, although this effect was less dramatic than after APAP (Fig. 3.4.1D). Overall, although AMAP has a reduced propensity to cause necrotic cell death compared to APAP, our data clearly show that it is toxic in PHH.

AMAP triggers mitochondrial dysfunction in PHH without JNK activation or P-JNK translocation to mitochondria

Given the importance of mitochondria in the toxicity after APAP in mice and humans, we hypothesized that AMAP caused mitochondrial dysfunction in PHH. To test this hypothesis, release of glutamate dehydrogenase (GDH), an enzyme localized in the

mitochondrial matrix that is released into the serum upon mitochondrial damage (McGill et al., 2012a), was evaluated. Significant release of GDH was observed in both AMAP- and APAP-treated hepatocytes at 48 h after treatment, and the effect of AMAP was dose-dependent (Fig. 3.4.3A,B). Consistent with that, the JC-1 assay revealed the significant loss of mitochondrial membrane potential after AMAP by a decline of the red-to-green fluorescence ratio, although the decrease was less extensive than after exposure to APAP (Fig. 3.4.3C). Importantly, compared with APAP, the less severe mitochondrial dysfunction after AMAP correlated with the lower ALT release and GSH depletion after AMAP compared to APAP (Fig. 3.4.1). JNK activation and translocation, which are critical amplifying events in APAP-induced hepatotoxicity, were also assessed after AMAP treatment (Fig. 3.4.4). At 6 and 24 h after AMAP exposure, an increase in total JNK compared to untreated cells was observed in the cytosol, which was paralleled by a modest increased expression of P-JNK (Fig. 3.4.4A). However, the ratio of P-JNK-to-total JNK did not change significantly before or after AMAP (Fig. 3.4.4C). Furthermore, there was no P-JNK translocation to mitochondria and only a minor JNK translocation at 24 h (Fig. 3.4.4B). Compared to APAP, JNK activation and P-JNK translocation to the mitochondria after AMAP is negligible (Fig. 3.4.4B). Taken together, AMAP causes significant mitochondrial dysfunction, but JNK activation and phospho-JNK translocation likely do not contribute to the cell death in PHH.

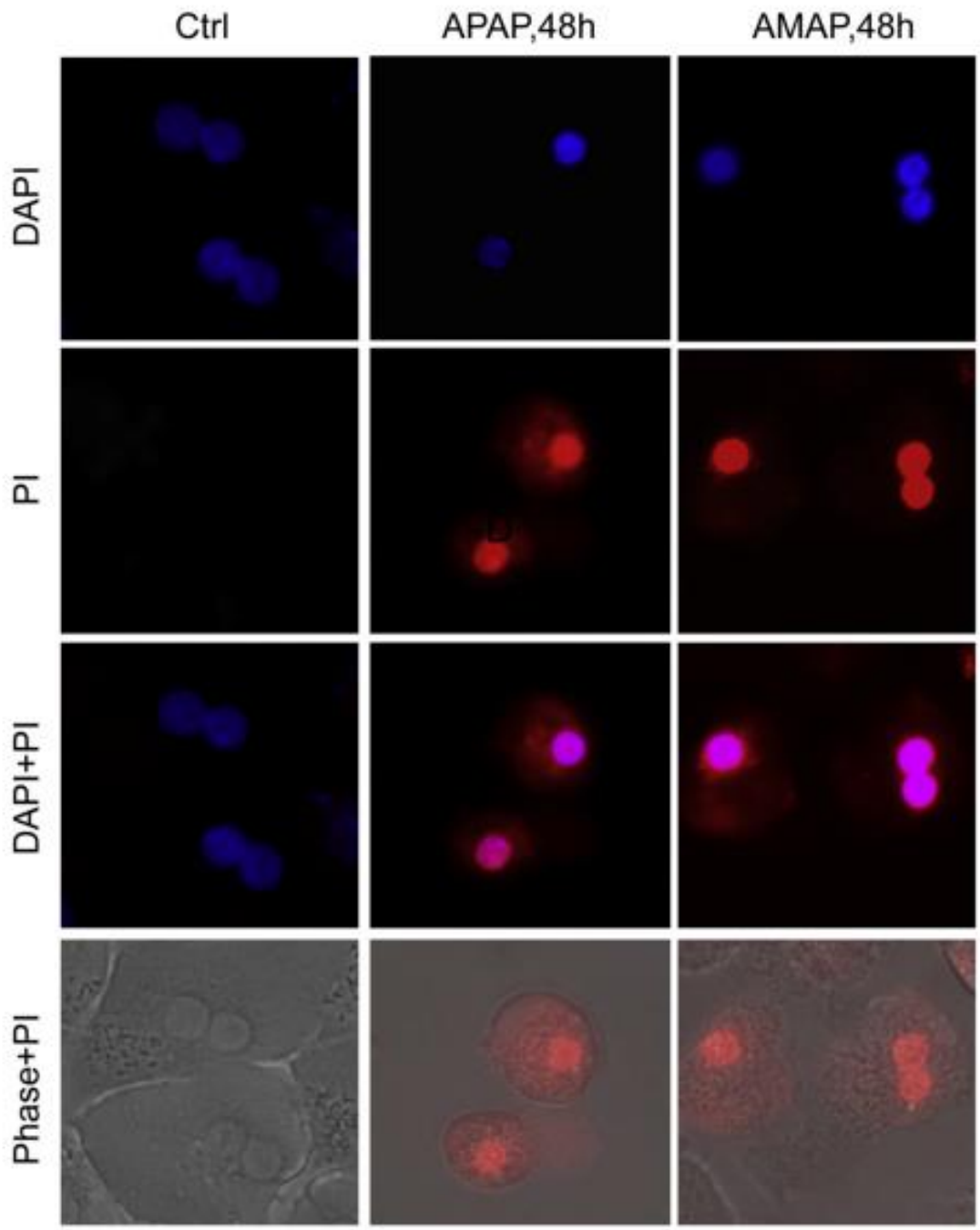


Figure. 3.4.2 AMAP and APAP induce necrosis in human hepatocytes.

Primary human hepatocytes (PHH) were treated with 10 mM of AMAP or APAP over a period of 48 h and necrotic cell death as indicated by nuclear PI staining was assessed. DAPI, 4′,6-Diamidino-2-Phenylindole (nuclear stain); PI, propidium iodide (nuclear stain of necrotic cells).

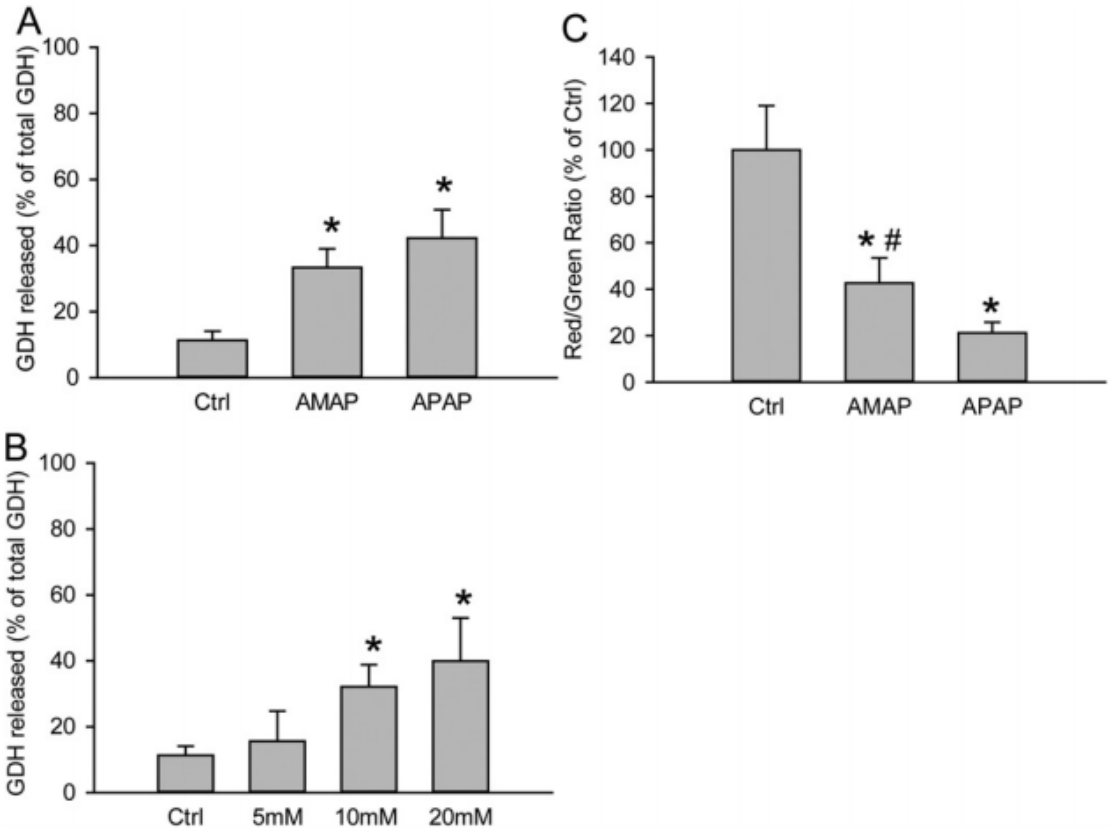


Figure 3.4.3 AMAP triggers mitochondrial dysfunction in PHH.

(A) Percentage of glutamate dehydrogenase (GDH) release into the culture medium at 48 h after 10 mM AMAP or APAP. (B) Dose– response of GDH release at 48 h after AMAP (5–20 mM). (C) Loss of mitochondria membrane potential after 24 h exposure to 10 mM AMAP or APAP as indicated by the decrease of the red/ green fluorescence ratio using the JC-1 assay. Data represent mean ± SE from experiments using cells from 8 donors. *P < 0.05 (compared with controls); # P < 0.05 (compared with APAP group).

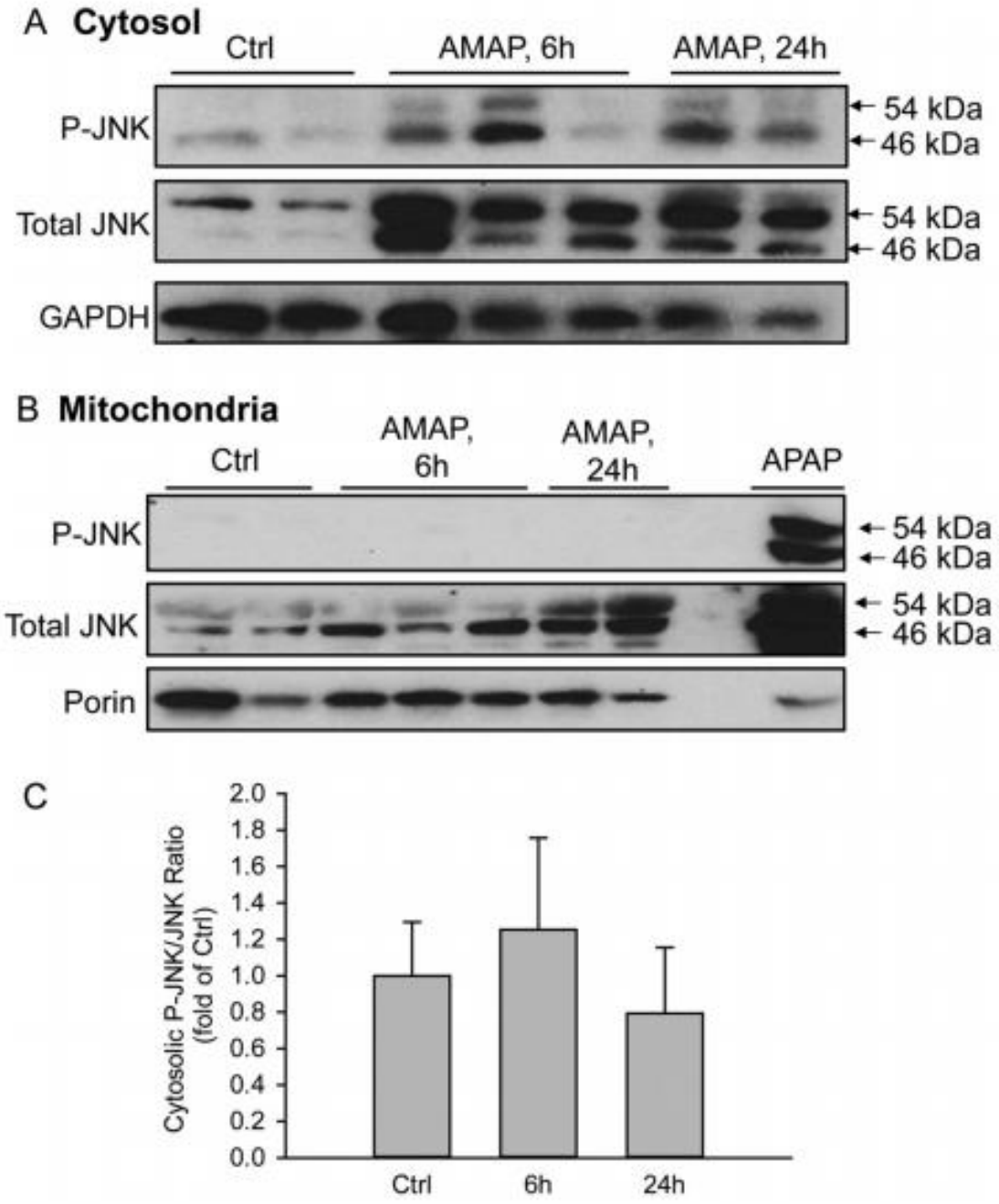


Figure 3.4.4 No JNK activation or mitochondrial translocation after AMAP in PHH.

JNK activation in the cytosol (A) and P-JNK translocation to the mitochondria (B) were evaluated by Western blotting. Mitochondrial fraction from APAP-treated PHH was used as positive control. (C) Densitometry of cytosolic JNK activation. Data represent mean ± SE from densitometry using cells from 3 donors.

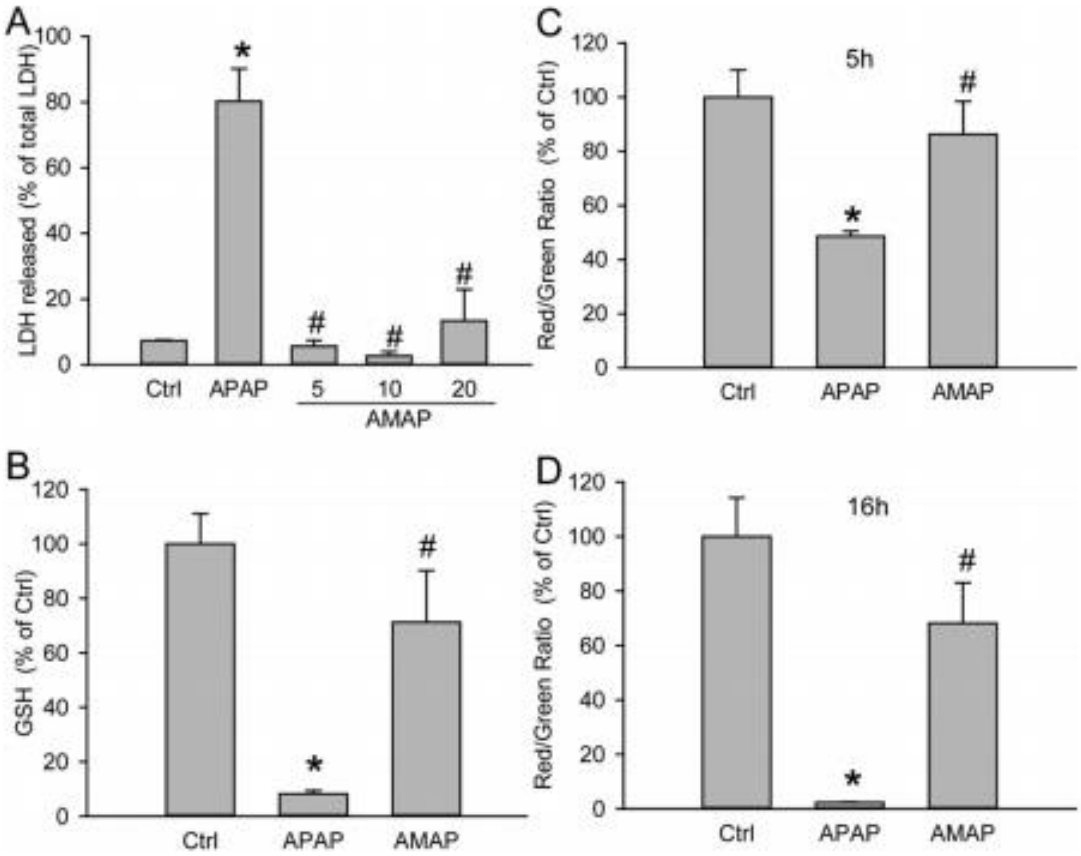


Figure 3.4.5 No significant mitochondrial dysfunction or cell death after AMAP in primary mouse hepatocytes (PMH).

(A) Lactate dehydrogenase was measured as an indicator of cell death after exposure to 5–20 mM AMAP or 5 mM APAP for 15 h. (B) GSH depletion at 5 h after 5 mM AMAP or APAP. Loss of mitochondrial membrane potential at 5 h (C) and 16 h (D) after AMAP and APAP were assessed by decrease of red/green fluorescence ratio using the JC-1assay. Data represent mean ± SE from experiments using primary mouse hepatocytes from 4 different isolations. *P < 0.05 (compared with controls); # P < 0.05 (compared with the APAP group).

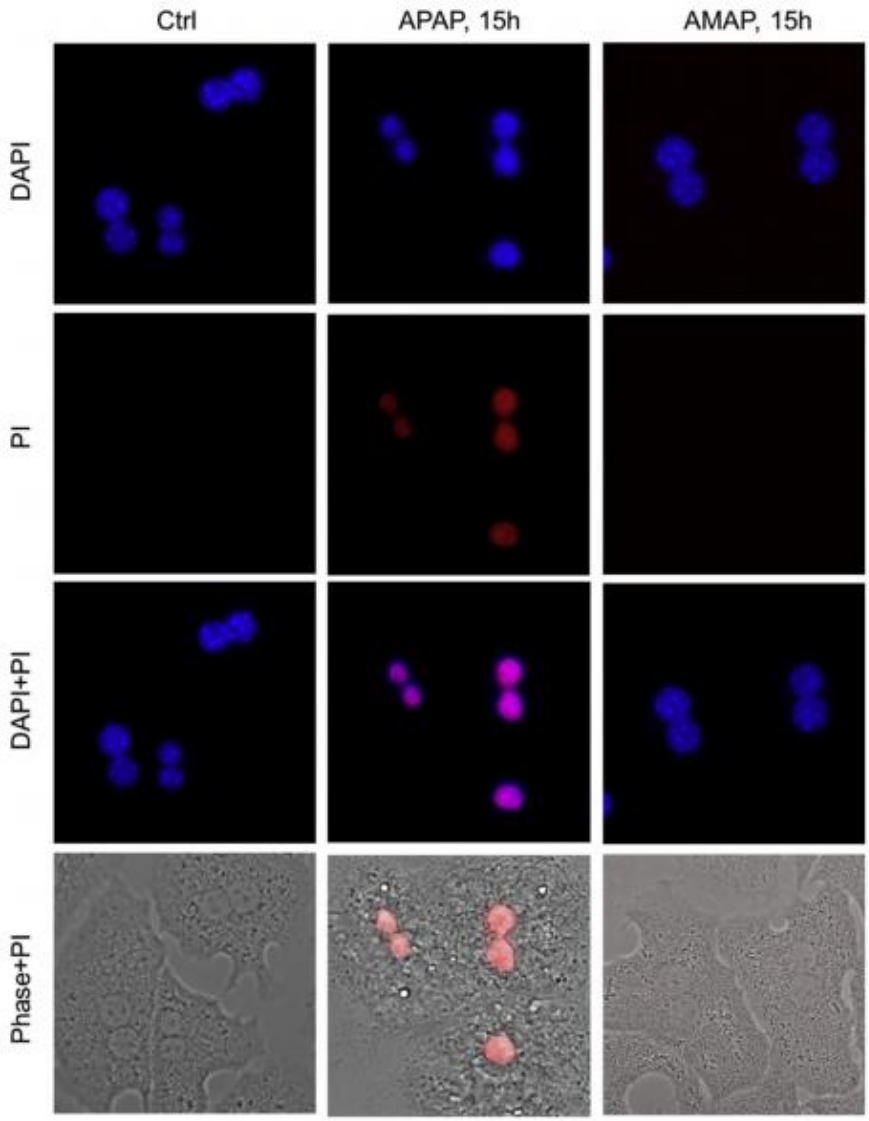


Figure 3.4.6 APAP but not AMAP caused cell necrosis in mouse hepatocytes.

Primary mouse hepatocytes (PMH) were treated with 5 mM of AMAP or APAP over a period of 15 h and necrotic cell death as indicated by nuclear PI staining was assessed. DAPI, 4′,6-Diamidino-2-Phenylindole (nuclear stain); PI, propidium iodide (nuclear stain of necrotic cells).

No injury or significant mitochondrial dysfunction after AMAP in primary mouse hepatocytes (PMH) under the conditions studied

For comparison, primary mouse hepatocytes were treated with 5 mM APAP or various concentrations of AMAP. Consistent with previous findings, AMAP was not significantly toxic even at 20 mM as demonstrated by the lack of lactate dehydrogenase (LDH) release.

However, 5 mM APAP resulted in extensive cell death (80% release of total LDH into culture medium) (Fig. 3.4.5A). The necrotic cell death after APAP was confirmed by cellular uptake of the red PI stain using immunofluorescent staining (Fig. 3.4.6). Consistent with the lack of LDH release, AMAP-treated cells did not show any cellular PI uptake. Overlay of PI with the nuclear stain DAPI or phase contrast images confirmed the nuclear localization of the PI stain in APAP but not AMAP-treated cells (Fig. 3.4.6). A similar trend was observed with GSH content, as exposure of AMAP had no significant effect on GSH levels while APAP caused 92% depletion of total GSH (Fig. 3.4.5B). In contrast to PHH, no mitochondrial dysfunction was observed in PMH at either 5 h or 16 h after AMAP (Fig. 3.4.5C, D). Based on these data we conclude that AMAP does not induce cell death or mitochondrial dysfunction in PMH under the conditions studied.

AMAP induces a significant increase of mitochondrial protein binding in PHH but not in PMH under the conditions studied

As mitochondrial protein binding is thought to be a key event in the initiation of APAP toxicity in mice, covalent binding in mitochondria from PHH was measured by immunoblotting for arylated proteins. An antibody against acetamidobenzoic acid was

used that can detect both APAP and AMAP protein adducts (Matthews et al., 1997 and Salminen et al., 1998). In PHH, extensive mitochondrial protein adducts formation was detected at 6 and 24 h after AMAP exposure (Fig. 3.4.7A,B). Note that the adduct bands consist of various proteins with molecular weights ranging from 30 to 250 kDa. A comparison to adduct detection in AMAP-treated PMH clearly indicates lower overall mitochondrial protein adducts formation in PMH than in PHH (Fig. 3.4.7A,B,C). On the other hand, a substantial number of mitochondrial protein adducts were observed in PMH after APAP treatment (Fig. 3.4.7A). The unexpected bands in the SDS control lane (Fig. 3.4.7A) were caused by SDS as this band was absent when only buffer was used (Supplemental Figure). Thus, despite less protein loading on the gel (as seen by the lower porin detection), APAP appeared to trigger much higher mitochondrial protein adducts formation than AMAP in PMH. Nevertheless, these data suggest that AMAP exposure results in significantly more mitochondrial covalent protein binding in PHH, which may be involved in AMAP-induced mitochondrial damage and hepatotoxicity in these cells.

3.5. Discussion

The aim of this study was to investigate the mechanisms of AMAP hepatotoxicity in freshly isolated PHH and PMH. In contrast to results in mice and PMH, we found that AMAP is toxic to PHH. Comparison between PHH and PMH suggested the importance of mitochondrial protein adducts formation and mitochondrial dysfunction in the toxicity.

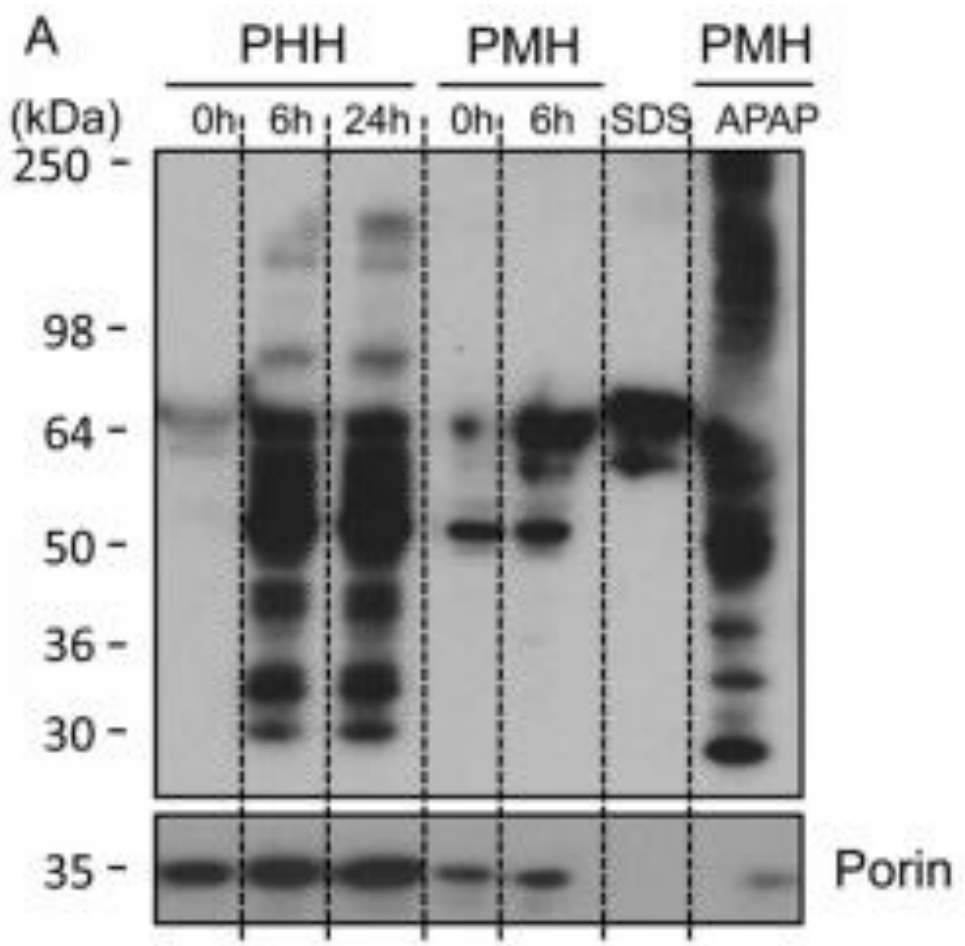


Figure 3.4.7 AMAP induces covalent protein binding in PHH but not PMH.

(A) Mitochondrial fractions were isolated from PHH and PMH which were treated with AMAP for 6 h or 24 h. Mitochondrial fraction from APAP-treated PMH was used as a positive control, and a blank (bl) lane loaded with only SDS loading buffer was used as a negative control. 0 h lanes represent mitochondria samples of control PHH or control PMH without any treatment. Porin was used as a loading control. (B) (C) Densitometry of adduct levels normalized to porin for PHH and PMH. The results of each 0 h lane were set to 1 and the data for AMAP-treated cells are expressed as fold increase over baseline. Data represent mean ± SE from experiments using primary human or mouse hepatocytes from 3 different isolations per group. *$P < 0.05$ (compared with corresponding controls).

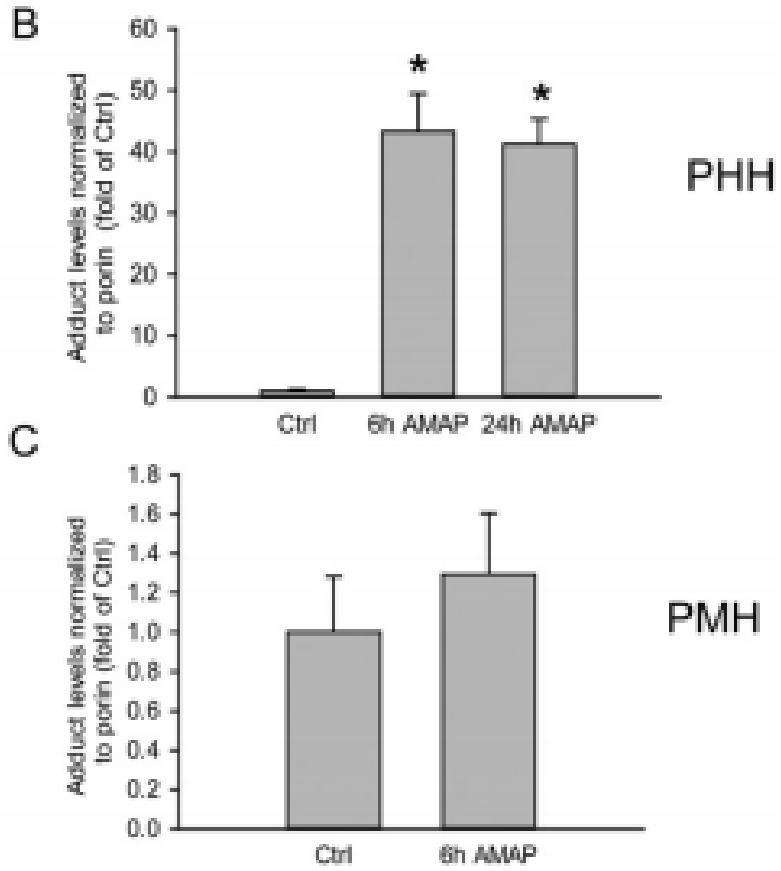
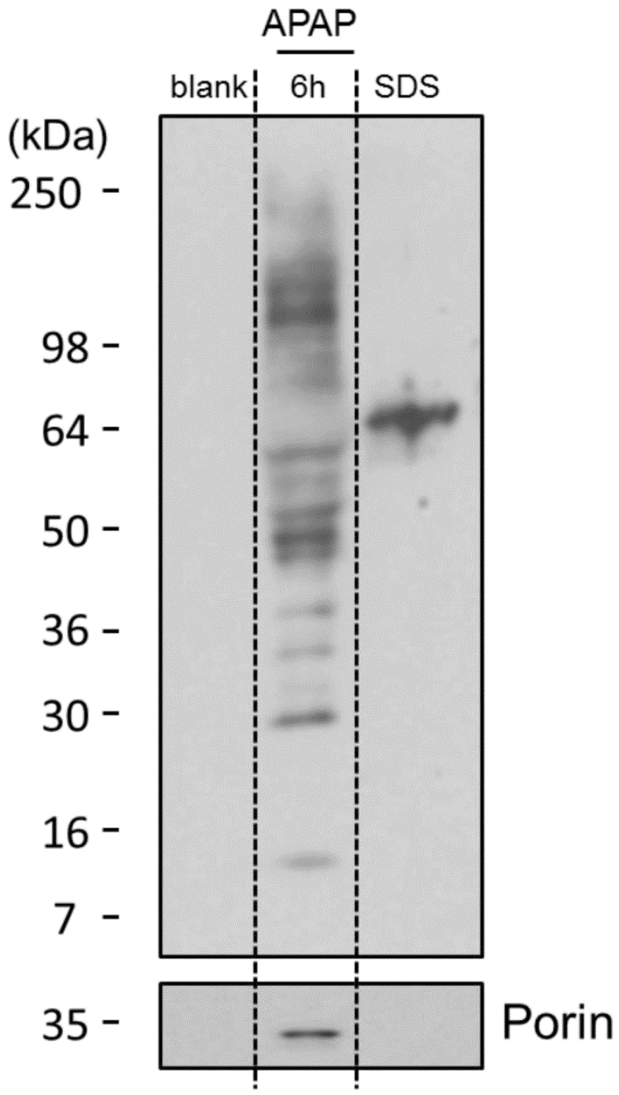


Figure 3.4.7 AMAP induces covalent protein binding in PHH but not PMH. (A) Mitochondrial fractions were isolated from PHH and PMH which were treated with AMAP for 6 h or 24 h. Mitochondrial fraction from APAP-treated PMH was used as a positive control, and a blank (bl) lane loaded with only SDS loading buffer was used as a negative control. 0 h lanes represent mitochondria samples of control PHH or control PMH without any treatment. Porin was used as a loading control. (B) (C) Densitometry of adduct levels normalized to porin for PHH and PMH. The results of each 0 h lane were set to 1 and the data for AMAP-treated cells are expressed as fold increase over baseline. Data represent mean ± SE from experiments using primary human or mouse hepatocytes from 3 different isolations per group. *$P < 0.05$ (compared with corresponding controls)



Supplemental Figure SDS buffer results in non-specific binding during western blotting.

To assess the cause of the non-specific bands observed in the SDS lane in Figure 7A, the western blot was repeated and SDS buffer was compared with SDS-free buffer. A mitochondrial fraction from primary mouse hepatocytes (PMH) treated with APAP for 6 h was loaded as a positive control.

Mitochondrial protein adducts formation in AMAP-induced hepatotoxicity

AMAP has long been considered a non-hepatotoxic analog of APAP. The main reason for the difference in toxicity in mice appears to be protein adducts formation. When directly compared to APAP, AMAP triggers comparable total protein adducts formation but induces significantly less mitochondrial adducts (Myers et al., 1995, Tirmenstein and Nelson, 1989 and Qiu et al., 2001). Therefore, it was concluded that mitochondrial protein adduct levels, rather than total cellular adduct levels, correlate with toxicity. The more recent data showing that AMAP can cause toxicity (ATP depletion) in human liver slices cast some doubt on this conclusion (Hadi et al., 2013). However, our data suggest that mitochondrial protein binding in fact correlates with toxicity in PHH, and the significant disparity in mitochondrial adduct levels readily explains the species difference in our study. Currently two approaches are commonly used for assessment of protein adducts formation after AMAP: detection of radiolabeled adducts and detection with antibodies. A comparison of the two methods indicate similar performance of adduct detection after AMAP either quantitatively or qualitatively. Importantly, both approaches suggest low levels of mitochondrial adducts in PMH (Myers et al., 1995). As antibody detection performs equally as well as radiolabeling, which is generally the gold standard method to detect protein adducts, the limited detection of mitochondrial protein adducts observed here in PMH after AMAP is most likely due to actually low levels of adducts. Admittedly, we cannot guarantee that this antibody is able to detect all mitochondrial adducts in mouse samples. However, since the antibody appears to detect large numbers of mitochondrial AMAP adducts in PHH, one would have to assume a dramatically different efficacy in detecting AMAP adducts in human versus mouse samples. This

appears unlikely. Nevertheless, alternative detection methods are necessary for further verification.

Although the mitochondrial adducts profile in PHH after AMAP treatment appears to be similar to that in PMH after APAP (Fig. 2.4.7), a more detailed analysis of the adducted proteins is needed to determine if similar proteins were adducted. Proteomic investigation in mice demonstrated that proteins modified by AMAP constitute a subgroup of proteins modified by APAP in that species (Fountoulakis et al., 2000). Other studies narrowed down the list of adducted proteins and indicated that microsomal proteins, especially Cyp2e1, are the main targets of AMAP (Myers et al., 1995, Matthews et al., 1997 and Halmes et al., 1998) (Halmes et al., 1998). However, that is beyond the scope of the current study. The fact that there appear to be more mitochondrial protein adducts after APAP correlates with the more severe mitochondrial dysfunction and higher cell death after APAP compared to AMAP under the conditions studied. In addition, previous data from a comparison of APAP toxicity in rats and mice suggest that the extent of mitochondrial protein binding correlates with liver injury (McGill et al., 2012b). Collectively, our data together with previous findings consistently support the idea that mitochondrial protein adduct levels correlate with hepatotoxicity.

Mitochondrial dysfunction in AMAP-induced hepatotoxicity

The critical role of mitochondria in APAP-induced hepatotoxicity has been well established (Jaeschke et al., 2012 and Jaeschke et al., 2013). Specifically, mitochondria are involved in APAP toxicity by covalent protein binding (Tirmenstein and Nelson, 1989 and McGill et al., 2012b), mitochondrial oxidative stress (Jaeschke, 1990) and

peroxynitrite formation (Cover et al., 2005), JNK activation and P-JNK translocation to mitochondria (Hanawa et al., 2008), oxidant stress amplification (Saito et al., 2010a), mitochondrial Bax pore formation (Bajt et al., 2008), the mitochondrial membrane permeability transition pore opening (Kon et al., 2004) and mitochondrial endonuclease translocation to the nucleus (Bajt et al., 2006). Similar mechanisms were observed in the metabolically competent human hepatoma cell line HepaRG (McGill et al., 2011) and in primary human hepatocytes (Xie et al., 2014). For AMAP, previous reports have shown a limited relevance of mitochondria. AMAP leads to microsomal protein binding rather than the mitochondrial protein binding observed after APAP (Tirmenstein and Nelson, 1989 and Rashed et al., 1990; Myers et.al., 1995). Compared with APAP, AMAP depletes total GSH and mitochondrial GSH to a lesser extent (Nelson 1980)(Nelson, 1980, Tirmenstein and Nelson, 1989, Rashed et al., 1990 and Hanawa et al., 2008), despite the fact that a number of glutathione conjugates were identified *in vitro* (Rashed and Nelson, 1989). In contrast to APAP, AMAP did not disrupt mitochondrial calcium homeostasis by impairing the capacity to sequester calcium (Tirmenstein and Nelson, 1989). Thus, consistent evidence in mice indicates that AMAP does not appear to target mitochondria in that species. However, pharmacological intervention using BSO to deplete mitochondrial GSH made mice more vulnerable to AMAP (Tirmenstein and Nelson, 1991). Under these conditions, there was more mitochondrial protein binding correlating with AMAP toxicity in mice (Tirmenstein and Nelson, 1991). Overall, this led to the hypothesis that metabolites of AMAP may be too reactive to reach mitochondria under physiological conditions in mice (Rashed et al., 1990 and Myers et al., 1995). Our data are consistent with the hypothesis that AMAP triggered mitochondrial dysfunction

as a result of mitochondrial protein binding in PHH and the resulting mitochondrial damage likely led to cell death. This suggests more of a species difference in AMAP toxicity rather than a totally different mechanism. In support of this hypothesis, we have previously shown that the species difference in the time course of APAP toxicity between mice and humans is caused by the much more delayed protein adducts formation, especially in mitochondria, leading to delayed oxidant stress and JNK activation (Xie et al., 2014). In addition, the apparently nontoxic APAP analog, 2,6-dimethyl APAP, (2,6-DMA) was cytotoxic in PMH from phenobarbital-pretreated mice (Birge et al., 1989). Although the authors did not specifically assess mitochondrial protein adducts, the cytotoxicity of 2,6-DMA in PMH correlated with enhanced protein binding (Birge et al., 1989). These examples suggest that the cytotoxicity of AMAP or 2,6-DMA in PMH is mainly dependent on the experimental conditions. However, whether these difference in AMAP toxicity is only caused by a different metabolite profile and spectrum of adducts formed in human compared to mouse cells or if there are other factors involved remains to be investigated.

The metabolism profile of AMAP is different between mice and humans. In mice, around 40% of AMAP is eliminated either unaltered or conjugated with glucuronic acid or sulfate (Rashed et al., 1990). The remaining AMAP is metabolized by P450 enzymes to 3 major proximate metabolites which are 2,5-diOH-acetanilide, 3-OH-APAP and 3-OMe-APAP. These hydroquinones can be conjugated with glucuronic acid or sulfate, or they could be further converted to reactive quinones, which bind to GSH or attack cellular proteins (Kenna 2013). In precision-cut liver slices, it was reported that sulfation is the predominant pathway for AMAP in mouse livers, while glucuronidation prevails in

human liver slices (Hadi et al., 2013). Besides, levels of three hydroquinones, which are precursors of the toxic quinones differ significantly in mice and humans (Hadi et al., 2013). It is also possible that different cytochrome P450s are involved in AMAP metabolism in different models. Future investigations regarding the species differences are necessary to elucidate the different mechanisms of actions.

JNK activation and AMAP-induced hepatotoxicity

Interestingly, no P-JNK translocation to the mitochondria was observed in PHH after AMAP treatment. After APAP overdose, JNK activation and mitochondrial P-JNK translocation are critical for the mechanism of liver injury in mice (Hanawa et al., 2008). In addition, knock-down or inhibition of many kinases, including apoptosis signal-regulating kinase 1 (Nakagawa et al., 2008 and Xie et al., 2015c), mixed-lineage kinase 3 (Sharma et al., 2012) and glycogen synthase kinase-3beta (Shinohara et al., 2010) has revealed that they are involved in activation of JNK and translocation of P-JNK to mitochondria, which are critical amplifying events for the initial oxidative stress. For AMAP, previous studies demonstrated the absence of JNK activation in other models after AMAP treatment. In TGF-alpha transgenic mouse hepatocytes (TAMH), which exhibited susceptibility to APAP and resistance to AMAP, AMAP induced less JNK and c-Jun activation compared to APAP (Stamper et al., 2010). In fact, JNK siRNA had no effect on cell death after AMAP while it was able to attenuate the cytotoxicity after APAP (Stamper et al., 2010). Similarly, in mice there is no JNK activation or mitochondrial translocation after AMAP (Hanawa et al., 2008). However, there was no toxicity after AMAP in these models. In contrast, our data indicate AMAP hepatotoxicity in PHH without relevant JNK activation and mitochondrial P-JNK translocation

suggesting cell death in the absence of JNK activation. Interestingly, even in APAP-induced liver injury, JNK activation is not always necessary for the cell death mechanisms. In HepaRG cells, APAP triggers mitochondrial dysfunction and cell death without JNK activation (McGill et al., 2011 and Xie et al., 2014). In PHH hepatocytes, APAP causes JNK activation but JNK inhibitors are only moderately protective (Xie et al., 2014). Similarly, PKC inhibitors attenuate APAP-induced liver injury in mice by preventing phosphorylation of AMPK and by activating autophagy in a JNK-independent way (Saberi et al., 2014). Together these data suggest that although JNK is important for most models of APAP hepatotoxicity, especially in mice, it does not contribute to cell death after AMAP in PHH.

In summary, our results demonstrated the hepatotoxicity of AMAP in PHH. The cell death was preceded by GSH depletion and loss of mitochondrial membrane potential. JNK activation was likely not involved in AMAP toxicity. In contrast, no GSH depletion, mitochondrial dysfunction or cell death was observed in PMH after AMAP. Comparison between PHH and PMH revealed that AMAP induces a significant increase of mitochondrial protein binding in PHH but not in PMH under the conditions studied. Our data emphasize the importance of mitochondrial dysfunction and sustained mitochondrial protein binding in determining hepatotoxicity in human hepatocytes after AMAP treatment, and also indicate that species differences in protein adduct formation should be considered when evaluating the toxic potential of a compound.

Chapter 4. Lack of Direct Cytotoxicity of Extracellular ATP against Hepatocytes: Role in the Mechanism of Acetaminophen Hepatotoxicity

This section is adapted from Xie et.al (2015), "Lack of direct cytotoxicity of extracellular ATP against hepatocytes: role in the mechanism of acetaminophen hepatotoxicity", Journal of clinical and translational research, 1(2): 100–106, with permission from the publisher

4.1 Abstract

Background: Acetaminophen (APAP) hepatotoxicity is a major cause of acute liver failure in many countries. Mechanistic studies in mice and humans have implicated formation of a reactive metabolite, mitochondrial dysfunction and oxidant stress as critical events in the pathophysiology of APAP-induced liver cell death. It was recently suggested that ATP released from necrotic cells can directly cause cell death in mouse hepatocytes and in a hepatoma cell line (HepG2).

Aim: To assess if ATP can directly cause cell toxicity in hepatocytes and evaluate their relevance in the human system. Methods: Primary mouse hepatocytes, human HepG2 cells, the metabolically competent human HepaRG cell line and freshly isolated primary human hepatocytes were exposed to 10-100 μM ATP or ATγPin the presence or absence of 5-10 mM APAP for 9-24 h.

Results: ATP or ATγP was unable to directly cause cell toxicity in all 4 types of hepatocytes. In addition, ATP did not enhance APAP-induced cell death observed in primary mouse or human hepatocytes, or in HepaRG cells as measured by LDH release and by propidium iodide staining in primary mouse hepatocytes. Furthermore, addition of ATP did not cause mitochondrial dysfunction or enhance APAP-induced mitochondrial dysfunction in primary murine hepatocytes, although ATP did cause cell death in murine RAW macrophages.

Conclusions: It is unlikely that ATP released from necrotic cells can significantly affect cell death in human or mouse liver during APAP hepatotoxicity. Relevance for patients: Understanding the mechanisms of APAP-induced cell injury is critical for identifying

novel therapeutic targets to prevent liver injury and acute liver failure in APAP overdose patients.

4.2 Introduction

Acetaminophen, a safe analgesic drug at therapeutic doses, can induce severe liver injury and even liver failure after overdose in experimental animals and in humans (Larson, 2007, McGill et al., 2012a). Although the focus of pathophysiological studies during the last four decades was on intracellular signaling mechanisms (Nelson, 1990; Cohen et al., 1997; Jaeschke et al., 2012a), in recent years a contribution of the innate immune response has been discussed (Jaeschke et al., 2012b). A sterile inflammatory response is activated by release of damage-associated molecular patterns (DAMPs) such as nuclear DNA fragments, mitochondrial DNA, high mobility group box protein 1 protein (HMGB1) and ATP from necrotic hepatocytes (McGill et al., 2012a; Martin-Murphy et al., 2010; Antoine et al., 2012). The activation of toll-like receptors on macrophages by DAMPs leads to the transcriptional activation of pro-inflammatory cytokines including pro-IL-1β. In addition, stimulation of purinergic receptors by ATP activates the Nalp3 inflammasome, triggering the activation of caspase-1 which processes the pro-IL-1β to the active cytokine (Hoque et al., 2012). Although we could not confirm a role of caspase-1, the Nalp3 inflammasome and IL-1β (Williams et al., 2010b, 2011), or the purinergic receptor P2X7 (Xie et al., 2013) during APAP hepatotoxicity, a recent paper introduced a novel hypothesis for the role of ATP released by necrotic cells. Amaral et al (2013) provided evidence that human hepatoma cells (HepG2) exposed to APAP release ATP into the culture supernatant. In addition, the authors further demonstrated that

primary mouse hepatocytes and HepG2 cells can be directly killed by exposure to ATP at concentrations between 10 and 100 μM (Amaral et al., 2013). Thus, in addition to activating the inflammasome, ATP may directly contribute to aggravation of APAP-induced liver injury as a cytotoxic agent (Amaral et al., 2013). This novel concept would support a previous hypothesis that "death factors" released by necrotic cells are involved in the expansion of the original injury (Mehendale, 2012). If this hypothesis can be supported, any ATP leaking out of necrotic cells could be potentially dangerous for neighboring cells. This novel hypothesis could have a substantial impact not only on the mechanism of APAP hepatotoxicity but also on a large number of other liver disease models, e.g. ischemia-reperfusion injury, where extensive necrosis occurs (Jaeschke, 2003). Therefore, the objective of the current study was to determine if ATP is directly cytotoxic in 4 different cell culture models: primary cultured mouse and human hepatocytes and a metabolically competent (HepaRG) and a metabolically deficient (HepG2) hepatoma cell line. Alternatively, it was hypothesized that ATP could aggravate cell death during APAP hepatotoxicity.

4.3 Materials and Methods

HepaRG and HepG2 cell lines

HepaRG cells were acquired from Biopredic International (Rennes, France).The detailed process of HepaRG cell culture was described previously (McGill et al., 2011). HepG2 cells obtained from ATCC (Manassas, VA, USA) were cultured in DMSO (dimethyl sulfoxide)-free Williams' E medium containing penicillin/streptomycin, insulin and 10% fetal bovine serum, and were 70%~80% confluent before treatment. Both cell lines were treated with 10 mM APAP (Sigma, St. Louis, MO, USA) dissolved in warm Williams' E medium. ATP (Sigma) was dissolved in saline and added to the cell culture medium to a final concentration of 10 μM or 100 μM. Twenty-four hours after treatment of either ATP and/or APAP, medium and cell fractions were harvested, and cell viability was determined using the lactate dehydrogenase (LDH) assay (Bajt et al., 2004).

Primary mouse hepatocytes

Primary mouse hepatocytes were isolated by a two-step isolation procedure as previously described in detail (Bajt, et.al, 2004). The animals used were 8 week old male C57Bl/6 mice (Jackson Laboratories, Bar Habor, Maine, USA). Animals were acclimatized for at least 3 days and fasted overnight before cell isolation. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. Generally, cell viability of each isolation was more than 90%, and cell purity was >95% hepatocytes. Cells were treated with 5 mM APAP, which was dissolved in warm Williams' E medium. ATP was dissolved in saline and added to the cell culture medium to a final concentration of 10 μM, 100 μM, 1 mM or 10 mM. All

cells and supernatants were harvested 9 h or 24 h after the treatment. The LDH assay was carried out to assess the cell death.

Primary human hepatocytes

Freshly isolated primary human hepatocytes were obtained from the Biospecimen Core in the Department of Pharmacology, Toxicology and Therapeutics at The University of Kansas Medical Center. The liver sources were either unused tissue from donor livers or material from liver resections. All human tissues were obtained with informed consent according to ethical and institutional guidelines. Hepatocytes were isolated as previously described (Gramignoli et. al, 2012) using a 3 step process and the dissociation enzymes collagenase (Vitacyte, Indianapolis, Indiana, USA) and protease (Vitacyte). Human hepatocytes were plated in Hepatocyte Maintenance Medium (HMM, Lonza Group, Basel, Switzerland) supplemented with dexamethasone, insulin and gentamicin/amphotericin-B (Lonza) and 5% newborn calf serum (Atlanta Biologicals, Lawrenceville, Georgia, USA) was used to maintain the hepatocytes. The hepatocytes were allowed to attach for 2 hours in a 37 ℃, 5% CO_2 incubator before treatment. Cells were treated with 10 mM APAP dissolved in warm HMM, and all cells were harvested at 24 h post-treatment. For ATP treatment, ATP was dissolved in saline and added to the cell culture medium to a final concentration of 10 μM or 100 μM. After cell harvesting, alanine aminotransferase (ALT) activities were measured in cells and the supernatant using an ALT reagent kit (Pointe Scientific, Michigan, USA).

Statistics

All results were expressed as mean ± SE. Comparisons between multiple groups were performed with one-way ANOVA followed by a *post hoc* Bonferroni test. If the data were not normally distributed, we used the Kruskal-Wallis Test (nonparametric ANOVA) followed by Dunn's Multiple Comparisons Test. $P < 0.05$ was considered significant.

4.4 Results

Primary mouse hepatocytes

Previous results showed that cultured hepatocytes release ATP into the supernatant after exposure to 20 mM APAP resulting in concentrations of up to 10 µM (Amaral et al., 2013). Therefore, primary mouse hepatocytes were exposed to 10 or 100 µM ATP in the presence or absence of 5 mM APAP for 9 h (Figure 1).

Based on LDH release as indicator for cell necrosis (Bajt et al., 2004), ATP alone did not cause more cell death compared to untreated mouse hepatocytes. In addition, ATP did not trigger additional necrosis in cells exposed to APAP (Figure 1A). Similar results were obtained when JC-1 fluorescence was measured as indicator of the mitochondrial membrane potential (Figure 1B). ATP alone did not cause mitochondrial dysfunction and ATP did not aggravate APAP-induced loss of the mitochondrial membrane potential (Figure 1B). These results were similar to experiments with ATP concentrations of up to 10 mM and longer time points (24 h) (data not shown). To confirm these results, propidium iodide staining was performed to assess the number of necrotic cells (Figure 2). No differences were observed between the ATP and control groups or the APAP and APAP + ATP groups; the overall cell death was consistent with previous results. Finally, a non-hydrolyzable form of ATP (ATγP) was used in place of ATP as described by Amaral et al. (2013) and LDH was measured to assess cell death (Figure 3). The use of the ATγP yielded the same results, as no increase in cell death was found with ATγP alone or with co-treatment of ATγP and APAP (Figure 3).

Primary human hepatocytes and hepatoma lines

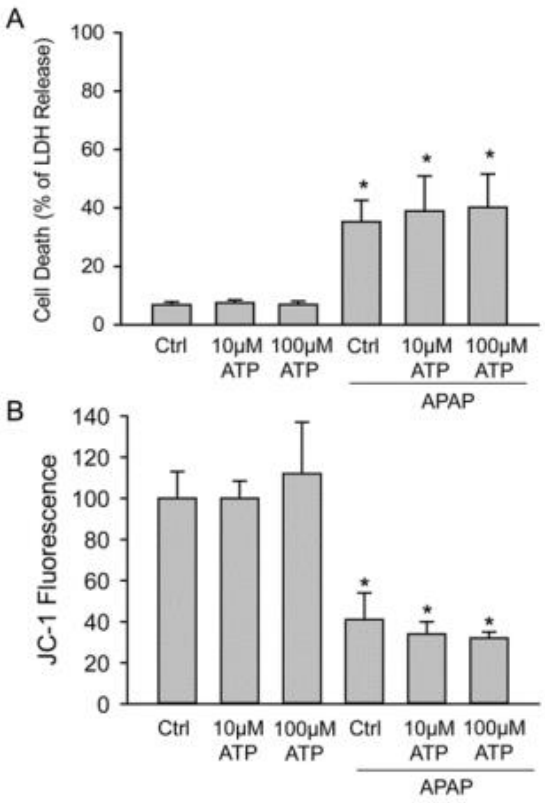


Figure 4.4.1 Effect of ATP on primary mouse hepatocytes.

Primary mouse hepatocytes were treated with 5 mM acetaminophen dissolved in Williams' E medium, and cell death was assessed by the percentage of lactate dehydrogenase (LDH) released into the medium from the cells at 9 h after the treatment (A). ATP was freshly dissolved in saline and added to the cell culture medium to a final concentration of 10 μM or 100 μM at the time of APAP treatment. JC-1 fluorescence was measured to assess mitochondrial dysfunction (B). Data represent mean ± SE of n=4 different mouse hepatocytes isolations *p <0.05 (compared to corresponding groups without APAP).

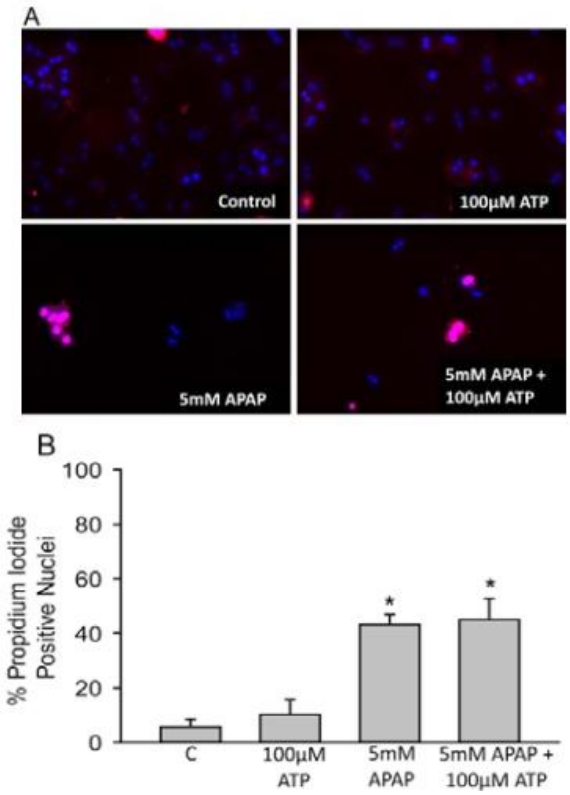


Figure 4.4.2 ATP does not increase cell death in murine hepatocytes exposed to APAP.

Primary mouse hepatocytes were treated with ATP in the presence or absence of 5 mM acetaminophen and cell death was assessed by propidium iodide/DAPI staining (A). Quantification was done by counting PI-positive cells in four different fields per well from different cell isolations. Data represent mean ± SE of n = 3 different mouse hepatocytes isolations *p <0.05 (compared to corresponding groups without APAP).

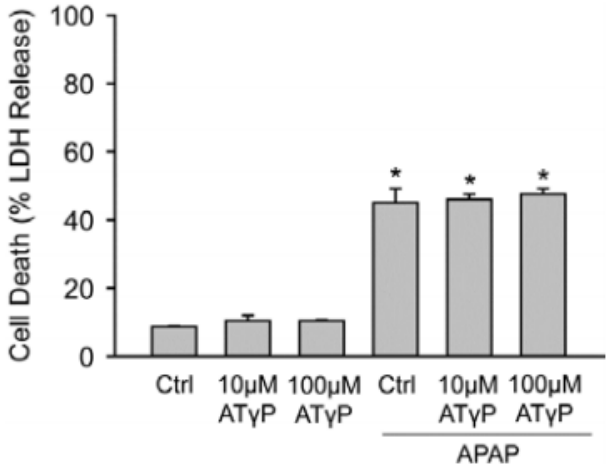


Figure 4.4.3 Non-hydrolyzable ATP (ATγP) does not increase cell death in hepatocytes.

Primary mouse hepatocytes were treated with 10 μM or 100 μM ATγP in the presence or absence of 5 mM acetaminophen and cell death was assessed by LDH release. Data represent mean ± SE of n = 3 different mouse hepatocytes isolations *$p<0.05$ (compared to corresponding groups without APAP).

Using freshly isolated primary human hepatocytes, a moderate degree of necrosis (ALT release) was observed after exposure to 10 mM APAP for 24 h as compared to untreated cells (controls) (Figure 4A). Similar to primary mouse hepatocytes, exposure to ATP did not cause any additional cell death in human hepatocytes compared to untreated cells or compared to APAP-treated cells (Figure 4A). The same results were obtained in the metabolically competent HepaRG cell line cultured with the same concentrations of APAP and ATP; again ATP failed to increase cell death in any treatment (Figure 4B). Because previous studies showed evidence of ATP cytotoxicity in HepG2 cells (Amaral et al., 2013), HepG2 cells were exposed to ATP in the presence or absence of 10 mM APAP. Similar to our previous findings (McGill et al., 2011), APAP did not cause cell death in HepG2 cells (Figure 4C). More importantly, neither 10 nor 100 μM ATP caused cell death in control HepG2 cells or in cells exposed to APAP (Figure 4C).

Cytotoxicity of ATP in the macrophage cell line RAW264.7

We measured ATP based-cytotoxicity in a macrophage cell line that expressed purinergic receptors (Kawano et al., 2012).Stimulating macrophages with ATP at concentrations up to 200 μM produced no toxicity with an incubation time of 9 h; however, longer incubation times (72 h) produced significant toxicity at a concentration of 200 μM (Figure 5). While these concentrations are extremely high and certainly well beyond even pathophysiological concentrations, these data do indicate that ATP is both stable and active *in vitro*, and can produce toxicity given long incubation times and higher concentrations. These data are supported by previous studies on the biological activity and the stability of ATP using a hexokinase assay (Heger et al., 2010; Xie 2015).

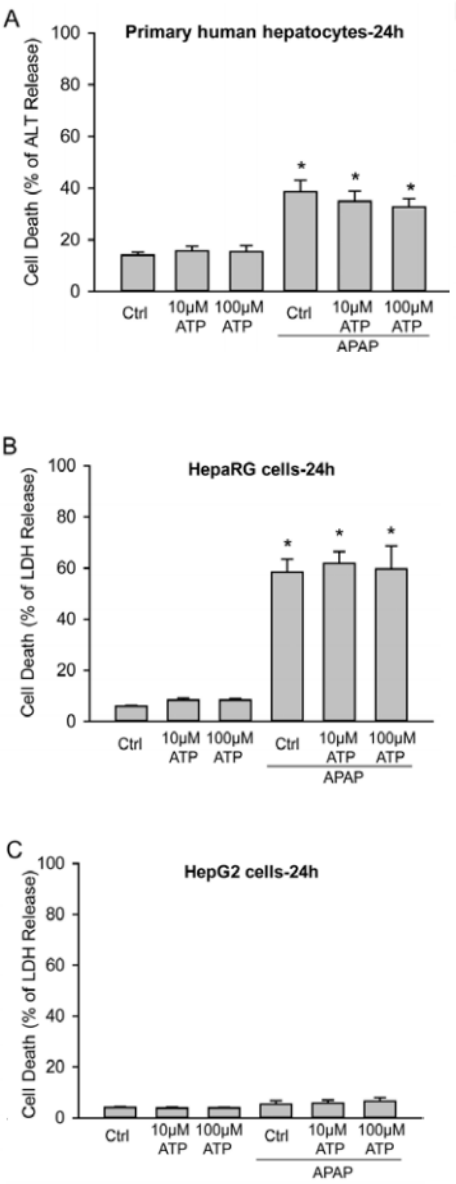


Figure 4.4.4 ATP does not increase cell death in human hepatocytes or hepatoma cell lines.

Primary human hepatocytes (A), HepaRG cells (B) or HepG2 cells were treated with ATP in the presence or absence of 5 mM acetaminophen and cell death was assessed by ALT or LDH release. Data represent mean ± SE of n=3 different HepaRG cell differentiations, n=3 HepG2 cell preparations and n=8 human hepatocyte isolations. * $p<0.05$ (compared to corresponding groups without APAP).

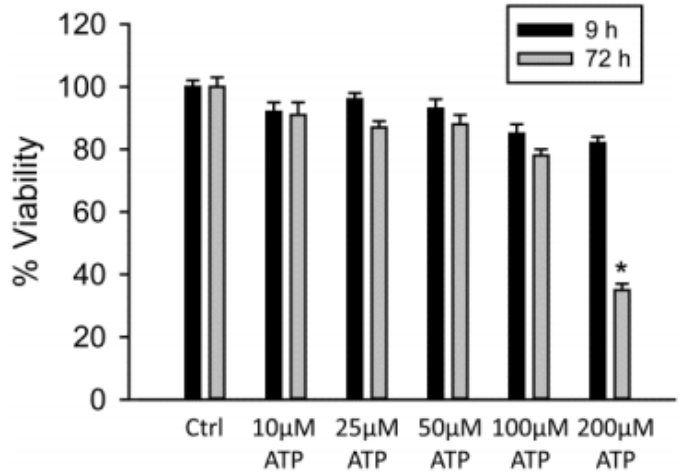


Figure 4.4.5 Cytotoxicity of ATP in RAW 264.7 macrophages.

RAW macrophages were exposed to increasing concentrations of ATP for 9 or 72 h, respectively, and then cell viability was measured via the SRB test. Data represent mean SE of n=4 different experiments. *$p<0.05$ (compared to Ctrl).

4.5 Discussion

The objective of this investigation was to determine if ATP can act as a direct cytotoxic agent against hepatocytes or can aggravate APAP-induced cell death. This hypothesis was tested in 4 different types of hepatocytes, i.e., primary mouse hepatocytes, primary human hepatocytes, a metabolically competent human hepatoma cell line (HepaRG) and a human hepatoma cell line that does not express CYPs or other drug metabolizing enzymes (HepG2). Consistent in all 4 cell types, various doses of ATP were unable to directly cause cell death and were also not able to enhance APAP-induced cell necrosis; ATγP, a non-hydrolyzable form of ATP, was equally ineffective to increase cell death in murine hepatocytes. In contrast to the different types of hepatocytes, ATP caused cell death in macrophages at high concentrations.

ATP as an activator of the inflammasome

The initial hypothesis was that ATP released during necrotic cell death can activate the Nalp3 inflammasome and caspase-1 through binding to the P2X7 receptor and thereby stimulate the processing of IL-1β, which then promotes neutrophil infiltration into the liver and an aggravation of liver injury by these inflammatory cells (Hoque et al., 2012). Although an activation of the Nalp3 inflammasome and a caspase-1-dependent processing of proIL1β could be confirmed (Williams et al. 2010a), there was no effect of endogenous IL-1β on the inflammatory response and liver injury after APAP overdose (Williams et al. 2010a). In addition, treatment with very high doses of exogenous IL-1β or endotoxin had no effect on APAP-induced liver injury (Williams et al. 2010a &b). Furthermore, IL-1 receptor-deficient mice showed similar injury as wild type animals

(William et al., 2010a) as did various KO mice of the Nalp3 inflammasome components (Williams et al., 2011a), animals deficient of various adhesion molecules (Cover et al., 2006) or NADPH oxidase-deficient mice (James et al., 2003; Williams et al., 2014) . Most importantly, the P2X7 inhibitor used by Hoque et al. was shown to inhibit P450 enzymes and thus protects because it prevents the initiation of injury rather than the activation of the inflammasome (Xie et al., 2013). Together, these data strongly argue against a relevant contribution of the ATP-activated inflammasome to the injury after APAP overdose. Consistent with these *in vivo* animal data, neutrophil activation in human APAP overdose patients occurs only during regeneration but not during the active injury phase (Williams et al., 2014) where most DAMPs including ATP are being released (McGill et al., 2013; Antoine et al., 2012). In addition, there is virtually no IL-1β formation in humans during APAP hepatotoxicity suggesting that also in humans the Nalp3 inflammasome is of limited relevance.

ATP as a direct cytotoxic agent

Recently, a second hypothesis was introduced. Amaral et al. reported that HepG2 cells release ATP after exposure to 20 mM APAP for 0.5-2 hours. The peak levels of ATP measured were approximately 10 μM at 0.5 hours (Amaral et al., 2013). Using 10 μM and 100 μM ATP, the authors then demonstrated that these doses caused 40% cell death in primary mouse hepatocytes isolated from C57Bl/6J mice and in HepG2 cells after 18-24 h exposure (Amaral et al., 2013). Based on these findings, the authors concluded that ATP, in addition to activating the inflammasome through purinergic receptors on macrophages, can also act as a direct cytotoxic agent against hepatocytes (Amaral et al., 2013). This cytotoxic effect was mediated through purinergic receptors, which are known

to be expressed on hepatocytes (Gonzales et al., 2007; Emmett et al., 2008). However, in our hands, ATP in concentrations from 10 μM up to 10 mM did not affect cell viability in primary mouse hepatocytes isolated from the same strain of mice, nor did treatment with a non-hydrolyzable ATP analogue (ATγP). Moreover, 5 mM APAP caused significant cell death at 9 h post-treatment in our primary mouse hepatocytes, but various concentrations of ATP or ATγP had no significant impact on this APAP-induced cell death. Cell death in these experiments as indicated by LDH or ALT release was confirmed by additional parameters such as propidium iodide staining, as measure of necrosis, and by the JC-1 assay, which indicates the mitochondrial membrane potential. Thus, ATP at levels that would be present in the extracellular milieu *in vivo* for only a short period of time and at concentrations which are one to several orders of magnitude beyond physiological levels could not kill mouse hepatocytes over a 24 h exposure time. Furthermore, there was no evidence that the same physiological or supraphysiological concentrations of ATP or ATγP could enhance APAP-induced cell death. Based on these findings, we have to conclude that ATP is not a direct cytotoxic agent during APAP-induced cell death of cultured murine hepatocytes. In contrast, some cytotoxicity was observed in murine macrophages exposed to supraphysiological concentrations of ATP confirming the idea that ATP could potentially be toxic *in vitro*, but only under circumstances largely irrelevant to human biology. Additional experiments with different hepatocyte cell types confirmed these findings. First, the metabolically competent human hepatocyte cell line HepaRG (Guillouzo et al., 2007), which is sensitive to APAP toxicity (MiGill et al., 2011), could not be killed by ATP alone, and ATP could not enhance APAP-induced cell death in these cells. Similarly, the viability of HepG2 cells, which are

known to have very low drug metabolizing enzyme levels (Wilkening et al., 2003) and are thus mostly resistant to APAP-induced necrosis (MiGill et al., 2011), was not affected by ATP alone or in combination with APAP. Finally, primary human hepatocytes, which were sensitive to APAP (Xie et al, 2014), did not show loss of cell viability with ATP alone and did not show more cell death when ATP was combined with APAP. In summary, our data consistently demonstrated in 4 different types of hepatocytes (human and mouse) that extracellular ATP was unable to directly cause cell death or aggravate APAP-induced cell death. Thus, it is unlikely that ATP released from necrotic cells can significantly affect cell death in the liver during APAP hepatotoxicity or other pathophysiologies that are characterized by extensive necrosis. APAP-induced cell death is clearly dominated by intracellular events caused by reactive metabolite formation, mitochondrial dysfunction and oxidant stress rather than extracellular mediators such as ATP. However, DAMP-induced cytokine formation *in vivo* can affect intracellular signaling events and enhance cell death (Bourdi et al., 2002) or recruit inflammatory cells in preparation for regeneration.

Chapter 5. Time Course of Acetaminophen-protein Adducts and Acetaminophen Metabolites in Circulation of Overdose Patients and in HepaRG Cells

This section is adapted from Xie et.al (2015), "Time course of acetaminophen-protein adducts and acetaminophen metabolites in circulation of overdose patients and in HepaRG cells", Xenobiotica, 45(10): 921–929, with permission from the publisher

5.1 Abstract

It has been suggested that acetaminophen (APAP)-protein adducts can be measured in circulation to diagnose APAP-induced liver injury. However, the full-time course of plasma adducts has not been studied specifically in early-presenting overdose patients. In fact, surprisingly little work has been done on the metabolism of APAP after overdose in general.

We measured APAP, five APAP metabolites and APAP-protein adducts in plasma samples from early- and late-presenting overdose patients, and APAP-protein adducts in culture medium from HepaRG cells.

In contrast to earlier rodents studies, we found that APAP-protein adducts were lower at early time points and peaked around the time of peak liver injury, suggesting that these adduct levels may take longer to become elevated or remain elevated than previously thought.

APAP and its major metabolites were elevated in plasma at early time points and rapidly decreased.

Although clinical measurement of APAP-protein adducts holds promise as a diagnostic tool, we suggest caution in its interpretation in very early-presenting patients. Our data also support the idea that sulfation is saturated even at low doses but glucuronidation has a much higher capacity, highlighting the importance of glucuronidation in APAP metabolism.

5.2 Introduction

At recommended doses, acetaminophen (APAP) is a safe and effective drug for the management of pain. However, overdose of APAP can cause severe acute liver injury and liver failure. It is responsible for almost 80 000 emergency department visits in the U.S. each year (Budnitz et al., 2011), as well as 26 000 hospitalizations and nearly 500 deaths (Nourjah et al., 2006) (Nourjah et al., 2006). In fact, APAP hepatotoxicity is one of the most common etiologies of acute liver failure (Lee, 2012).

The metabolism and pharmacokinetics of APAP after therapeutic doses were first studied in detail in the 1960s; our understanding of APAP metabolism has grown considerably since that time (McGill & Jaeschke, 2013). The bioavailability of orally administered APAP is about 80%. The drug is rapidly absorbed from the lumen of the small intestine, achieving maximum plasma concentration within 0.5–1.5 h in fasted subjects. Glucuronidation and sulfation are the major elimination pathways. Sulfation is saturated even at therapeutic doses (Clements et al., 1984; Prescott, 1980), so glucuronidation is dominant. In total, about 50–70% of a pharmacological dose is glucuronidated, while 25–35% undergoes sulfation. A much smaller percentage of the dose (5–10%) is converted to the reactive intermediate N-acetyl-p-benzoquinone imine (NAPQI) by cytochrome P450 enzymes. NAPQI binds to the cysteine sulfhydryl group on glutathione (GSH) and can also bind to protein sulfhydryl groups, even after low doses (Heard et al., 2011; McGill et al. 2013). Most of the APAP-GSH is broken down to mercapturic acid and cysteine conjugates and the resulting APAP-N-acetylcysteine (APAP-NAC), APAP-cysteine (APAP-cys), as well as APAP-protein adducts can be measured in plasma and urine as surrogates of NAPQI formation. Any remaining APAP (<5%) is excreted unchanged.

It has been suggested that APAP overdose can be rapidly diagnosed by measuring protein-derived APAP-cysteine adducts (hereafter referred to as "APAP-protein adducts") in serum from both adults (Davern et al., 2006) and children (James et al., 2008), but some aspects of the time course and mechanism of release of these adducts have not yet been investigated in humans. In particular, time course experiments in mice suggest that the concentration of APAP-protein adducts in circulation increases rapidly after APAP treatment and peaks even before the onset of liver injury (McGill et al., 2013). However, the full-time course of circulating adducts, beginning before the peak of injury, has not been characterized in APAP overdose patients. Also, despite our extensive knowledge of APAP metabolism after therapeutic doses, surprisingly few studies have specifically examined APAP metabolism after overdose.

To better understand the appearance of APAP-protein adducts in circulation and APAP metabolism after overdose, we collected plasma samples from patients with a recent history of APAP overdose. Patients were grouped as either early or late presenters on the basis of plasma APAP levels. We measured the five major metabolites of APAP, APAP-glucuronide (APAP-gluc), APAP-sulfate (APAP-sulf), APAP-GSH, free APAP-cys, and APAP-NAC, as well as APAP-protein adducts, in these samples using mass spectrometry. Our results suggest that APAP-protein adducts increase later than expected in humans and that clinical measurements of these adducts should be interpreted with caution. The data also reinforce the importance of glucuronidation in APAP metabolism.

5.3 Methods

Patient enrollment and categorization

Patients presenting to the University of Kansas Hospital in Kansas City, Kansas were studied prospectively. The diagnosis of APAP overdose was made by a physician. All patients or next of kin were informed of the study purpose and protocol and signed a consent form. The criteria for study inclusion were reported history of APAP overdose, high plasma APAP levels, or evidence of liver injury (ALT > 1000 U/L and prothrombin time $\geqslant$ 18 s). All patients met at least two of these criteria. Patients were excluded if there was reasonable evidence that their liver injury was due to some other etiology (e.g. viral hepatitis, alcoholic hepatitis, etc.). Because APAP has a short half-life, the patients were grouped into early or late time points on the basis of plasma APAP concentration. Patients considered "early" had a reported history of APAP overdose and relatively high plasma APAP levels (>25 μg/mL; the top of the therapeutic range) with little or no evidence of liver injury (<100 U/L ALT) at the time of presentation, which is known to develop at later time points in humans (McGill et al., 2012; Singer et al., 1995). Patients considered "late" had lower plasma APAP levels (1–25 μg/mL), coupled with evidence of liver injury (ALT $\geqslant$ 100 U/L in the first study sample, peak ALT > 1000 U/L and peak prothrombin time $\geqslant$18 s). A total of 25 μg/mL was chosen as our cut-off, because it is at the top of the range of serum concentrations that is normally seen after therapeutic doses of APAP. Our assumption is that patients with higher levels likely exceeded the maximum recommended dose of APAP. All patients were treated with either oral or i.v. NAC. All study procedures were done in accordance with the Declaration of Helsinki and

were approved by the internal review board (IRB) of the University of Kansas Medical Center.

Patient sample collection and handling

Blood samples were drawn in vacuum tubes dry-coated with heparin or EDTA. Plasma was obtained by centrifugation and aliquots were stored at −80 °C for later analysis.

Biochemistry

Clinical parameters, including alanine aminotransferase (ALT), INR, bilirubin and creatinine, were measured and calculated in the clinical lab at the University of Kansas Hospital using standard methods and reagents. Lactate dehydrogenase (LDH) release was measured as previously described (Bajt et al., 2004).

HepaRG cells

HepaRG cells were obtained from Biopredic International (Rennes, France). The cells were grown and maintained as previously described (McGill et al., 2011). For time course experiments, the cells were washed with 1 × PBS to remove the maintenance medium and treated with 20 mM APAP dissolved in DMSO-free Williams' E medium containing 10% FBS, insulin and penicillin/streptomycin.

Analysis of APAP and metabolites in human plasma and HepaRG cells

Protein-derived APAP-cysteine adducts (APAP-protein adducts) were measured in HepaRG cells as previously described (McGill et al., 2011). Before measurement of APAP-protein adducts in plasma or cell culture medium, the samples were prepared as previously described (McGill et al., 2013). Briefly, low-molecular weight compounds,

including free APAP-cys, were removed by dialysis followed by filtration through size exclusion columns to obtain total plasma protein. The proteins were then digested overnight using a mixture of proteases, followed by precipitation of the proteases and filtration to ensure that the samples were clean enough for HPLC. A detailed description of the basic method for APAP-protein adduct measurement has recently been published (Cook et al., 2015).

For plasma samples, the following reference standards and deuterated internal standards were obtained from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada): 4-acetamidophenyl β-D-glucuronide sodium salt (APAP-gluc, 98%), 4-acetaminophen sulfate potassium salt (APAP-sulf, 98%), 3-(N-acetyl-l-cystein-S-yl)-acetaminophen disodium salt (APAP-NAC, 95%), 3-cysteinylacetaminophen trifluoroacetic acid salt (APAP-cys, 95%), acetaminophen glutathione disodium salt (APAP-glut, 95%), acetaminophen-d4 (APAP-d4, 98% chemical purity, 99% isotopic purity), and 4-acetaminophen-d3 sulfate (APAP-d3-sulf, 98% chemical purity, 99% isotopic purity). APAP (analytical standard) and ammonium acetate (⩾98%) were obtained from Sigma-Aldrich Co. (St. Louis, MO). LC-MS grade acetonitrile and methanol were obtained from Honeywell Burdick and Jackson (Morristown, NJ). Glacial acetic acid was obtained from Spectrum Chemical Manufacturing Corp. (New Brunswick, NJ). Formic acid (88%) was obtained from Fisher Scientific (Pittsburgh, PA). Ultrapure water (18.2 MΩ) was obtained by passage of deionized water through a Milli-Q Plus filtration system (Millipore, Billerica, MA).

Stock and working solutions of reference standards and internal standards were prepared in methanol-water (1:1, v/v). Reference standard working solutions of 0.01, 0.1, 1, and

10 µg/mL were prepared by serial dilution. Specified concentrations refer to the free acid of salt reference standards. Calibration standards and quality control (QC) samples were prepared concurrently with study samples by fortification of 10 µL of analyte-free, heparinized human plasma with reference standard working solutions. The calibration curve ranged from 0.01 to 100 µg/mL, and QC samples of 0.05, 0.5, 5, and 50 µg/mL were included for assessment of accuracy. If necessary, patient samples were diluted for quantification within the curve range.

To maintain equivalence in preparation, methanol-water (1:1, v/v) was used to bring all study samples (10-µL aliquots) and fortified calibration and QC samples to a total volume of 110 µL. Study samples, calibrators, and QC samples were then fortified with 40 µL of internal standard working solution containing 2.5 and 25 µg/mL of APAP-d4 and APAP-d3-sulf, respectively. Plasma proteins were precipitated by the addition of acetonitrile (600 µL), and each sample was vortex mixed for 30 s before 15 min of centrifugation at 1100 × g. Sample supernatants were transferred to clean tubes, placed in a 35 °C water bath, and evaporated to dryness under a 10–15 psi air stream in a Zymark TurboVap LV Evaporator. Sample residues were reconstituted in 500 µL of 0.1% aqueous formic acid, vortex mixed for 30 s, and centrifuged for 5 min at 1100 × g. The supernatants (200 µL) were then carefully removed and transferred to a autosampler vials and held at 5 °C until the time of analysis. A 25 µL injection volume was used for analysis.

Sample analysis was performed on an Agilent 1200 Infinity Series HPLC system equipped with an Agilent Poroshell 120 EC-C18 column (2.1 × 100 mm ID, 2.7-µm particle size), which was interfaced with an Agilent 6460 triple quadrupole mass

spectrometer (Agilent Technologies, Santa Clara, CA) or on a Waters Acquity UPLC system equipped with a Waters C18 column and interfaced with a Waters Quattro Premier XE triple quadrupole mass spectrometer (Waters Corp., Milford, MA). Sample preparation and measurement were the same in both cases. Standard curves were used to quantify samples in every run. Chromatographic separation was achieved using 10-mM aqueous ammonium acetate, pH 3.5 (A) and methanol (B) at a flow rate of 0.25 mL/min. Mobile phase was maintained at 3% B for the first 6.0 min, increased linearly to 35% B at 9.0 min, maintained at 95% B from 9.1 to 12.0 min, decreased linearly to 3% B at 12.5 min, and then re-equilibrated at 3% B until 20.0 min. The column compartment was maintained at 40 °C, and prepared samples were stored in the autosampler at 5 °C during analysis. The mass spectrometer was operated in electrospray ionization mode with MRM data acquisition. APAP-sulf and APAP-d3-sulf were monitored in negative ionization mode; all other analytes and APAP-d4 were monitored in positive ionization mode. The following source conditions were applied: 350 °C gas temperature, 10 L/min gas flow, 30 psi nebulizer pressure, 350 °C sheath gas temperature, 9 L/min sheath gas flow, 3500 V capillary voltage, and 500 V nozzle voltage. Fragmentor voltage and collision energy were optimized for each MRM transition. The following MRM transitions (precursor m/z → product m/z) were monitored for quantification: 152.1 → 110.0 for APAP, 328.1 → 152.1 for APAP-gluc, 230.1 → 150.1 for APAP-sulf, 313.1 → 208.0 for APAP-NAC, 271.1 → 140.0 for APAP-cys, 457.1 → 140.0 for APAP-GSH, 156.1 → 114.1 for APAP-d4, and 233.1 → 153.1 for APAP-d3-sulf. Additional secondary transitions were monitored for qualitative confirmation of analyte presence. Data analysis was performed with MassHunter Workstation Quantitative Analysis

software (v. B.04.00, Agilent Technologies, Santa Clara, CA). Calibration curves were constructed by plotting the analyte to internal standard chromatographic peak area ratio against the known analyte concentration in each calibration standard. APAP-d4 was used as the internal standard for APAP, and APAP-d3-sulf was used as the internal standard for all other analytes. Linear curve fits were applied for all analytes except APAP-NAC, for which a quadratic curve fit was used. Curves were weighted in proportion to the reciprocal of the squared analyte concentration.

Statistics

Normality was assessed using the Shapiro–Wilk test. For normally distributed data, significance was tested using the Student's t-test or one-way analysis of variance (ANOVA) for experiments involving two or more groups, respectively. For non-normally distributed data, significance was tested using the Mann–Whitney U-test or the Kruskal–Wallis test for experiments involving two or more groups, respectively. Multiple comparisons were performed post-hoc using either Tukey's test or Dunn's test, depending upon whether the data were normal or non-normal, respectively. Fisher's Exact test was used for categorical values.

5.4 Results

To begin studying the time course of APAP-protein adducts and metabolism of APAP in overdose patients, we used mass spectrometry to measure APAP, five major APAP

metabolites and APAP-protein adducts in plasma from nine APAP overdose patients who presented before the peak of injury as indicated by plasma ALT levels. APAP-GSH concentrations were below the lower limit of detection in most samples, indicating that most of this conjugate is quickly broken down to APAP-NAC and APAP-cys. However, we were able to detect and quantify all of the other metabolites. Consistent with previous results, we found that the major metabolite of APAP was APAP-gluc, followed by APAP-sulf, APAP-cys and finally by APAP-NAC (Figure 1A and C). APAP and all four metabolites were high at the time of study admission, but rapidly decreased (Figure 1A, C, E). Surprisingly, although APAP-protein adduct concentrations were already detectable at the time of study admission, the peak of circulating adducts was not observed until later time points, around the time of peak ALT (Figure 1F). This is different from what has been observed in mice (McGill et al., 2013).

We then sorted 17 APAP overdose patients into early- and late-presenting groups. Patients were categorized on the basis of plasma APAP levels because it was difficult to ascertain the time of ingestion for many of the patients. APAP has a short circulating half-life, and liver injury develops relatively late after APAP overdose. Thus, patients presenting early after APAP ingestion with a reported history of overdose will have higher plasma APAP concentrations and little or no evidence of liver damage in the first sample obtained after study enrollment. On the other hand, patients with a reported history of APAP overdose who have lower plasma APAP but signs of liver injury in the first study sample are likely to be later presenters. When patient reported time of overdose was available, it was found to be consistent with our patient grouping. The time from overdose to hospital admission was known for three of the early-presenting patients

(mean: 10 h, range: 5–16 h) and for 10 of the late-presenting patients (mean: 83 h, range: 3–336 h). Although one patient in the latter group presented to the hospital within 3 h of

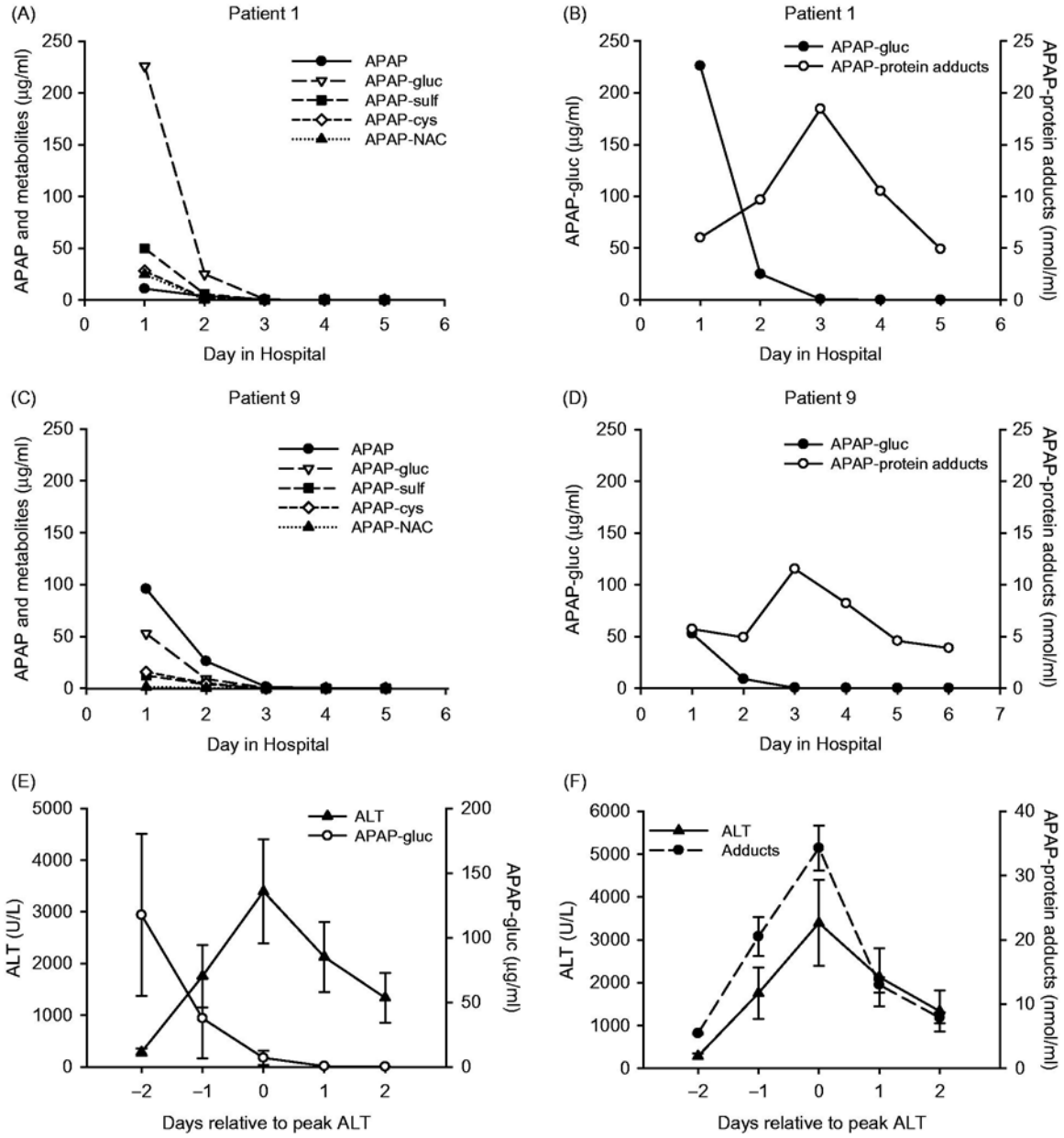


Figure 5.4.1 Time courses of APAP, APAP metabolites, and APAP-protein adducts in early-presenting overdose patients.

Plasma APAP, metabolites, and protein adducts were retrospectively measured in plasma from APAP overdose patients who presented at least 2 d before the peak of liver injury indicated by ALT. (A,C) Plasma concentrations of APAP and APAP metabolites from a representative patient. (B,D) Plasma levels of APAP-gluc and APAP-protein adducts

from a representative patient. (E) Mean plasma levels of APAP-gluc and ALT in all patients presenting at least 2 d before the peak of liver injury. (F) Mean APAP-protein adducts and ALT in all patients presenting at least 2 d before the peak of liver injury. Data for multiple patients are expressed as mean ± SEM for n = 9.

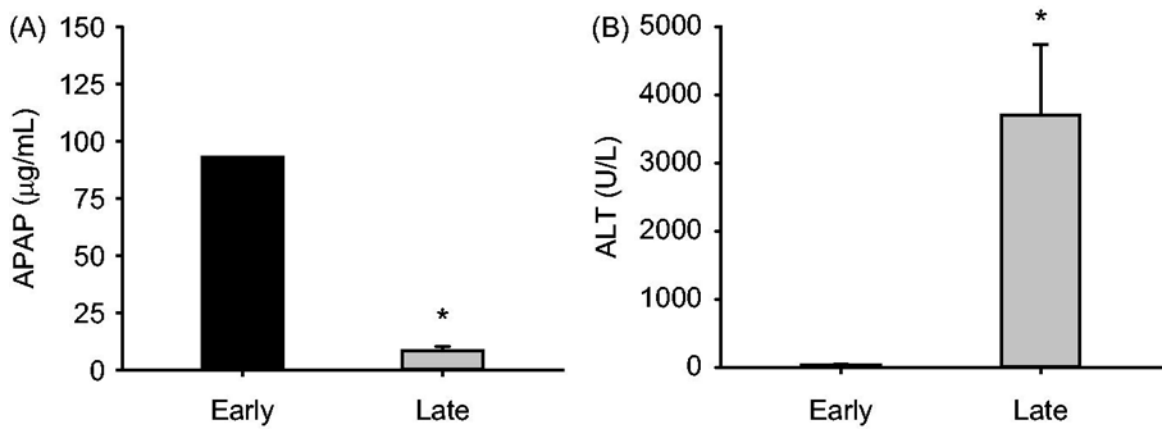


Figure 5.4.2 Plasma APAP and ALT concentrations in the early and late groups.

Plasma APAP and ALT were measured in the first sample available after study enrollment. (A) Plasma APAP. (B) Plasma ALT. Data expressed as mean ± SEM for 6 early patients and 11 late patients. *$p < 0.05$.

Table 5.4.1 Patient characteristics

Parameter	Early group	Late group	*p* Value
N	6	11	
Age (years)[a]	31, 18–56	35, 19–46	>0.5
Sex (% female)	50	73	>0.5
Survival	5 / 6	11 / 11	>0.5
Admission INR[a,b]	1.2, 1.1–1.3	3.3, 1.6–5.7	0.018
Admission bilirubin (mg/dL)[a,b]	1.0, 0.50–1.3	3.9, 1.3–7.9	0.0090
Admission creatinine (mg/dL)[a,b]	1.0, 0.71–1.6	2.2, 0.72–5.7	0.062

[a]Data are presented as mean, range.
[b]When available.

the reported time of overdose, it is important to note that this particular patient did not enroll in our study for another 24 h. The remaining patients in the late group for whom this information is available presented much later. In every case, treatment with NAC was immediately started. Although the time frame of APAP use was not available for all patients, the patients in the early group tend to be classified by the diagnosing physician as acute overdoses (duration of APAP use <48 h) while the late group included a mix of acute and chronic (duration of APAP use >48 h) cases. The average plasma APAP and ALT levels in the first plasma samples available after the time of study enrollment are shown in Figure 2. Other clinical and demographic information for these patients are listed in Table 5.4.1.

We found that the average plasma concentration of APAP-sulf was not significantly different between our early and late patients (Figure 3A). Moreover, the APAP-sulf levels in these overdose patients were not remarkably different from those previously reported in individuals receiving subtoxic or even just therapeutic doses of APAP (Gelotte et al., 2007; Prescott, 1980) . On the other hand, APAP-gluc levels were much higher in the early patients (Figure 3B) and were elevated approximately 20-fold compared with previously reported values for APAP-gluc after subtoxic doses. Together, these results are in agreement with the earlier findings that both sulfation and renal elimination of APAP-sulf are saturated after APAP ingestion and remain so until later time points (Clements et al., 1984; Prescott, 1980), while glucuronidation and APAP-gluc elimination are not so easily saturated even after overdose because the APAP-gluc levels in serum seem to scale with the concentration of APAP. Similar to APAP-sulf, the APAP-NAC and free APAP-cys breakdown products of APAP-GSH, were not

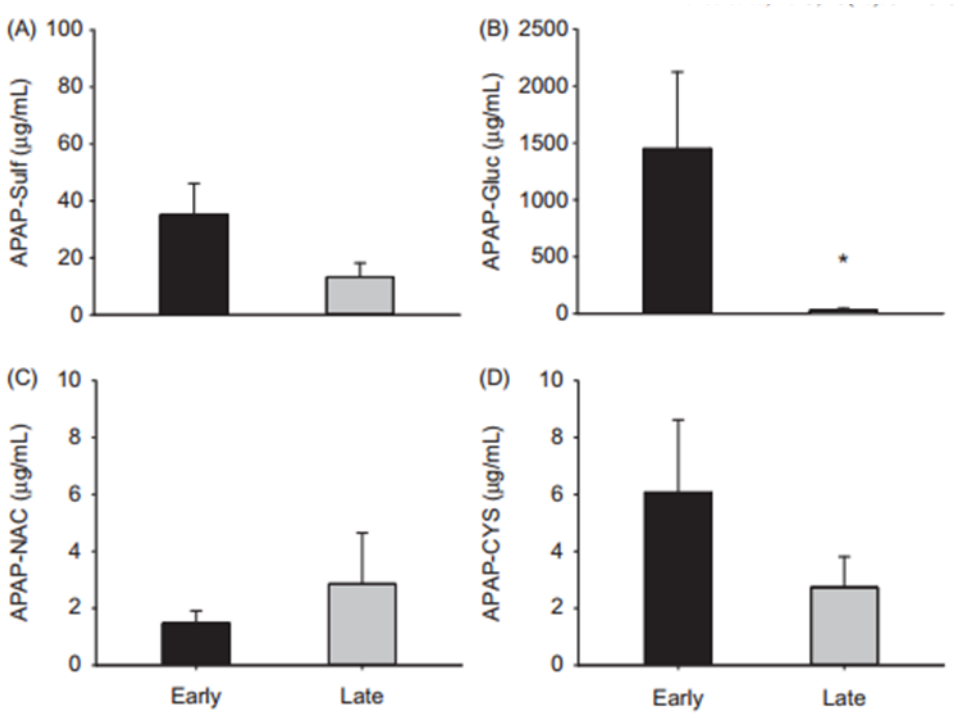


Figure 5.4.3 Plasma APAP metabolite concentrations in the early and late groups.

Plasma APAP metabolites were measured in the first sample available after study enrollment. (A) Plasma APAP-sulf. (B) Plasma APAP-gluc. (C) Plasma APAP-NAC. (D) Plasma free APAP-cys. Data expressed as mean ± SEM for 6 early patients and 11 late patients. *$p<0.05$.

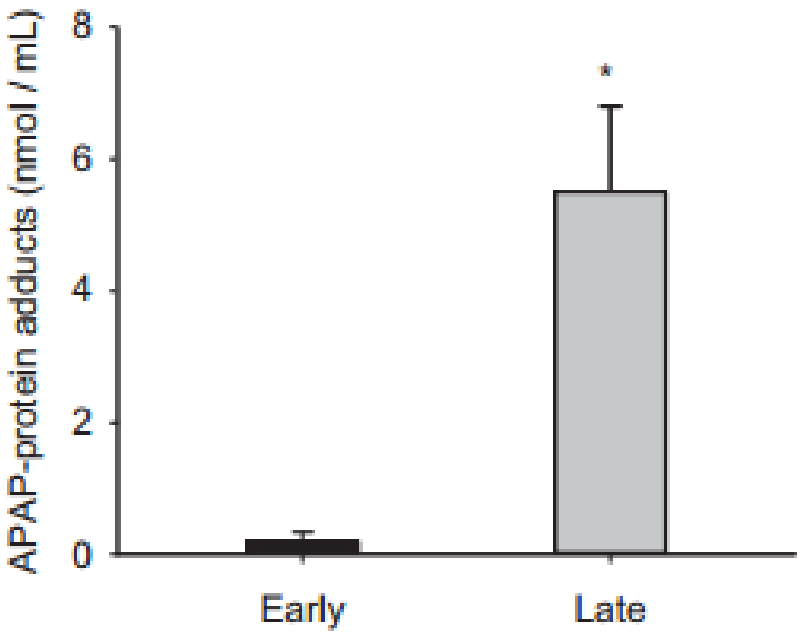


Figure 5.4.4 Plasma APAP-protein adduct concentrations in the early and late groups.

Plasma APAP-protein adducts were measured in the first sample available after study enrollment. Data are expressed as mean ± SEM for 6 early patients and 11 late patients. *$p<0.05$.

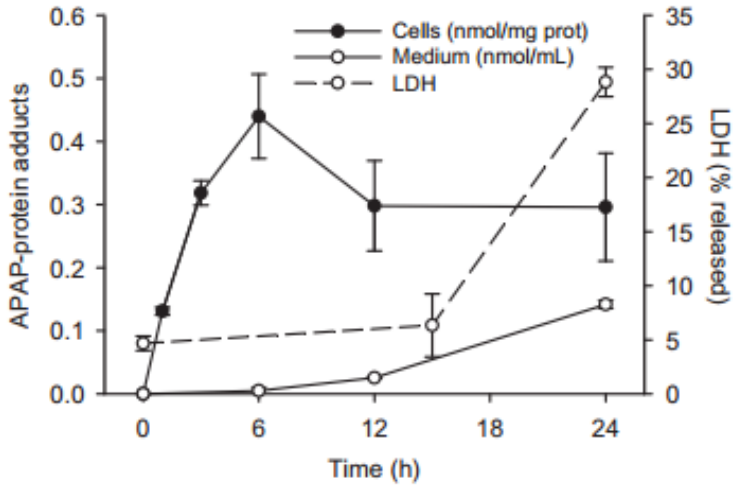


Figure 5.4.5 Time course of APAP-protein adduct release and cell death in HepaRG cells.

HepaRG cells were treated with 20 mM APAP for the indicated times. APAP-protein adducts were measured in both cell lysates and in the cell culture medium. Cell death was assessed by lactate dehydrogenase (LDH) release, as described in the methods section. Data are expressed as mean ± SEM for three to six independent experiments.

significantly different between groups (Figure 3C and D). Because one would expect a saturated metabolic pathway to plateau and the levels of its metabolite products to remain unchanged for some time, the latter finding may be in agreement with the idea that the GSH pathway is also overwhelmed.

As we predicted from our time course data, protein adduct levels in plasma were much higher in the late-presenting group (Figure 4). These data suggest that the appearance of peak levels of APAP-protein adducts may be delayed or evolve over hours or even days in humans after overdose, in contrast to rodents which have elevated plasma adducts well before any detectable increase in ALT (within 15–30 min after APAP treatment; McGill et al., 2013). It is possible to argue that the lower plasma APAP-protein adducts level in the early group is due to earlier treatment with NAC, but the fact that APAP-NAC and free APAP-cys levels did not differ between groups suggest that this is unlikely to be the case. Moreover, these data are from the first available samples obtained after study enrollment. Generally, the patients were enrolled on the day of admission and therefore had not been receiving NAC for long. Furthermore, we also observed a late increase in APAP-protein adducts in culture medium from the human liver cell line HepaRG (Figure 5), which also differs from what has been reported for primary mouse hepatocytes (McGill et al., 2013). Interestingly, however, the adduct concentration in the culture medium still increased before an increase in enzyme release (Figure 5), consistent with the idea that adducts are released through a mechanism other than simply cell necrosis.

5.5 Discussion

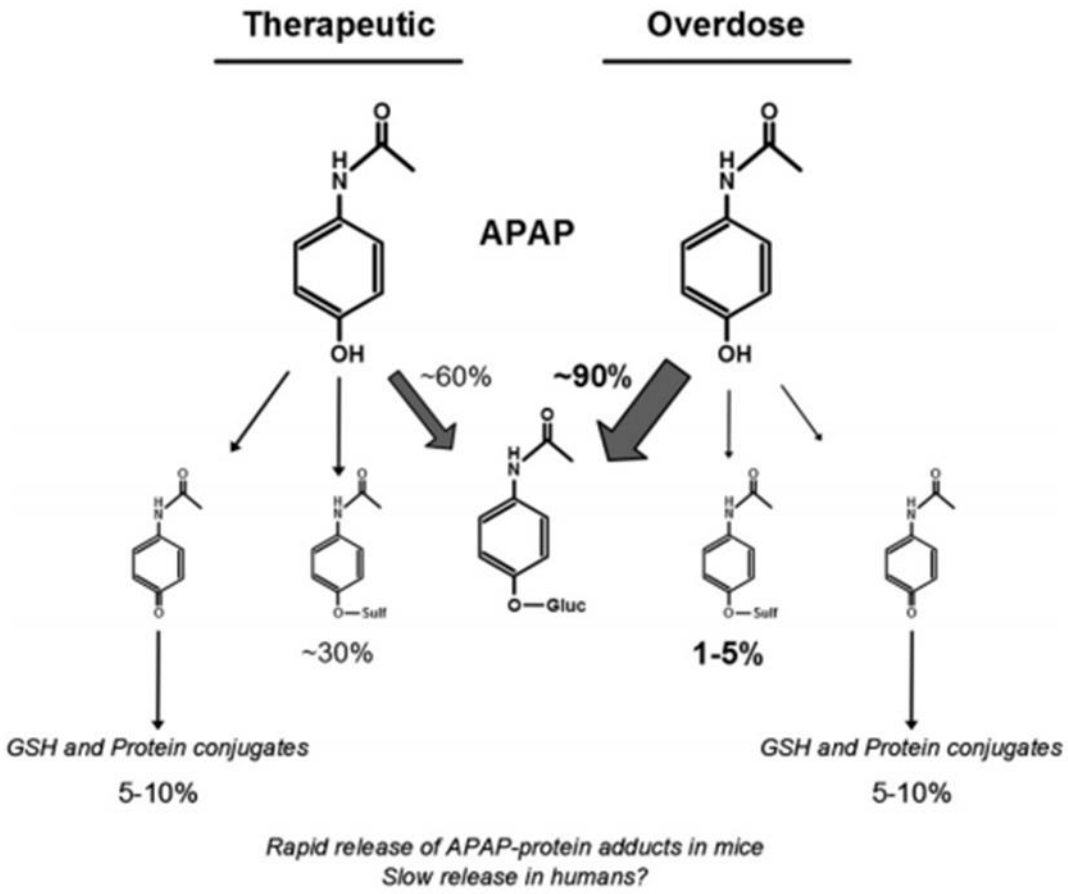


Figure 5.4.6 Schematic of APAP metabolism and APAP-protein adducts release.

At therapeutic doses, roughly a third of the dose is eliminated by sulfation while two-thirds is eliminated by glucuronidation. After overdose, sulfation is saturated and a much higher percentage is glucuronidated. Furthermore, our data suggest that APAP-protein adducts are released more slowly in humans than in mice.

The metabolism and disposition of APAP in humans after therapeutic doses has been studied for quite some time. Recently, several groups have even succeeded with studies designed to explore the mechanisms of APAP hepatotoxicity in humans (Antoine et al., 2012; McGill et al., 2012, 2014a,b; Weerasinghe et al., 2014; Williams et al., 2014) (Williams et al., 2014). One early advance in this area was the discovery that APAP-protein adducts can be detected in humans (Hinson et al., 1990; Muldrew et al., 2002; Webster et al., 1996). Since then, it has been suggested that the presence of APAP-protein adducts in serum can be used to diagnose APAP overdose (Davern et al., 2006; James et al., 2008). However, there are still gaps in our knowledge about the mechanism of appearance of APAP-protein adducts in serum and even the basic metabolism of APAP after overdose. It is clear that glucuronidation, sulfation, glutathione conjugation, and protein binding all occur in overdose patients, but the kinetics and toxicological significance of each is less well understood. Our goal with this study was to develop a better understanding of the full-time course of APAP-protein adduct formation and APAP metabolism after overdose in humans. A summary of our findings are schematically shown in Figure 6.

Kinetics of plasma APAP-protein adducts after overdose

In mice, APAP-protein adducts rapidly appear in plasma after APAP treatment (McGill et al., 2013). Although it is important to keep in mind that APAP studies in mice are usually done under fasting conditions to deplete GSH more quickly and this could affect the results, it has also been shown APAP-protein adducts are detectable in serum from humans as well as mice even after low doses, which do not cause injury (Heard et al., 2011; McGill et al., 2013). Despite the earlier suggestion that APAP-protein adducts are

released by cell lysis (Davern et al., 2006; James et al., 2009), it is clear from these studies that liver injury is not required for the appearance of adducts in circulation. Furthermore, APAP-protein adducts form rapidly in human hepatocytes after APAP treatment (Xie et al., 2014). Thus, it would seem likely that plasma adducts would increase early in humans after APAP overdose even before the injury begins. If true, then the rapid determination of APAP-protein adducts upon patient presentation using a point-of-care assay could hold considerable clinical value for the early diagnosis of APAP-induced liver injury. However, the detailed time course of circulating APAP-protein adducts had not previously been measured specifically in a cohort of early-presenting patients who went on to develop high ALT and other signs of severe liver injury. Although individual early-presenting patients have sometimes been included, most work to date has focused on later time points in order to determine the half-life of these adducts in circulation (James et al., 2009; Heard et al., 2011). Perhaps, the most interesting finding from our data is that plasma APAP-protein adducts may rise slowly in humans after overdose. This is consistent with the earlier observation that APAP-protein adduct peak levels correlate well with ALT (James et al., 2009). Based on this, it is possible that APAP overdose patients presenting early will have low plasma APAP-protein adduct concentrations. If treatment decisions are made solely on the basis of a rapid point-of-care assay performed at the time of presentation, such low levels may prevent the proper treatment of some patients. Thus, circulating adduct levels should be interpreted carefully. Additionally, because plasma APAP and its metabolites are higher at early time points, we suggest that the plasma level of the parent drug or a metabolite should also be measured in patients with suspected overdose in order to confirm a negative result from

adduct measurement. If both adducts and the APAP or APAP metabolite levels are low, then the result is likely to be a true negative. Although this approach may not aid diagnosis in every possible case (Bebarta et al., 2014), it is likely to be effective in most cases. The results of previous work that suggested that serum APAP levels (i.e. the Rumack-Matthews nomogram) are more useful for diagnosis than APAP-protein adducts at early time points are in agreement with this idea (James et al., 2008).

Little is known about the mechanisms of APAP-protein adduct release into circulation. It has been shown that APAP-protein adducts appear in the medium of primary mouse hepatocytes cultured in the absence of extracellular protein and before the onset of cell injury, suggesting that adducts are actively secreted out of hepatocytes into the extracellular space, not simply passively released during cell death. However, the actual route of secretion is unknown. Furthermore, although it is clear that the release of APAP-protein adducts into circulation does not require cell death (Heard et al., 2011; McGill et al., 2013), there is evidence that cell death can further increase APAP-protein adduct levels (McGill et al., 2013). Thus, it seems that the mechanism of adduct release is complex. Additional study is needed to better understand this phenomenon.

Attempts have been made to develop models to determine the time and dose of an APAP overdose (Remien et al., 2012). Although it was suggested that APAP-protein adduct levels in serum would likely not improve these models (Remien et al., 2012), our data showing a clear decrease in APAP and its metabolites while adduct levels were increasing (Figure 1) may indicate that the relative amounts of metabolites and adducts can provide some indication of the timing of overdose relative to presentation on their own.

Importance of glucuronidation after overdose

It has been known for some time that sulfation is saturated by APAP even after doses in the therapeutic range (Clements et al., 1984; Gelotte et al., 2007; Prescott, 1980) . Our data support this finding in overdose patients. At therapeutic doses, about one-third of the drug undergoes sulfation, while roughly two-third is glucuronidated. The remainder is either converted to NAPQI and binds to GSH or proteins, or is excreted unchanged. Though it is common to cite these percentages in articles even when referring to overdose patients, our results show that glucuronidation plays an even larger role after overdose due to saturation of the sulfation pathway. It seems that glucuronidation is not easily saturated even after overdose. While the APAP-sulf concentrations measured in our patients did not differ much from what has previously been reported after therapeutic doses, the APAP-gluc levels were much higher. It is commonly thought that both of these phase II elimination pathways, along with glutathionylation, are saturated after APAP overdose and that the APAP is then converted to its reactive metabolite (Chun et al., 2009; McClain et al., 1999; Srivastava et al., 2010). However, it is now clear that the reactive metabolite forms and binds to proteins even after therapeutic doses at which glucuronidation and glutathionylation are certainly not saturated (Heard et al., 2011; McGill et al., 2013). The latter strongly suggests that saturation of these pathways is not necessary for NAPQI formation to occur. Nevertheless, glucuronidation is quite important.

The significance of the glucuronidation pathway of APAP elimination has been studied in both rodents and humans, though the results have been controversial. Glucuronidation-deficient Gunn rats have been shown to be much more sensitive to the toxicity of APAP

than other rat strains (de Morais & Wells, 1989). Consistent with this, early experiments with low doses in humans revealed that individuals with Gilbert's syndrome do not glucuronidate APAP as well and have higher concentrations of APAP-GSH in urine (de Morais et al., 1992). However, these results are in conflict with an earlier experiment involving Gilbert's syndrome patients that yielded opposite result(Ullrich et al., 1987). The difference in findings between these studies may have been due to differences in dose normalization and route of exposure, although later work using an experimental design similar to that used in the no-effect study provided support for the idea that Gilbert's patients may be at increased risk of APAP hepatotoxicity (Esteban & Pérez-Mateo, 1999). More recently, Court et al. (2013) found that liver samples from humans with polymorphisms in the 3′ untranslated region of UGT1A, particularly rs8330, had higher APAP glucuronidation activity. Importantly, they also found that there were fewer individuals with these alleles among patients with acute liver failure caused by unintentional APAP overdose than among those with acute liver failure from other etiologies (Court et al., 2013), which may suggest that a greater ability to glucuronidate APAP can protect against the hepatotoxicity caused by overdose. Overall, there is a strong case that glucuronidation is the most important route of elimination of APAP, such that Gilbert's syndrome patients are likely to be more susceptible to APAP toxicity while patients who are better at glucuronidation are less likely to develop APAP-induced liver injury. Our data support this conclusion.

Potential weaknesses

Our grouping of patients based on serum APAP and ALT levels is a potential weakness of this study. Although our grouping was consistent with the available information for

time from overdose to time of presentation, we did not have complete data. Furthermore, we did not collect data concerning the time from overdose to NAC treatment or other basic patient information, such as the dose of APAP taken, which could affect the metabolite and adduct levels that we measured. This should be kept in mind when interpreting the results presented in this article.

Our data suggest that plasma APAP-protein adducts increase slowly in humans in contrast to mice, and this may have important implications for the rapid clinical measurement of APAP-protein adducts in early-presenting patients. Moreover, our data confirm that the sulfation pathway is quickly saturated after exposure to APAP, but indicate that glucuronidation is a much higher capacity system. Overall, however, the results of this study provide additional support for the idea that glucuronidation is the most critical elimination pathway for APAP.

Chapter 6. Inhibitor of Apoptosis Signal-regulating Kinase 1 Protects Against Acetaminophen-induced Liver Injury

This section is adapted from Xie et.al (2015), "Inhibitor of apoptosis signal-regulating kinase 1 protects against acetaminophen-induced liver injury", Toxicology and Applied Pharmacology, 286(1): 1–9, with permission from the publisher

6.1 Abstract

Metabolic activation and oxidant stress are key events in the pathophysiology of acetaminophen (APAP) hepatotoxicity. The initial mitochondrial oxidative stress triggered by protein adduct formation is amplified by c-jun-N-terminal kinase (JNK), resulting in mitochondrial dysfunction and ultimately cell necrosis. Apoptosis signal-regulating kinase 1 (ASK1) is considered the link between oxidant stress and JNK activation. The objective of the current study was to assess the efficacy and mechanism of action of the small-molecule ASK1 inhibitor GS-459679 in a murine model of APAP hepatotoxicity. APAP (300 mg/kg) caused extensive glutathione depletion, JNK activation and translocation to the mitochondria, oxidant stress and liver injury as indicated by plasma ALT activities and area of necrosis over a 24 h observation period. Pretreatment with 30 mg/kg of GS-459679 almost completely prevented JNK activation, oxidant stress and injury without affecting the metabolic activation of APAP. To evaluate the therapeutic potential of GS-459679, mice were treated with APAP and then with the inhibitor. Given 1.5 h after APAP, GS-459679 was still protective, which was paralleled by reduced JNK activation and p-JNK translocation to mitochondria. However, GS-459679 treatment was not more effective than N-acetylcysteine, and the combination of GS-459679 and N-acetylcysteine exhibited similar efficacy as N-acetylcysteine monotherapy, suggesting that GS-459769 and N-acetylcysteine affect the same pathway. Importantly, inhibition of ASK1 did not impair liver regeneration as indicated by PCNA staining. In conclusion, the ASK1 inhibitor GS-459679 protected against APAP toxicity by attenuating JNK activation and oxidant stress in mice and may have therapeutic potential for APAP overdose patients.

6.2 Introduction

Acetaminophen (APAP) is a widely used analgesic and antipyretic. A therapeutic dose of APAP is safe and effective while an overdose can cause severe liver injury in animals and humans (Larson, 2007 and McGill et al., 2012a). Hepatotoxicity is initiated by formation of a reactive metabolite, N-acetyl-p-benzoquinone imine, which is detoxified by glutathione but also binds to cellular proteins (Cohen et al., 1997). Although formation of protein adducts, especially in mitochondria, is a critical event in the pathophysiology (Tirmenstein and Nelson, 1989), it is not sufficient to cause cell death (Jaeschke and Bajt, 2006). The current hypothesis is that protein binding induces mitochondrial dysfunction including inhibition of mitochondrial respiration (Meyers et al., 1988) and formation of reactive oxygen species (ROS) (Jaeschke, 1990) and peroxynitrite (Hinson et al., 1998 and Cover et al., 2005). This oxidant stress together with lysosomal iron taken up into the mitochondria by the Ca2 + uniporter (Kon et al., 2010), leads to the opening of the mitochondrial membrane permeability transition (MPT) pore and collapse of the membrane potential (Kon et al., 2004, Reid et al., 2005, Ramachandran et al., 2011a and LoGuidice and Boelsterli, 2011). The massive loss of mitochondrial function triggers necrotic cell death (Gujral et al., 2002). A number of therapeutic interventions that accelerated the recovery of mitochondrial glutathione and improved the detoxification of mitochondrial ROS and peroxynitrite provided clear evidence for the critical role of the mitochondrial oxidant stress in the mechanism of cell death (Knight et al., 2002, James et al., 2003, Ramachandran et al., 2011b and Saito et al., 2010b). However, it remained unclear how this extensive mitochondrial oxidant stress ultimately can be induced.

Studies during the last decade aimed at elucidating intracellular signaling events have demonstrated a critical role of the mitogen-activated protein kinase (MAPK) c-jun-N-terminal kinase (JNK) in APAP-induced cell death in mice (Gunawan et al., 2006, Henderson et al., 2007 and Latchoumycandane et al., 2007) and in human hepatocytes (Xie et al., 2014). Importantly, JNK was not only activated (phosphorylated) in the cytosol, p-JNK also translocated to the mitochondria (Hanawa et al., 2008) and amplified the mitochondrial oxidant stress (Saito et al., 2010a) through binding to an anchor protein in the outer mitochondrial membrane (Win et al., 2011). It was also shown that an early mitochondrial oxidant stress triggered JNK activation (Hanawa et al., 2008 and Saito et al., 2010a). However, JNK is not a redox-sensitive kinase. In fact, the upstream kinases, apoptosis signal-regulating kinase 1 (ASK1) (Nakagawa et al., 2008) and mixed-lineage kinase 3 (MLK3) (Sharma et al., 2012) have been identified as critical upstream regulators of JNK activation. Oxidant stress can release ASK1 from its complex with reduced thioredoxin by oxidation of thioredoxin resulting in the liberation of the active kinase (Saitoh et al., 1998). Because ASK1 is thought to be mainly responsible for sustained activation of JNK, the substantial reduction in APAP-induced liver injury in ASK1-deficient mice demonstrated the critical role of ASK1 in the pathophysiology (Nakagawa et al., 2008). Recently a potent and selective small molecule inhibitor of ASK1 with properties amenable for *in vivo* efficacy testing in rodents was developed (Gerczuk et al., 2012 and Toldo et al., 2012). This inhibitor enabled us to test if pharmacologic inhibition of ASK has therapeutic potential for treating APAP hepatotoxicity and to study the mechanisms of protection.

6.3 Materials and methods

Animals

8 week old male C57Bl/6 mice were acquired from Jackson Laboratories for the experiments. Animals were housed in a controlled environment with a 12 hour light/dark cycle and free access to food and water. Animals were acclimatized for at least 3 days and fasted overnight before experiments. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center.

Inhibitors

GS-459679 and GS-444217 are potent and selective ATP competitive inhibitors of ASK1 developed by Gilead Sciences, Inc., Foster City, CA (Breckenridge et al., manuscript in preparation). In a competitive, time-resolved fluorescence resonance energy transfer immunoassay, GS-459679 and GS-444217 directly inhibit ASK1 kinase activity with IC50 (concentration that inhibits ASK1 kinase by 50%) values of 6.1 and 2.9 nM, respectively. One micromolar GS-459679 results in 99% inhibition of the ASK1 kinase but does not inhibit 20 other kinases implicated in cellular stress signaling (Gerczuk et al., 2012).

Experimental design

For pre-treatment studies, animals were administered either the ASK1 inhibitor GS-459679 (ASK1i) (10 or 30 mg/kg), or vehicle (55% PEG in H2O) 30 min prior to administration of APAP (300 mg/kg, i.p.). Animals were euthanized 0.5, 6 or 24 h after APAP. For post-treatment experiments, animals were administered APAP (300 mg/kg, i.p.) followed by ASK1i (30 mg/kg, i.p.), N-acetylcysteine (NAC) (500 mg/kg, i.p.),

ASK1i + NAC or vehicle (saline) at 1.5 or 3 h after APAP. Animals were allowed access to regular food at 6 h after APAP treatment; the animals were euthanized at 24 h. For 48 h experiments evaluating liver regeneration, animals were given back food (control diet or diet containing 0.2% of the ASK1 inhibitor GS-444217) 6 h after APAP administration. At the end of the experiments, animals were anesthetized with isoflurane and blood was drawn from vena cava into heparinized syringes for determination of alanine aminotransferase (ALT) activity. The liver was excised and rinsed in saline before being divided for histology and subcellular fractionation, and the rest being snap frozen in liquid nitrogen and subsequently stored at − 80 °C.

Histology

Formalin-fixed tissue samples were embedded in paraffin and 4 μm sections were cut. Replicate sections were stained with hematoxylin and eosin (H&E) to evaluate necrosis as described (Gujral et al., 2002).

Measurement of GSH and GSSG

Total soluble GSH and GSSG were determined in the liver homogenate with a modified method of Tietze (Jaeschke and Mitchell, 1990). In brief, the frozen tissue was homogenized at 0 ℃ in 3% sulfosalicylic acid containing 0.1 mM EDTA. For measurement of GSSG, GSH was trapped with 10 mM N-ethylmaleimide. The sample was centrifuged after dilution with 0.01 N HCl and the supernatant was further diluted with 100 mM potassium phosphate buffer (KPP), pH 7.4. The samples were then assayed using dithionitrobenzoic acid. All data are expressed as GSH-equivalents.

Isolation of subcellular fractions and western blotting

Mitochondria and cytosolic fractions were isolated using differential centrifugation as described (Xie et al., 2013). Western blots were performed as described in detail (Bajt et al., 2000 and Du et al., 2013), using the following antibodies: a rabbit anti-JNK antibody (Cell signaling Technology, Danvers, MA) and a rabbit anti-phospho-JNK antibody #4668, which recognizes Thr183/Tyr185 phosphorylated JNK1/2 protein (Cell Signaling Technology, Danvers, MA). A horseradish peroxidase-coupled donkey anti-rabbit IgG (Santa Cruz) was used as secondary antibody. Proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech. Inc., Piscataway, NJ).

Statistics

All data are presented as mean ± SE. For normally distributed data, comparisons were made between two groups using Student's t-test, or between three or more groups using one-way analysis of variance (ANOVA) with Tukey's post-hoc test. For non-normally distributed data, comparisons were made between two groups using the Mann–Whitney U-test, or between three or more groups using the Kruskal–Wallis test with Dunn's multiple comparisons. All statistical tests were performed using SigmaPlot software (Systat, San Jose, CA). $P < 0.05$ was considered significant.

6.4 Results

Pharmacological inhibition of ASK1 protects against APAP-induced liver injury

As an initial attempt to investigate the effect of ASK1 inhibition on APAP hepatotoxicity, mice were treated with 10 or 30 mg/kg of GS-459679 (ASK1i), 30 min prior to administration of 300 mg/kg APAP. Liver injury was then examined 6 and 24 h later. APAP induced significant liver injury as indicated by the increase in plasma ALT activities, which were elevated at 6 h and further increased by 24 h (Fig. 1A). This was accompanied by development of centrilobular necrosis (Figs. 1B, C). Pretreatment with 10 mg/kg ASK1i did not achieve significant protection against APAP hepatotoxicity up to 24 h. However, pre-treatment with 30 mg/kg ASK1i resulted in a pronounced protection as indicated by the 93% (6 h) and 95.2% (24 h) reduction in plasma ALT activities (Fig. 1A) and 100% (6 h) and 84% (24 h) less centrilobular necrosis (Figs. 1B, C), indicating that pharmacological blockage of ASK1 activity could prevent APAP-induced liver injury. Evaluation of plasma concentrations of ASK1i at 6 h post-APAP administration confirmed a higher exposure of ASK1i in animals administered 30 mg/kg compared to 10 mg/kg (Supplemental Fig. 1). Since the 30 mg/kg dose seemed to be most effective, this dose was used for further experiments. In order to investigate whether the ASK1i treatment protects against the injury by inhibiting drug metabolism, short term experiments were carried out by measuring the depletion of liver GSH at 30 min after APAP as a surrogate marker for NAPQI formation. APAP caused 86% depletion of hepatic GSH levels within 30 min. The initial loss of liver GSH was comparable in animals treated with APAP alone or in combination with either the vehicle or ASK1i, suggesting that the inhibitor did not affect metabolic activation of APAP (Fig. 2).

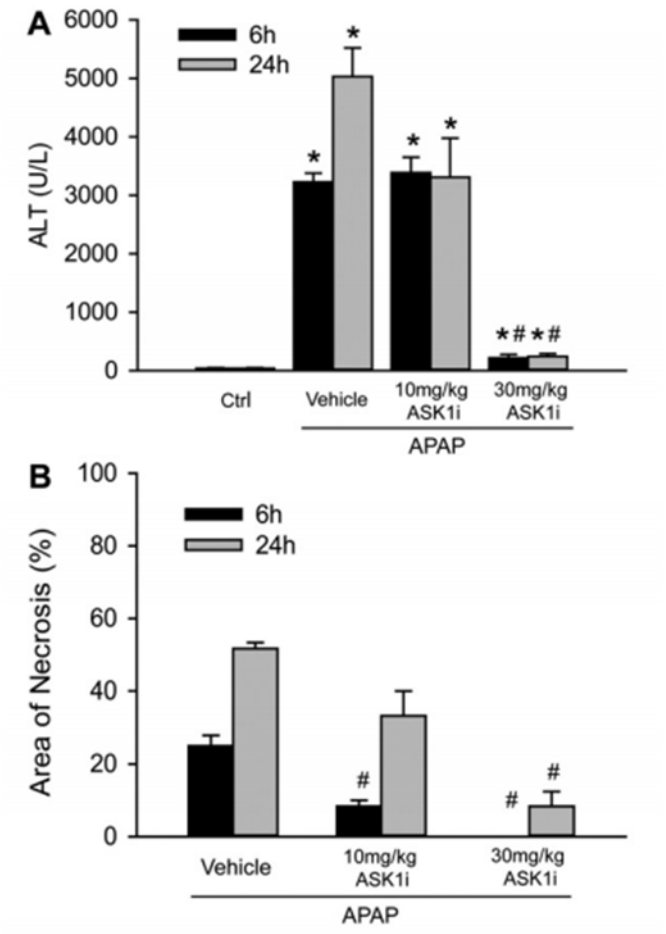


Figure 6.4.1 Protective effect of the ASK1 inhibitor GS-459679 against APAP-induced liver injury.

C57BL/6 mice were pretreated with either 10 or 30 mg/kg GS-459679 or an equivalent dose of the vehicle (55% PEG in water) 30 min before 300 mg/kg APAP administration. (A) Plasma alanine aminotransferase (ALT) activities measured at 6 and 24 h after APAP treatment. (B) Quantitation of the areas of necrosis in H&E-stained liver sections. (C) Representative sections from each group with H&E staining. Data represent means ± SE of n = 4–11 animals per group. *P < 0.05 compared with control (Ctrl) groups, # P < 0.05 compared with corresponding APAP + vehicle groups.

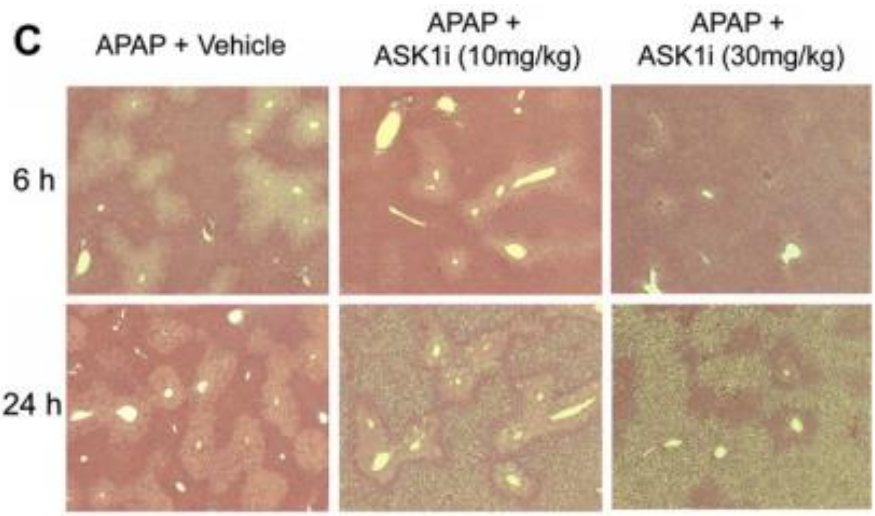


Figure 6.4.1 Protective effect of the ASK1 inhibitor GS-459679 against APAP-induced liver injury.

C57BL/6 mice were pretreated with either 10 or 30 mg/kg GS-459679 or an equivalent dose of the vehicle (55% PEG in water) 30 min before 300 mg/kg APAP administration. (A) Plasma alanine aminotransferase (ALT) activities measured at 6 and 24 h after APAP treatment. (B) Quantitation of the areas of necrosis in H&E-stained liver sections. (C) Representative sections from each group with H&E staining. Data represent means ± SE of n = 4–11 animals per group. *P < 0.05 compared with control (Ctrl) groups, # P < 0.05 compared with corresponding APAP + vehicle groups.

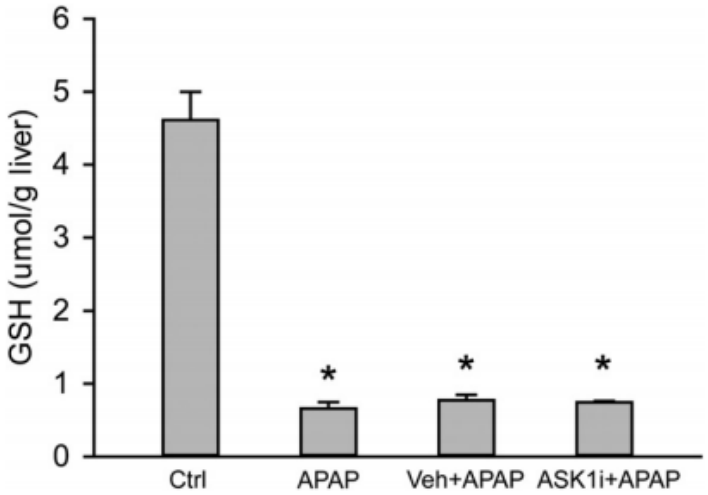


Figure 6.4.2 Effect of ASK1 inhibitor GS-459679 on APAP-induced GSH depletion.

Mice were treated with 30 mg/kg GS-459679 or equivalent amount of vehicle (55% PEG in water) 30 min before 300 mg/kg APAP administration. GSH levels (μmol/g liver) were measured 30 min after APAP treatment. Data represent means ± SE on n = 3–4 animals per group. *P b 0.05 compared with the control group.

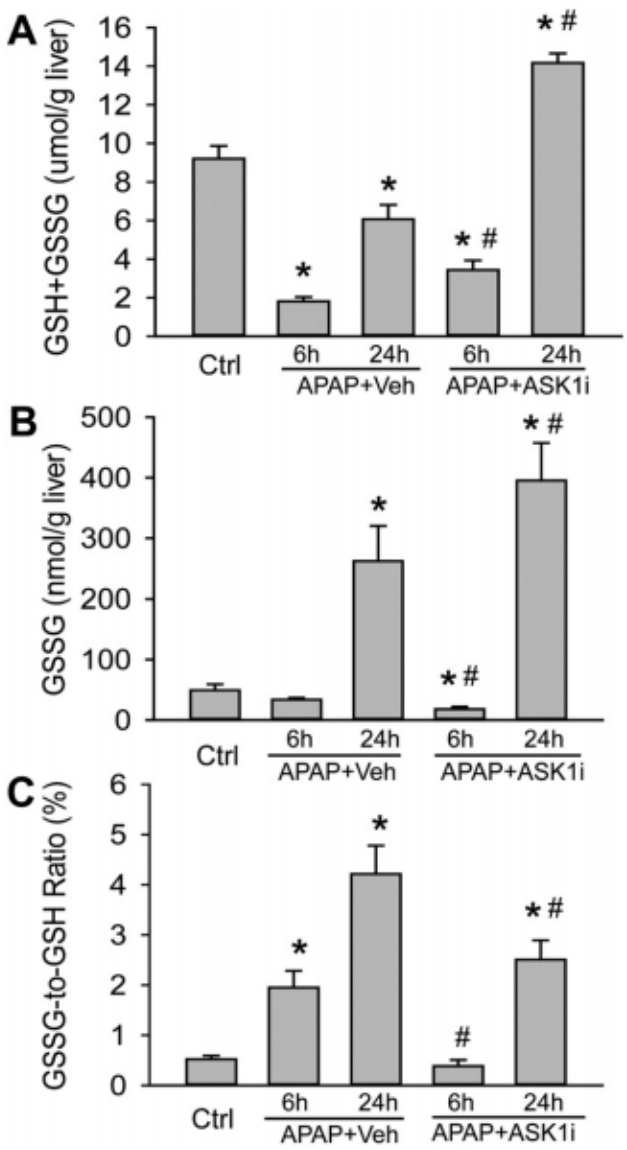


Figure 6.4.3 Effect of the ASK1 inhibitor GS-459679 on hepatic GSH and GSSG levels after APAP treatment.

Mice were treated with 30 mg/kg GS-459679 or equivalent amount of vehicle (55% PEG in water) 30 min before 300 mg/kg APAP administration. GSH levels were measured either 6 or 24 h after APAP treatment. (A) Total liver glutathione (GSH + GSSG) in μmol/g liver. (B) Glutathione disulfide (GSSG) in nmol/g liver. (C) GSSG-to-GSH ratio. Data represent means ± SE of n = 4–7 animals per group.*P < 0.05 compared with control (Ctrl) groups, # P b 0.05 compared with APAP + vehicle groups.

Assessment of GSH content and oxidant stress (GSSG-to-GSH ratio) after APAP treatment indicated a partial recovery of hepatic GSH levels at 6 and 24 h (Fig. 3A), a significant increase in GSSG levels at 24 h (Fig. 3B) and a significantly higher GSSG-to-GSH ratio at 6 and 24 h (Fig. 3C). Treatment with 30 mg/kg ASK1i remarkably enhanced the recovery of GSH levels (Fig. 3A) and prevented (6 h) or attenuated the oxidant stress (24 h) (Fig. 3C).

ASK1 inhibition prevents activation of JNK subsequent to APAP overdose

JNK activation has been implicated in APAP hepatotoxicity and ASK1 is suggested to function upstream of JNK in this pathological context (Nakagawa et al., 2008). To confirm the mechanism of action of the pharmacological ASK inhibitor, the next series of experiments evaluated JNK activation after APAP overdose. APAP treatment caused significant JNK activation as demonstrated by elevated levels of the Thr183/Tyr185 phosphorylated protein, which peaked by 6 h and decreased by 24 h (Fig. 4A). Pretreatment with the ASK1i almost completely prevented JNK activation at 6 h (Fig. 4A). The changes in JNK phosphorylation were not due to alterations in total JNK protein, since these were unaltered at both 6 and 24 h after APAP (Fig. 4A). Quantitative densitometric analysis confirmed the changes in the p-JNK and total JNK levels (Figs. 4B, C).

Inhibition of ASK1 prevents APAP-induced translocation of JNK to mitochondria

JNK translocation to mitochondria is a key mechanistic feature of APAP-induced hepatotoxicity and mitochondrial permeability transition (Hanawa et al., 2008). Since administration of ASK1i prevented JNK activation, the effect of this intervention on

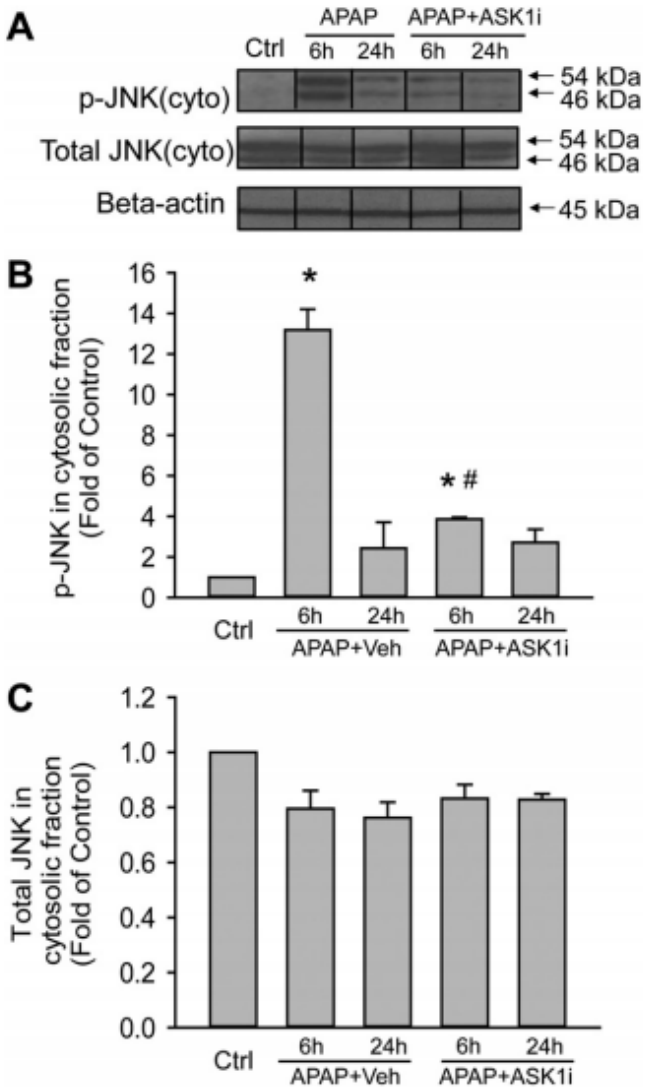


Figure 6.4.4 Effect of the ASK1 inhibitor on JNK activation.

Western blot analysis (A) of p-JNK and total JNK in the cytosolic fraction of livers from untreated mice (Ctrl), mice treated with 30 mg/kg ASK1 inhibitor or mice treated with the same volume of vehicle (55% PEG in water) 30 min prior to 300 mg/kg of APAP administration. Liver samples were obtained 6 or 24 h after APAP treatment. Densitometric quantification was performed on bands of p-JNK (B) and total JNK (C). Data represent means ± SE of n = 4 animals. *$P < 0.05$ compared with control groups. # $P < 0.05$ compared with APAP + vehicle group.

mitochondrial JNK translocation was examined. Treatment with APAP resulted in translocation of activated JNK (p-JNK) to the mitochondria by 6 h and this was significantly inhibited by ASK1i pretreatment (Fig. 5). The APAP-induced elevation in p-JNK was accompanied by increases in total JNK in mitochondria and this was prevented by administration of ASK1i (Fig. 5). This suggests that JNK translocation to mitochondria occurs subsequent to activation, implying that ASK1 inhibition in the context of APAP overdose prevents JNK activation and downstream translocation of p-JNK to mitochondria.

ASK1i administration can protect against liver injury even when given after APAP overdose

To examine if ASK1i administration was also effective in a therapeutic setting, the next series of experiments compared ASK1i with N-acetylcysteine, which is the current standard of care in the clinic for APAP overdose. Animals treated with a single dose of 500 mg/kg NAC 1.5 h after APAP were significantly protected against liver injury at 24 h as indicated by both ALT activities and area of necrosis (Fig. 6A). However, this protection was significantly eroded when NAC was administered 3 h after APAP, although ALT levels were still significantly less than controls (Fig. 6A). Animals treated with ASK1i 1.5 h after APAP also showed significant protection against liver injury when compared to vehicle-treated animals but when ASK1i administration was delayed to 3 h post APAP, no protection against injury was evident (Fig. 6B). When animals were treated with both NAC and ASK1i, protection was almost complete when the combination was given at 1.5 h after APAP (Fig. 6C). However, delaying the drug administration to either 3 h (Fig. 6C) or 2 h and 15 min (data not shown) after APAP

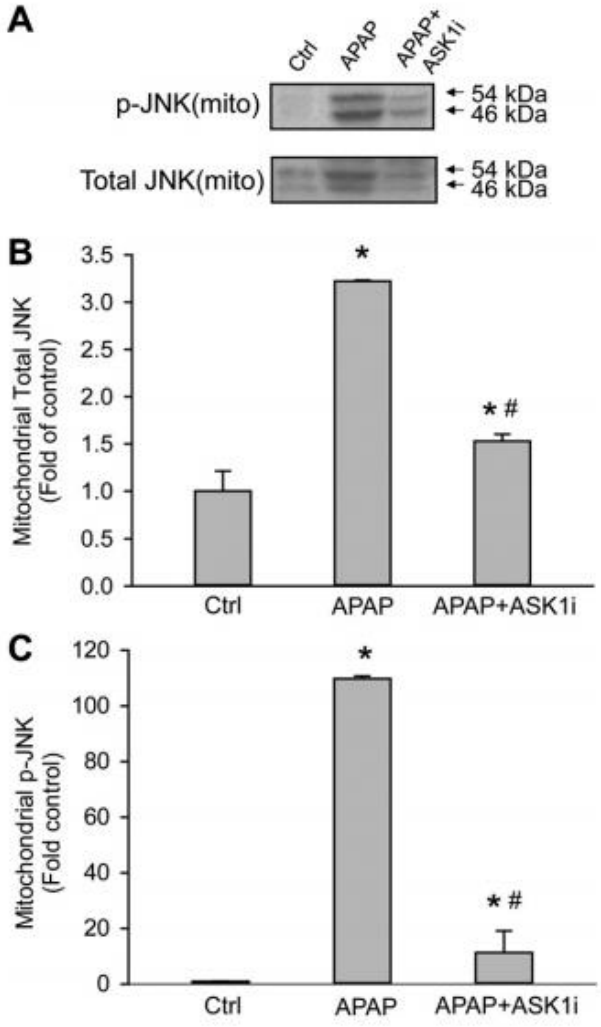


Figure 6.4.5 Western blots and densitometric analysis of p-JNK translocation to the mitochondria at 6 h.

Mice were treated with 30 mg/kg ASK1 inhibitor GS-459679 or the vehicle (55% PEG in water) 30 min before 300 mg/kg APAP injection. Western blotting was performed for p-JNK and total JNK in mitochondria (A). Densitometric quantification was carried out on band intensity of both total JNK (B) and p-JNK (C). Data represent means ± SE of n = 4 animals per group. *P < 0.05 compared with control (Ctrl) group, # P < 0.05 compared with APAP + vehicle group.

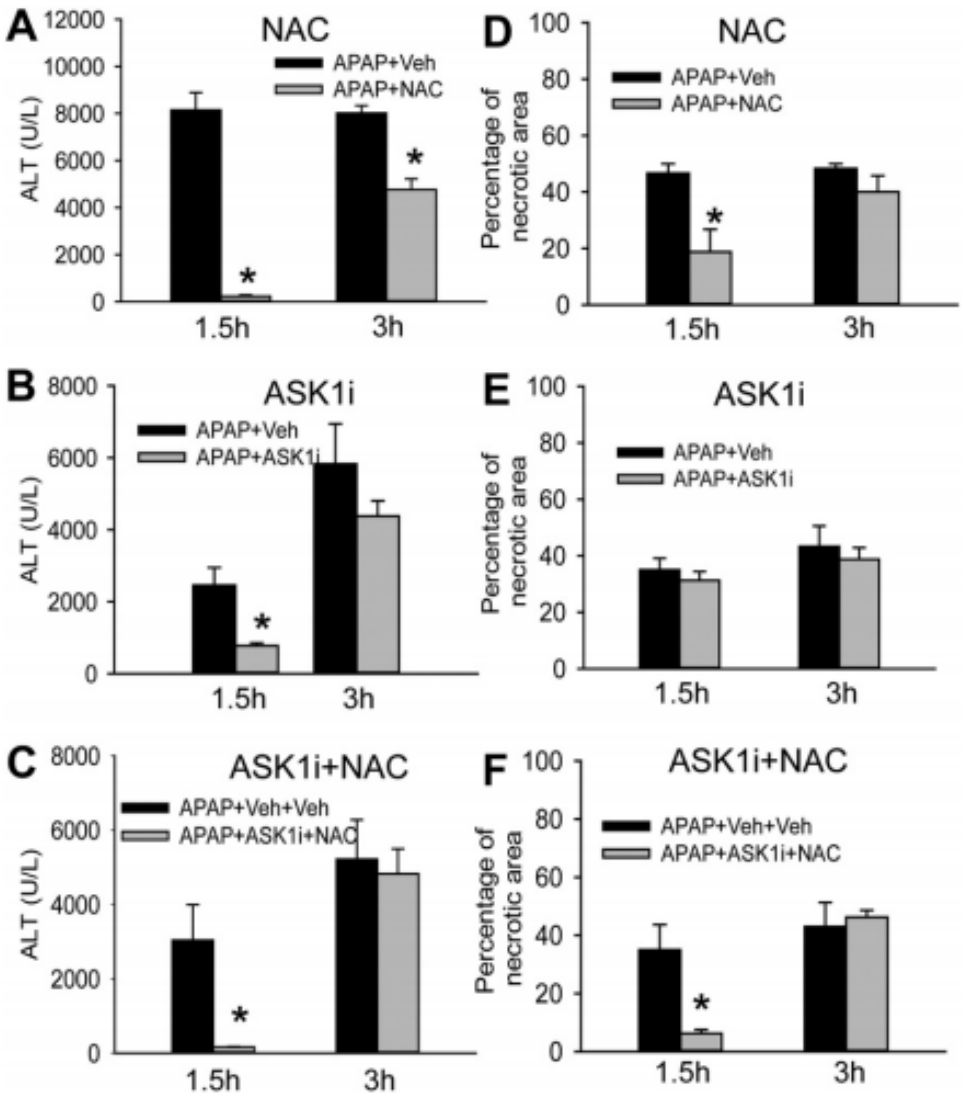


Figure 6.5.6 Effect of ASK1 inhibitor GS-459679 post-treatment against APAP hepatotoxicity at 24 h.

Mice were treated with 500 mg/kg N-acetylcysteine (NAC) (A), 30 mg/kg ASK1i (B) and the combination of both (C) either 1.5 or 3 h after 300 mg/kg of APAP administration. The vehicle for NAC was PBS, for ASK1i was 55% PEG, and for ASK1i + NAC it was PBS + 55% PEG. Plasma ALT activities and the areas of necrosis were quantified. Data represent means ± SE of 3–5 animals per group and time point. *P b 0.05 compared with the respective APAP + vehicle treated group

negated this protection. This suggests that ASK1 inhibition is only effective early after APAP administration (within 1.5 h in the mouse) and loses its efficacy relatively rapidly after that time.

ASK1i administration after APAP overdose can also prevent JNK activation in the cytosol and its translocation to the mitochondria. To examine if the mechanism of protection by ASK1i 1.5 h post treatment also involved prevention of JNK activation, cytosolic JNK activation and its translocation to mitochondria were examined by western blotting at 6 h after APAP. The APAP-induced JNK activation in the cytosol was significantly reduced by NAC, ASK1i or NAC + ASK1i post treatment, all of which also effectively prevented p-JNK translocation to mitochondria (Fig. 7). To evaluate the reason for the limited therapeutic window for the ASK1i inhibitor, early JNK activation and mitochondrial p-JNK translocation were determined (Fig. 8). Limited APAP-induced JNK activation in the cytosol was observed as early as 1 h with almost no p-JNK translocation to the mitochondria. However, by 2 h, massive JNK activation and extensive mitochondrial p-JNK translocation had occurred (Fig. 8). This suggests that ASK1 inhibition is only effective if it occurs before mitochondrial p-JNK translocation.

ASK1 inhibitor administration does not impair liver regeneration after APAP

A major concern about inhibition of the JNK pathway is the fact that it may lead to impairment of liver regeneration (Schwabe et al., 2003). To test whether pharmacological inhibition of ASK1 reduces the regenerative capacity of the liver, expression of proliferating cell nuclear antigen (PCNA), which is a marker of regeneration, was assessed. APAP hepatotoxicity caused significant up-regulation of PCNA expression at

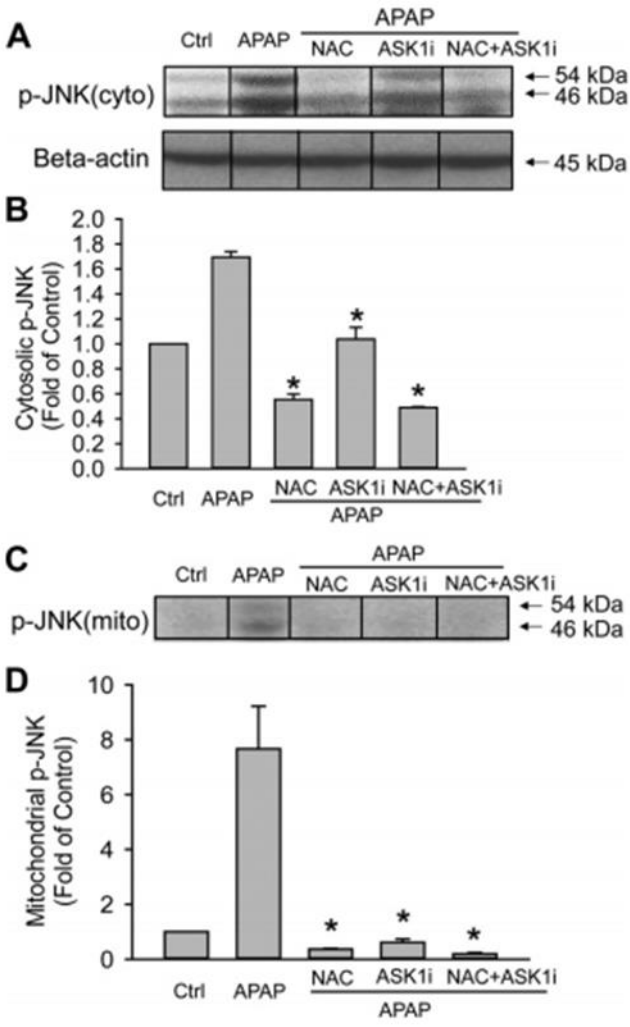


Figure 6.5.7 Effect of ASK1 inhibitor GS-459679 post-treatment on APAP-induced JNK activation.

Western blots (A) and densitometry analysis of p-JNK in the cytosolic (B) and mitochondrial (C) fractions. Mice were treated with 500 mg/kg N-acetylcysteine (NAC) (A), 30 mg/kg ASK1i (B) and the combination of both (C) 1.5 h after administration of 300 mg/kg APAP. The vehicle for NAC was PBS, for ASK1i was 55% PEG in water, and for ASK1i + NAC it was PBS + 55% PEG. Data represent means ± SE of 3–5 animals per group. *P < 0.05 compared with APAP + vehicle treated group.

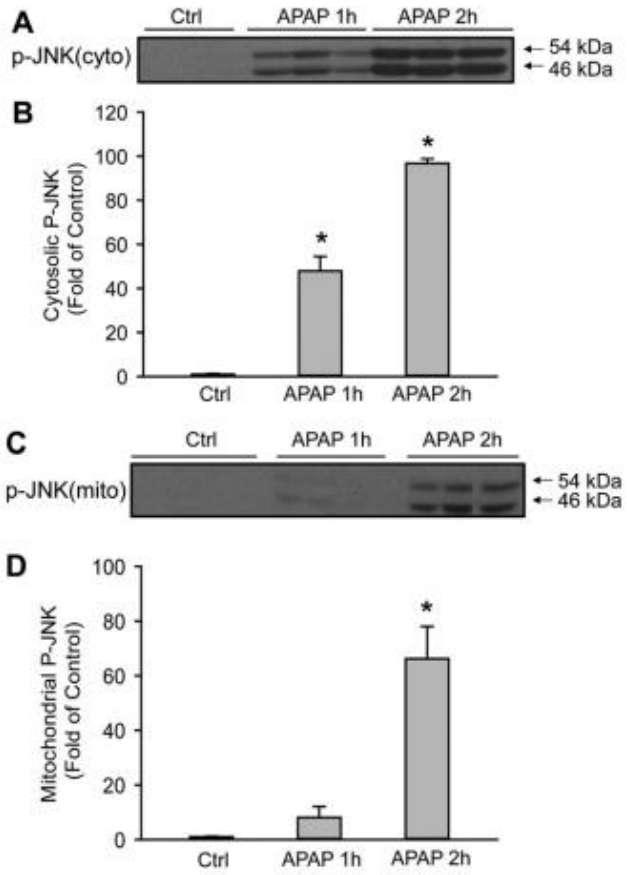


Figure 6.5.8 Time course of JNK activation after APAP treatment.

P-JNK was determined in cytosol (A, B) and in mitochondrial fraction (C, D) after treatment with 300 mg/kg APAP. Data represent means ± SE of 3 animals per group. *P < 0.05 compared with control (Ctrl).

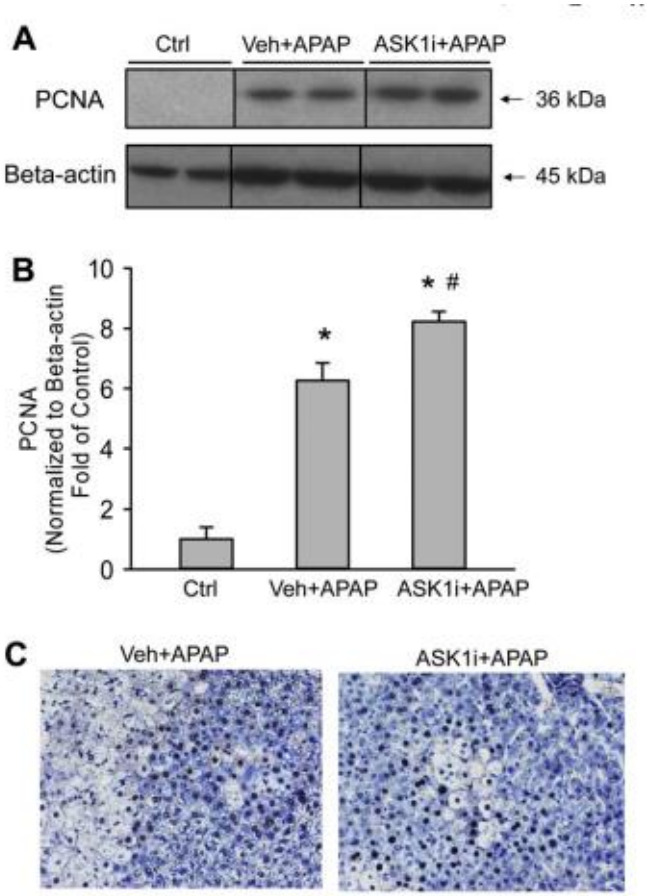


Figure 6.5.9 Effect of the ASK1 inhibitor on the regeneration response after APAP-induced liver injury.

Proliferating cell nuclear antigen (PCNA) levels was determined by western blotting (A) and immunohistochemistry (C) 24 h after APAP in animals treated either with vehicle (55% PEG in water) or 30 mg/kg ASK1 inhibitor 1.5 h after APAP. Densitometry data (B) represent means ± SE of 4 animals per group. *P < 0.05 compared with control (Ctrl) group, # P < 0.05 compared with APAP + vehicle group

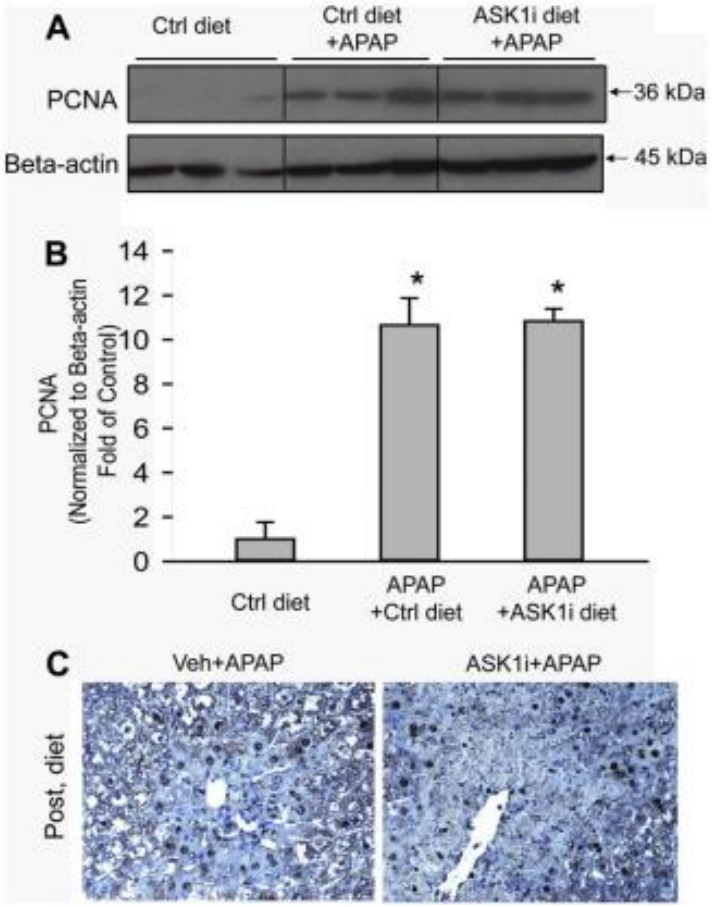


Figure 6.5.10 Effect of chronic ASK1 inhibitor (GS-444217) treatment on the regeneration response after APAP-induced liver injury.

Proliferating cell nuclear antigen (PCNA) levels were determined by western blotting (A) and immunohistochemistry (C) 48 h after APAP in animals fed an ASK1 inhibitor-containing diet or control diet beginning at 6 h after 300 mg/kg APAP. Densitometry data (B) represent means ± SE of 4 animals per group. *P < 0.05 compared with control (Ctrl) group, #P < 0.05 compared with APAP + control diet.

24 h, an effect which was amplified by administration of ASK1i either 1.5 h post-treatment (Figs. 9A, B) or 30 min pretreatment (data not shown). Histological assessment of PCNA staining confirmed the western blot data (Fig. 9C). We also evaluated if continuous inhibition of ASK1 following APAP-induced liver injury impaired liver regeneration. To that end, animals were administered APAP and then 6 h later provided food with 0.2% by weight of the ASK1 inhibitor GS-444217 and PCNA staining was evaluated 48 h post-APAP administration. GS-444217 was utilized for this study because it has excellent absorption properties such that high steady state plasma concentrations are achieved when it is administered in rodent chow (Supplemental Fig. 2 and Gilead unpublished data). Consistent with the data from pretreatment or post-treatment of ASK1i (GS-459679), the increased PCNA expression was not impaired by GS-444217 in the diet following APAP administration (Figs. 10A, B). These results were confirmed by PCNA staining in histological sections (Fig. 10C). We confirmed that GS-444217 had a similar protective effect on APAP-induced liver injury as GS-459679 when administered 30 min prior to APAP (Supplemental Fig. 3). Together, these data suggest that ASK1 inhibition does not interfere with the regeneration process after APAP hepatotoxicity.

6.5 Discussion

The objective of the investigation was to evaluate the protective effect of the ASK1 inhibitor GS-459679 in a murine model of APAP hepatotoxicity. Our data showed that pretreatment with the ASK1 inhibitor and treatment during a limited therapeutic window after APAP overdose attenuated liver injury.

The ASK1 inhibitor protects against APAP hepatotoxicity by inhibition of JNK

ASK1 is a serine/threonine protein kinase, which can activate the MAPK cascade resulting in the activation of p38 and JNK. ASK1 is expressed in both cytoplasm and mitochondria and can be activated by ROS or cytokines such as tumor necrosis factor-α (Ichijo et al., 1997, Nishitoh et al., 1998 and Matsuzawa et al., 2005). Under basal conditions, ASK1 is bound and repressed by the reduced form of thioredoxin (Saitoh et al., 1998). However, during oxidative stress, ASK1 is released from the oxidized form of thioredoxin and activated by an autocatalytic mechanism (Liu et al., 2000), allowing it to induce the phosphorylation of MAP2 kinases that phosphorylate and activate downstream targets such as JNK. It has been demonstrated that mice deficient in ASK1 were significantly protected against APAP hepatotoxicity (Nakagawa et al., 2008). Our data show that pharmacological inhibition of ASK1 with a potent and highly selective small molecule ASK1 inhibitor can attenuate APAP-induced liver injury as indicated by reduced JNK activation, lower oxidant stress and ALT release and limited centrilobular necrosis.

The importance of JNK activation and p-JNK translocation to the mitochondria as a mechanism to amplify the mitochondrial oxidant stress has been well established in mice

(Hanawa et al., 2008 and Saito et al., 2010a) and also recently in primary human hepatocytes (Xie et al., 2014). The fact that the ASK1 inhibitor attenuated JNK activation in the cytosol, largely prevented the translocation of p-JNK to the mitochondria and reduced the mitochondrial oxidant stress was consistent with an effect upstream of JNK activation. Several other kinases including mixed-lineage kinase 3 (MLK3) (Sharma et al., 2012) and glycogen synthase kinase 3β (GSK3β) (Shinohara et al., 2010) were shown to be involved in JNK activation during APAP-induced liver injury. Although MLK3 can also be activated by oxidant stress, MLK3 is responsible for GSK3β and JNK activation (Sharma et al., 2012). In contrast, ASK1 is considered to be mainly involved in the prolonged late JNK activation (> 4 h post-APAP administration) (Nakagawa et al., 2008). The importance of the prolonged JNK activation for APAP-induced liver injury is supported by studies in JNK-deficient mice (Gunawan et al., 2006 and Henderson et al., 2007), other studies demonstrating that liver injury is decreased when oxidative stress and sustained JNK activation are decreased; for example in female mice (Du et al., 2014), in Fas receptor-deficient lpr mice (Williams et al., 2013), or in mice treated with allopurinol (Williams et al., 2014). In addition, the increased liver injury after APAP overdose in animals deficient in phosphatases, which are known to counteract JNK phosphorylation, further supports the critical role of the ASK1-induced JNK activation cascade at least in the mouse (Mobasher et al., 2013 and Wancket et al., 2012).

The role of ASK1 and JNK activation during APAP overdose in humans remains unclear. More recent translational studies support the hypothesis that protein adduct formation and mitochondrial dysfunction are important for the human pathophysiology (Davern et al., 2006, McGill et al., 2012a and McGill et al., 2014c). Although APAP causes extensive

JNK activation and mitochondrial p-JNK translocation in primary human hepatocytes, the protection by inhibition of JNK was modest (Xie et al., 2014). In contrast, APAP did not induce JNK activation in the metabolically competent human hepatoma cell line HepaRG (Xie et al., 2014), which is, however, still susceptible to APAP-induced cell injury involving oxidant stress and mitochondrial dysfunction (McGill et al., 2011). Thus, whereas the relevance of ASK1 and JNK activation is well documented in the murine system, the importance of ASK1 in the human pathophysiology of APAP-induced liver injury remains to be further investigated.

The therapeutic window of pharmacological ASK1 inhibition in APAP hepatotoxicity

In addition to the efficacy of pretreatment with the ASK1 inhibitor, our study also demonstrated that administration of the ASK1 inhibitor during a narrow therapeutic window after APAP overdose can still be effective. However, the ASK1 inhibitor was not more effective than NAC treatment, the standard of care in patients. In addition, the combined treatment of NAC and ASK1 inhibitor did not show a relevant additive effect. These results indicate that both interventions target the same mechanism in the pathophysiology. The faster recovery of hepatic GSH levels mediated by treatment of NAC after the metabolism phase leads to enhanced scavenging of reactive oxygen and peroxynitrite (Knight et al., 2002, James et al., 2003 and Saito et al., 2010b). Inhibition of ASK1 reduces JNK activation, which is critical for the amplification of the mitochondrial oxidant stress (Hanawa et al., 2008 and Saito et al., 2010a). Thus, both interventions attenuate the mitochondrial oxidant stress, which is responsible for the MPT pore opening and necrosis (Kon et al., 2004 and Reid et al., 2005).

The limited therapeutic window of ASK1 inhibition in mice may be related to the rapid JNK activation and subsequent translocation to the mitochondria. The ASK1 inhibitor was effective only when it was administered before significant p-JNK had translocated to the mitochondria, suggesting the primary mechanism of action of the ASK1 inhibitor was to prevent JNK activation and translocation. After p-JNK has translocated to the mitochondria, the amplification of the oxidant stress may involve other kinases in a feed-forward mechanism, which no longer depends on ASK1 alone or may involve JNK-independent mechanisms (Saberi et al., 2014). In either case, these data are not consistent with the original hypothesis that ASK1 is only responsible for the late JNK activation. Our data suggest that ASK1 inhibition is required relatively early (< 1.5 h after APAP) in order to be effective.

The limited therapeutic window for ASK1 inhibition in mice may not translate to the human pathophysiology, which is generally delayed compared to mice with peak injury at 36–48 h in humans and around 12 h in mice. The reason for the delay in human hepatocytes appears to be related to delay in mitochondrial adduct formation, JNK activation and p-JNK translocation to the mitochondria after APAP overdose (Xie et al., 2014). As a consequence, NAC treatment is still 100% protective even if initiated 6 h after APAP and still partially effective when given 15 h after APAP exposure (Xie et al., 2014). In addition, a JNK inhibitor was still effective when administered 3 h after APAP in human hepatocytes (Xie et al., 2014). Thus, the therapeutic window for the ASK1 inhibitor needs to be assessed in humans but it can be expected to be substantially larger than in mice.

Effect of the ASK1 inhibitor on regeneration

Although JNK activation has been associated with mechanisms of liver injury in many disease models including APAP hepatotoxicity (Czaja, 2003 and Gunawan et al., 2006), JNK can phosphorylate c-jun and activate the transcription factor AP-1, which can promote regeneration by Cyclin D1 gene expression (Schwabe et al., 2003). Because regeneration is critical for recovery and survival after APAP-induced liver injury (Apte et al., 2009, Bajt et al., 2003 and Bhushan et al., 2013), and because other kinases can activate JNK independently of ASK1, it was important to evaluate if the ASK1 inhibitor affects regeneration as assessed by the expression of PCNA in the recovering liver. Similar to previous interventions such as GSH or NAC (Bajt et al., 2003), which reduced APAP-induced liver injury, the ASK1 inhibitor actually enhanced PCNA expression. However, using a second ASK1 inhibitor with high oral availability in the diet starting 6 h after APAP had no effect of PCNA expression during the recovery phase at 48 h. Thus, our data demonstrated that 2 different ASK1 inhibitors protected against APAP hepatotoxicity and did not inhibit regeneration. Second, the inhibitor GS-444217, which has better oral availability than GS-459679, allowed continuous inhibition of ASK1 for several days. The fact that no effect on regeneration was observed supports the hypothesis that this inhibitor could be used therapeutically. Together, these findings indicate that inhibition of ASK1-mediated JNK activation can attenuate liver injury but does not affect cell cycle activation and regeneration after APAP-induced liver injury.

In summary, our data demonstrate that the specific and selective ASK1 inhibitor GS-459679 protected against APAP-induced liver injury by inhibiting JNK activation, p-JNK translocation to the mitochondria and oxidant stress. The inhibitor was effective in this murine model as pretreatment and during a limited therapeutic window also when

administered after APAP. In addition, the ASK1 inhibitor did not inhibit regeneration after APAP-induced liver injury. Because of the delayed JNK activation and delayed cell injury process in humans, small molecule ASK1 inhibitors may be suitable as therapeutic interventions against APAP-induced liver injury.

Chapter 7. Discussion and Future Directions

7.1 Discussion- testing interventions on APAP-induced hepatotoxicity

Exploring superior therapeutic options is central in APAP toxicity studies, and research on protective interventions has boomed in recent years. Overall, targeting every single step of the injury process holds promise for a potential therapy of APAP-induced liver injury. However, several concerns need to be addressed during the mechanistic testing.

First, are we targeting relevant cellular events? The failed attempts on caspase inhibitors serve as a good example, as apoptosis does not play a crucial role in the pathogenesis of APAP toxicity (Lawson et al., 1999; Williams et al., 2010). Similarly, interventions on sterile inflammation pathways are also unable to provide satisfactory results (Jaeschke et al., 2012).

Second, are there any pharmacological molecules available to abrogate the contributing event? Genetic models such as gene knockout mice could provide valuable mechanistic insights; however, it is the functional and specific small molecule inhibitors, which may translate findings in animals to humans.

Third, are those interventions practically useful? For examples, inhibiting APAP metabolism seems the perfect strategy to prevent the hepatotoxicity as it solves the root of the problem. However, drug metabolism happens within the first few hours after drug ingestion while the overdose patients usually seek for medical attention when symptoms of toxicity developed, i.e. mainly after drug metabolism. With that, blocking APAP metabolism is not a realistic approach to prevent APAP-induced liver injury.

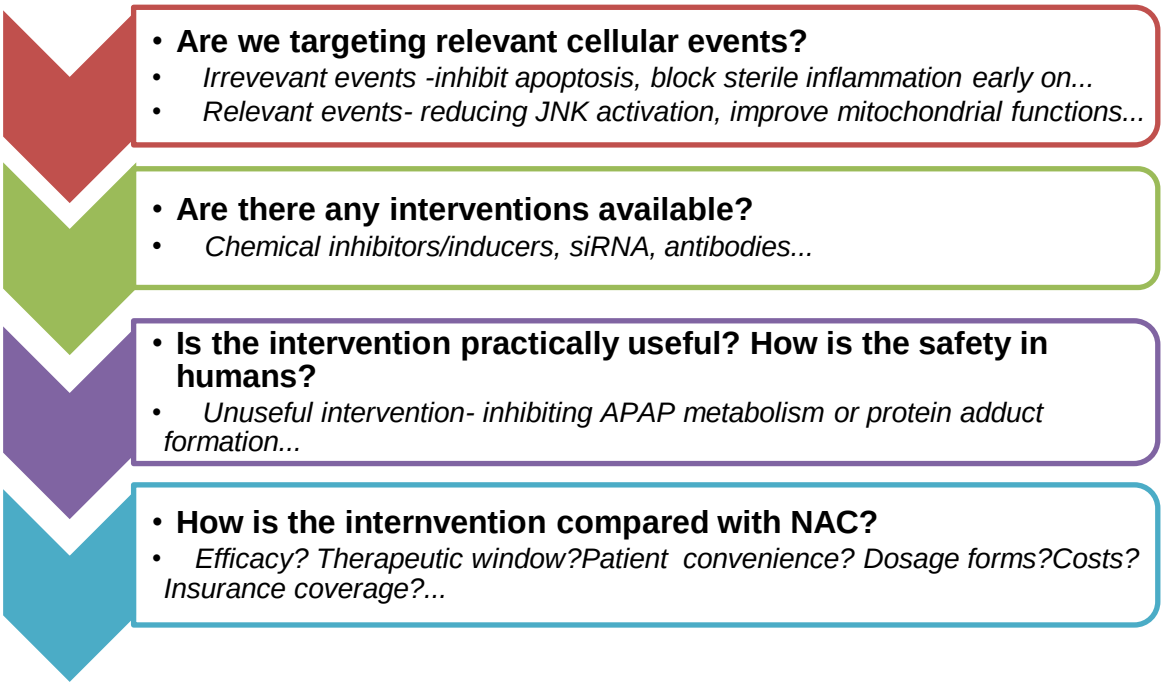


Figure 7.1 A mechanistic evaluation algorithm for potential interventions on APAP hepatotoxicity

Last but not least, how is the intervention compared with NAC? No matter what the protective mechanisms of the potential therapies are, it will be compared to the standard of care -NAC treatment. For any potential therapeutic option, a superior efficacy and less toxicity than NAC would argue for its market approval by FDA. However, unlike cancer treatments, APAP toxicity does not affect as many patients and the costs of developing a new drug are prohibitive. Therefore, the best chance of introducing a new therapeutic intervention against APAP toxicity would be to repurpose an existing drug approved for human use. With that, comparisons with existing treatment are absolutely critical. Although molecules like ASKi and JNKi meet all qualifications mentioned above and could alleviate APAP-induced liver injury *in vivo*, however, the inferior efficacies compared with NAC pose an obstacle for their translation to clinical trials and market approval (Xie et al., 2014; Xie et al., 2015c). Future efforts are warranted for exploration of treatment options which requires legitimate mechanistic studies of APAP hepatotoxicity.

As summarized in Table 7.1, an evaluation algorithm could be applied when new interventions are tested in the APAP hepatotoxicity model. Among the extensive studies on APAP toxicity, the majority fail to validate one or more steps in the proposed algorithm.

7.2 Future directions

APAP overdose patients represent the largest group of both acute liver failure and drug-induced liver injury. Therefore, further understanding of the mechanistic events is not only critical for clinical diagnosis and treatment, but may also reveal novel therapeutic intervention strategies that could be targeted by drugs. A few directions worth exploring in the future are discussed here.

First, further investigations are required on the cellular signaling pathways contributing to the pathogenesis after APAP. Although a basic signaling network has been established, however, knowledge gaps still exist (see above in Chapter 1"Introduction"). In retrospect, the exploration of contributing signaling pathways after APAP is not a random shot, and several hints have led to the identification of potential targets. Among those hints, one is the extrapolation from classical cell death/survival models like TNF-α mediated signal transductions to the APAP model. This extrapolation has led to the research on JNK and subsequently a series of upstream and downstream mediators including ASK1, MLK3, GSK3β, Sab, RIP1, and RIP3. Another lead is mitochondrial dysfunction. The mitochondrion is the most critical target among all organelles in APAP, and it controls cellular activities including energy supply, cell death, ROS formation, Ca^{2+} homeostasis, while it also undergoes mitochondrial dynamics including mitochondrial fission, fusion and removal by processes like mitophagy. All these events are closely related to liver pathogenesis after APAP and other liver diseases. Future efforts could continue to be devoted to mitochondrial biogenesis and programmed necrosis pathways which encompass a number of potential targets. Undoubtedly, more and more signaling

pathways and mediators will be revealed in the future, which will facilitate the identification of new potential therapeutic targets.

Second, improvement of study models is warranted. As there is no perfect model but only useful models, all the current *in vivo* or *in vitro* systems for studying APAP hepatotoxicity bear certain advantages and disadvantages as summarized in Table 7.2.1. However, approaches to improve existing models are ongoing. For example, primary human hepatocytes (PHH) are the most preferred *in vitro* model to test drug toxicity; however, two major obstacles prevent the widespread usage of PHH, which are limited availability and cell dedifferentiation over time. To overcome the former, cryopreserved PHH were introduced which allows better and more controlled access to PHH when needed. For the latter, more advanced PHH culture systems like 3D culture or sandwich culture systems are adapted. Although problems exist as to the improved systems, more relevant models could reveal different aspects of the injury mechanisms.

Third, the search for mechanism-based biomarkers needs to be continued. The importance of biomarkers is generally acknowledged for both clinical applications and drug development. Traditional clinical endpoints for liver dysfunction are unspecific and are unable to predict patient outcomes. Previous efforts have led to the discovery of a battery of biomarkers, including HMGB1 (Antonie et al., 2009& 2012), keratin-18 (Antonie et al., 2009& 2012), micro RNAs (Ward et al., 2014; Vliegenthart et al., 2015), APAP-protein adduct (Daven et al., 2006; McGill et al., 2013), mtDNA, nuclear DNA (McGill et al., 2012a &2014c), glutamate dehydrogenase (GDH) (McGill et al., 2012a), argininosuccinate synthetase (McGill et al., 2014a), acylcarnitines (McGill et al., 2014b), glycodeoxycholic acid (Woolbright et al., 2014) and others. However, several

disadvantages exist, including the still undefined mechanisms in humans, lack of prognostic information or limited improvement over currently used clinical panels, all of which largely restrict their clinical utility. Therefore, the translational applicability of theses existing biomarkers require further testing. Additional explorations of biomarkers based on thorough understanding of injury mechanisms are undoubtedly beneficial for either clinical applications or drug development.

Fourth and as mentioned above in 7.1, the pursuit of novel therapeutic options for APAP are critical, which requires better understanding of the injury process from every aspect. As discussed in detail in 1.4, current treatment strategies include enhance antioxidant defense, targeting signaling pathways, promoting liver regeneration, enhancing autophagy, blocking mitochondria MPT and so on. However, none of them have managed to outperform the standard antidote NAC. Given that NAC is most effective if given early on after APAP ingestion, further investigations of the late injury mechanisms and liver regeneration are indispensable for providing new therapeutic targets and better treatment options.

Other future directions include the exploration of macromolecules adducted by NAPQI, as no specific proteins have been found to be fully responsible for the injury.

The importance of APAP-induced hepatotoxicity has gone beyond the toxicity of a single OTC, as it also constitutes the most prevalent type of ALF clinically in the US, a classic reference model of DILI for industrial drug development, and a human-relevant system to study cellular events like ROS, mitochondrial dysfunction and protein adduct formation.

With that, every step towards a better understating of the pathogenesis after APAP is beneficial for all sides.

Table 7.2.1 Advantages and disadvantages of models currently used for studies of APAP-induced hepatotoxicity

Models	Advantages	Disadvantages
Mice	Accessible; Relevant	Gender differences; Accelerated progression of injury
Rats	Accessible	Highly resistant to APAP
HepaRG cell line	Relevant, express various drug-metabolizing enzymes	From a single donor; Still a cancer cell line
HepG2 cell line	Accessible	Low levels of CYPs; Still a cancer cell line
Cryopreserved human hepatocytes	Large quantities; accessible	Low viability after thawing
Plasma samples from APAP overdose patients	Relevant	Limited number of patients; No experimental intervention
Primary human hepatocytes	Gold standard to study DILI; Exp intervention	Sporadic availability; High costs

Chapter 8. References

References

Abdelmegeed, M. A., Jang, S., et al. (2013). "Robust protein nitration contributes to acetaminophen-induced mitochondrial dysfunction and acute liver injury." Free Radic Biol Med **60**: 211-222.

Allen, A. L. (2003). "The diagnosis of acetaminophen toxicosis in a cat." Canadian Veterinary Journal-Revue Veterinaire Canadienne **44**(6): 509-510.

Amaral, S. S., Oliveira, A. G., et al. (2013). "Altered responsiveness to extracellular ATP enhances acetaminophen hepatotoxicity." Cell Commun Signal **11**(1): 10.

Antoine, D. J., Jenkins, R. E., et al. (2012). "Molecular forms of HMGB1 and keratin-18 as mechanistic biomarkers for mode of cell death and prognosis during clinical acetaminophen hepatotoxicity." J Hepatol **56**(5): 1070-1079.

Antoine, D. J., Williams, D. P., et al. (2009). "High-mobility group box-1 protein and keratin-18, circulating serum proteins informative of acetaminophen-induced necrosis and apoptosis in vivo." Toxicol Sci **112**(2): 521-531.

Antoniades, C. G., Quaglia, A., et al. (2012). "Source and characterization of hepatic macrophages in acetaminophen-induced acute liver failure in humans." Hepatology **56**(2): 735-746.

Apte, U., Singh, S., et al. (2009). "Beta-catenin activation promotes liver regeneration after acetaminophen-induced injury." Am J Pathol **175**(3): 1056-1065.

Bajt, M. L., Cover, C., et al. (2006). "Nuclear translocation of endonuclease G and apoptosis-inducing factor during acetaminophen-induced liver cell injury." Toxicol Sci **94**(1): 217-225.

Bajt, M. L., Farhood, A., et al. (2008). "Mitochondrial bax translocation accelerates DNA fragmentation and cell necrosis in a murine model of acetaminophen hepatotoxicity." J Pharmacol Exp Ther **324**(1): 8-14.

Bajt, M. L., Knight, T. R., et al. (2004). "Acetaminophen-induced oxidant stress and cell injury in cultured mouse hepatocytes: protection by N-acetyl cysteine." Toxicol Sci **80**(2): 343-349.

Bajt, M. L., Lawson, J. A., et al. (2000). "Protection against Fas receptor-mediated apoptosis in hepatocytes and nonparenchymal cells by a caspase-8 inhibitor in vivo: evidence for a postmitochondrial processing of caspase-8." Toxicol Sci **58**(1): 109-117.

Bajt, M. L., Ramachandran, A., et al. (2011). "Apoptosis-inducing factor modulates mitochondrial oxidant stress in acetaminophen hepatotoxicity." Toxicol Sci **122**(2): 598-605.

Baker, J. A., Marshall, P. G., et al. (1963). "Some Antipyretics Related to Aspirin and Phenacetin." Journal of Pharmacy and Pharmacology **15**: T97-&.

Bebarta, V. S., Shiner, D. C., et al. (2014). "A case of moderate liver enzyme elevation after acute acetaminophen overdose despite undetectable acetaminophen level and normal initial liver enzymes." Am J Ther **21**(3): e82-84.

Bernal, W., Auzinger, G., et al. (2010). "Acute liver failure." Lancet **376**(9736): 190-201.

Bertolini, A., Ferrari, A., et al. (2006). "Paracetamol: new vistas of an old drug." CNS Drug Rev **12**(3-4): 250-275.

Bhushan, B., Borude, P., et al. (2013). "Role of bile acids in liver injury and regeneration following acetaminophen overdose." Am J Pathol **183**(5): 1518-1526.

Bhushan, B., Walesky, C., et al. (2014). "Pro-Regenerative Signaling after Acetaminophen-Induced Acute Liver Injury in Mice Identified Using a Novel Incremental Dose Model." American Journal of Pathology **184**(11): 3013-3025.

Birge, R. B., Bartolone, J. B., et al. (1989). "Selective Protein Arylation by Acetaminophen and 2,6-Dimethylacetaminophen in Cultured-Hepatocytes from Phenobarbital-Induced and Uninduced Mice - Relationship to Cytotoxicity." Biochem Pharmacol **38**(24): 4429-4438.

Bojar, H., Basler, M., et al. (1976). "Preparation of parenchymal and non-parenchymal cells from adult human liver--morphological and biochemical characteristics." J Clin Chem Clin Biochem **14**(11): 527-532.

Botting, R. M. (2000). "Mechanism of action of acetaminophen: is there a cyclooxygenase 3?" Clin Infect Dis **31 Suppl 5**: S202-210.

Bourdi, M., Davies, J. S., et al. (2011). "Mispairing C57BL/6 Substrains of Genetically Engineered Mice and Wild-Type Controls Can Lead to Confounding Results as It Did in Studies of JNK2 in Acetaminophen and Concanavalin A Liver Injury." Chem Res Toxicol **24**(6): 794-796.

Bourdi, M., Korrapati, M. C., et al. (2008). "Protective role of c-Jun N-terminal kinase 2 in acetaminophen-induced liver injury." Biochem Biophys Res Commun **374**(1): 6-10.

Bourdi, M., Masubuchi, Y., et al. (2002). "Protection against acetaminophen-induced liver injury and lethality by interleukin 10: role of inducible nitric oxide synthase." Hepatology **35**(2): 289-298.

Brok, J., Buckley, N., et al. (2006). "Interventions for paracetamol (acetaminophen) overdose." Cochrane Database Syst Rev(2): CD003328.

Budnitz, D. S., Lovegrove, M. C., et al. (2011). "Emergency department visits for overdoses of acetaminophen-containing products." Am J Prev Med **40**(6): 585-592.

Cerec, V., Glaise, D., et al. (2007). "Transdifferentiation of hepatocyte-like cells from the human hepatoma HepaRG cell line through bipotent progenitor." Hepatology **45**(4): 957-967.

Chalasani, N. and Bjornsson, E. (2010). "Risk factors for idiosyncratic drug-induced liver injury." Gastroenterology **138**(7): 2246-2259.

Chambers, J. W., Cherry, L., et al. (2011). "Selective inhibition of mitochondrial JNK signaling achieved using peptide mimicry of the Sab kinase interacting motif-1 (KIM1)." ACS Chem Biol **6**(8): 808-818.

Cheung, C., Yu, A. M., et al. (2005). "The cyp2e1-humanized transgenic mouse: role of cyp2e1 in acetaminophen hepatotoxicity." Drug Metab Dispos **33**(3): 449-457.

Chiu, H., Gardner, C. R., et al. (2003). "Role of tumor necrosis factor receptor 1 (p55) in hepatocyte proliferation during acetaminophen-induced toxicity in mice." Toxicol Appl Pharmacol **193**(2): 218-227.

Chun, L. J., Tong, M. J., et al. (2009). "Acetaminophen hepatotoxicity and acute liver failure." J Clin Gastroenterol **43**(4): 342-349.

Clements, J. A., Critchley, J. A., et al. (1984). "The role of sulphate conjugation in the metabolism and disposition of oral and intravenous paracetamol in man." Br J Clin Pharmacol **18**(4): 481-485.

Cohen, S. D., Pumford, N. R., et al. (1997). "Selective protein covalent binding and target organ toxicity." Toxicol Appl Pharmacol **143**(1): 1-12.

Cook, S. F., King, A. D., et al. (2015). "Quantification of a biomarker of acetaminophen protein adducts in human serum by high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry: clinical and animal model applications." J Chromatogr B Analyt Technol Biomed Life Sci **985**: 131-141.

Corcoran, G. B., Racz, W. J., et al. (1985). "Effects of N-acetylcysteine on acetaminophen covalent binding and hepatic necrosis in mice." J Pharmacol Exp Ther **232**(3): 864-872.

Corcoran, G. B. and Wong, B. K. (1986). "Role of glutathione in prevention of acetaminophen-induced hepatotoxicity by N-acetyl-L-cysteine in vivo: studies with N-acetyl-D-cysteine in mice." J Pharmacol Exp Ther **238**(1): 54-61.

Court, M. H., Duan, S. X., et al. (2001). "Interindividual variability in acetaminophen glucuronidation by human liver microsomes: identification of relevant acetaminophen UDP-glucuronosyltransferase isoforms." J Pharmacol Exp Ther **299**(3): 998-1006.

Court, M. H., Freytsis, M., et al. (2013). "The UDP-glucuronosyltransferase (UGT) 1A polymorphism c.2042C>G (rs8330) is associated with increased human liver acetaminophen glucuronidation, increased UGT1A exon 5a/5b splice variant

mRNA ratio, and decreased risk of unintentional acetaminophen-induced acute liver failure." J Pharmacol Exp Ther **345**(2): 297-307.

Cover, C., Liu, J., et al. (2006). "Pathophysiological role of the acute inflammatory response during acetaminophen hepatotoxicity." Toxicol Appl Pharmacol **216**(1): 98-107.

Cover, C., Mansouri, A., et al. (2005). "Peroxynitrite-induced mitochondrial and endonuclease-mediated nuclear DNA damage in acetaminophen hepatotoxicity." J Pharmacol Exp Ther **315**(2): 879-887.

Coyle, P., Philcox, J. C., et al. (2002). "Metallothionein: the multipurpose protein." Cell Mol Life Sci **59**(4): 627-647.

Crome, P., Vale, J. A., et al. (1976). "Oral methionine in the treatment of severe paracetamol (Acetaminophen) overdose." Lancet **2**(7990): 829-830.

Cubero, F. J., Zoubek, M. E., et al. (2015). "Combined Activities of JNK1 and JNK2 in Hepatocytes Protect Against Toxic Liver Injury." Gastroenterology.

Dahlin, D. C., Miwa, G. T., et al. (1984). "N-acetyl-p-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen." Proc Natl Acad Sci U S A **81**(5): 1327-1331.

Davern, T. J., 2nd, James, L. P., et al. (2006). "Measurement of serum acetaminophen-protein adducts in patients with acute liver failure." Gastroenterology **130**(3): 687-694.

Davidson, D. G. and Eastham, W. N. (1966). "Acute liver necrosis following overdose of paracetamol." Br Med J **2**(5512): 497-499.

de Morais, S. M., Uetrecht, J. P., et al. (1992). "Decreased glucuronidation and increased bioactivation of acetaminophen in Gilbert's syndrome." Gastroenterology **102**(2): 577-586.

de Morais, S. M. and Wells, P. G. (1989). "Enhanced acetaminophen toxicity in rats with bilirubin glucuronyl transferase deficiency." Hepatology **10**(2): 163-167.

Donahower, B., McCullough, S. S., et al. (2006). "Vascular endothelial growth factor and hepatocyte regeneration in acetaminophen toxicity." Am J Physiol Gastrointest Liver Physiol **291**(1): G102-109.

Donahower, B. C., McCullough, S. S., et al. (2010). "Human recombinant vascular endothelial growth factor reduces necrosis and enhances hepatocyte regeneration in a mouse model of acetaminophen toxicity." J Pharmacol Exp Ther **334**(1): 33-43.

Du, K., McGill, M. R., et al. (2015). "Resveratrol prevents protein nitration and release of endonucleases from mitochondria during acetaminophen hepatotoxicity." Food Chem Toxicol **81**: 62-70.

Du, K., Williams, C. D., et al. (2014). "Lower susceptibility of female mice to acetaminophen hepatotoxicity: Role of mitochondrial glutathione, oxidant stress and c-jun N-terminal kinase." Toxicol Appl Pharmacol **281**(1): 58-66.

Du, K., Williams, C. D., et al. (2013). "The gap junction inhibitor 2-aminoethoxy-diphenyl-borate protects against acetaminophen hepatotoxicity by inhibiting cytochrome P450 enzymes and c-jun N-terminal kinase activation." Toxicol Appl Pharmacol **273**(3): 484-491.

Du, K., Xie, Y., et al. (2015). "Pathophysiological significance of c-jun N-terminal kinase in acetaminophen hepatotoxicity." Expert Opin Drug Metab Toxicol: 1-11.

Dubach, U. C., Rosner, B., et al. (1991). "An epidemiologic study of abuse of analgesic drugs. Effects of phenacetin and salicylate on mortality and cardiovascular morbidity (1968 to 1987)." N Engl J Med **324**(3): 155-160.

Emmett, D. S., Feranchak, A., et al. (2008). "Characterization of ionotrophic purinergic receptors in hepatocytes." Hepatology **47**(2): 698-705.

Ferreira, S. H., Moncada, S., et al. (1997). "Prostaglandins and the mechanism of analgesia produced by aspirin-like drugs. 1973." Br J Pharmacol **120**(4 Suppl): 401-412; discussion 399-400.

Franc, J. E., Kollia, G., et al. (1997). "Avitriptan coadministration delays acetaminophen absorption but does not alter its metabolism." Clinical Pharmacology & Therapeutics **61**(2): Pi22-Pi22.

Fremont, L. (2000). "Biological effects of resveratrol." Life Sci **66**(8): 663-673.

Gelotte, C. K., Auiler, J. F., et al. (2007). "Disposition of acetaminophen at 4, 6, and 8 g/day for 3 days in healthy young adults." Clin Pharmacol Ther **81**(6): 840-848.

Godoy, P., Hewitt, N. J., et al. (2013). "Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME." Archives of Toxicology **87**(8): 1315-1530.

Gonzales, E., Prigent, S., et al. (2007). "Rat hepatocytes express functional P2X receptors." FEBS Lett **581**(17): 3260-3266.

Grimes, C. A. and Jope, R. S. (2001). "The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling." Prog Neurobiol **65**(4): 391-426.

Guillouzo, A., Corlu, A., et al. (2007). "The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics." Chem Biol Interact **168**(1): 66-73.

Gujral, J. S., Knight, T. R., et al. (2002). "Mode of cell death after acetaminophen overdose in mice: apoptosis or oncotic necrosis?" Toxicol Sci **67**(2): 322-328.

Gunawan, B. K., Liu, Z. X., et al. (2006). "c-Jun N-terminal kinase plays a major role in murine acetaminophen hepatotoxicity." Gastroenterology **131**(1): 165-178.

Hadi, M., Dragovic, S., et al. (2013). "AMAP, the alleged non-toxic isomer of acetaminophen, is toxic in rat and human liver." Archives of Toxicology **87**(1): 155-165.

Halmes, N. C., Samokyszyn, V. M., et al. (1998). "The acetaminophen regioisomer 3 '-hydroxyacetanilide inhibits and covalently binds to cytochrome P450 2E1." Toxicol Lett **94**(1): 65-71.

Hanawa, N., Shinohara, M., et al. (2008). "Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury." J Biol Chem **283**(20): 13565-13577.

Harrison, P. M., Keays, R., et al. (1990). "Improved outcome of paracetamol-induced fulminant hepatic failure by late administration of acetylcysteine." Lancet **335**(8705): 1572-1573.

Heard, K. J., Green, J. L., et al. (2011). "Acetaminophen-cysteine adducts during therapeutic dosing and following overdose." BMC Gastroenterol **11**: 20.

Heger, M., Heemskerk, A. A., et al. (2010). "Absence of 633-nm laser irradiation-induced effects on glucose phosphorylation by hexokinase." J Photochem Photobiol B **98**(3): 216-222.

Henderson, N. C., Pollock, K. J., et al. (2007). "Critical role of c-jun (NH2) terminal kinase in paracetamol- induced acute liver failure." Gut **56**(7): 982-990.

Hinson, J. A., Roberts, D. W., et al. (1990). "Mechanism of paracetamol toxicity." Lancet **335**(8691): 732.

Hinz, B., Cheremina, O., et al. (2008). "Acetaminophen (paracetamol) is a selective cyclooxygenase-2 inhibitor in man." FASEB J **22**(2): 383-390.

Hoffmann, K. J., Streeter, A. J., et al. (1985). "Identification of the major covalent adduct formed in vitro and in vivo between acetaminophen and mouse liver proteins." Mol Pharmacol **27**(5): 566-573.

Holme, J. A., Hongslo, J. K., et al. (1991). "Comparative cytotoxic effects of acetaminophen (N-acetyl-p-aminophenol), a non-hepatotoxic regioisomer acetyl-m-aminophenol and their postulated reactive hydroquinone and quinone metabolites in monolayer cultures of mouse hepatocytes." Biochem Pharmacol **42**(5): 1137-1142.

Hong, H. Y. and Kim, B. C. (2007). "Mixed lineage kinase 3 connects reactive oxygen species to c-Jun NH2-terminal kinase-induced mitochondrial apoptosis in genipin-treated PC3 human prostate cancer cells." Biochem Biophys Res Commun **362**(2): 307-312.

Hoque, R., Sohail, M. A., et al. (2012). "P2X7 receptor-mediated purinergic signaling promotes liver injury in acetaminophen hepatotoxicity in mice." Am J Physiol Gastrointest Liver Physiol **302**(10): G1171-1179.

Hu, B. and Colletti, L. M. (2008). "Stem cell factor and c-kit are involved in hepatic recovery after acetaminophen-induced liver injury in mice." Am J Physiol Gastrointest Liver Physiol **295**(1): G45-G53.

Hughes, R. D., Gazzard, B. G., et al. (1977). "Controlled trial of cysteamine and dimercaprol after paracetamol overdose." Br Med J **2**(6099): 1395.

Ichijo, H., Nishida, E., et al. (1997). "Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways." Science **275**(5296): 90-94.

IMS (2008). " IMS National Sales Perspectives."

IMS (2014). "National Prescription Audit."

Jaeschke, H. (1990). "Glutathione disulfide formation and oxidant stress during acetaminophen-induced hepatotoxicity in mice in vivo: the protective effect of allopurinol." J Pharmacol Exp Ther **255**(3): 935-941.

Jaeschke, H. (2015). "Acetaminophen: Dose-Dependent Drug Hepatotoxicity and Acute Liver Failure in Patients." Dig Dis **33**(4): 464-471.

Jaeschke, H., Cover, C., et al. (2006). "Role of caspases in acetaminophen-induced liver injury." Life Sci **78**(15): 1670-1676.

Jaeschke, H., Fisher, M. A., et al. (1998). "Activation of caspase 3 (CPP32)-like proteases is essential for TNF-alpha-induced hepatic parenchymal cell apoptosis

and neutrophil-mediated necrosis in a murine endotoxin shock model." Journal of Immunology **160**(7): 3480-3486.

Jaeschke, H., McGill, M. R., et al. (2011). "Pathophysiological relevance of proteomics investigations of drug-induced hepatotoxicity in HepG2 cells." Toxicol Sci **121**(2): 428-430; author reply 431-423.

Jaeschke, H., McGill, M. R., et al. (2012). "Oxidant stress, mitochondria, and cell death mechanisms in drug-induced liver injury: Lessons learned from acetaminophen hepatotoxicity." Drug Metab Rev **44**(1): 88-106.

Jaeschke, H., McGill, M. R., et al. (2011). "Current issues with acetaminophen hepatotoxicity--a clinically relevant model to test the efficacy of natural products." Life Sci **88**(17-18): 737-745.

Jaeschke, H. and Mitchell, J. R. (1990). "Use of Isolated-Perfused Organs in Hypoxia and Ischemia-Reperfusion Oxidant Stress." Methods Enzymol **186**: 752-759.

Jaeschke, H., Williams, C. D., et al. (2013). "Models of drug-induced liver injury for evaluation of phytotherapeutics and other natural products." Food Chem Toxicol **55**: 279-289.

Jaeschke, H., Williams, C. D., et al. (2012). "Acetaminophen hepatotoxicity and repair: the role of sterile inflammation and innate immunity." Liver Int **32**(1): 8-20.

James, L. P., Capparelli, E. V., et al. (2008). "Acetaminophen-associated hepatic injury: evaluation of acetaminophen protein adducts in children and adolescents with acetaminophen overdose." Clin Pharmacol Ther **84**(6): 684-690.

James, L. P., Lamps, L. W., et al. (2003). "Interleukin 6 and hepatocyte regeneration in acetaminophen toxicity in the mouse." Biochem Biophys Res Commun **309**(4): 857-863.

James, L. P., Letzig, L., et al. (2009). "Pharmacokinetics of acetaminophen-protein adducts in adults with acetaminophen overdose and acute liver failure." Drug Metab Dispos **37**(8): 1779-1784.

James, L. P., McCullough, S. S., et al. (2003). "Acetaminophen toxicity in mice lacking NADPH oxidase activity: role of peroxynitrite formation and mitochondrial oxidant stress." Free Radic Res **37**(12): 1289-1297.

Kanebratt, K. P. and Andersson, T. B. (2008). "HepaRG cells as an in vitro model for evaluation of cytochrome p450 induction in humans." Drug Metabolism and Disposition **36**(1): 137-145.

Kawano, A., Tsukimoto, M., et al. (2012). "Regulation of P2X7-dependent inflammatory functions by P2X4 receptor in mouse macrophages." Biochem Biophys Res Commun **420**(1): 102-107.

Kenna, J. G. (2013). "A new twist to an old tale: novel insights into the differential toxicities of acetaminophen and its regioisomer N-acetyl-meta-aminophenol (AMAP)." Archives of Toxicology **87**(1): 15-18.

Knight, T. R., Fariss, M. W., et al. (2003). "Role of lipid peroxidation as a mechanism of liver injury after acetaminophen overdose in mice." Toxicol Sci **76**(1): 229-236.

Knight, T. R., Ho, Y. S., et al. (2002). "Peroxynitrite is a critical mediator of acetaminophen hepatotoxicity in murine livers: Protection by glutathione." Journal of Pharmacology and Experimental Therapeutics **303**(2): 468-475.

Knight, T. R., Kurtz, A., et al. (2001). "Vascular and hepatocellular peroxynitrite formation during acetaminophen toxicity: Role of mitochondrial oxidant stress." Toxicological Sciences **62**(2): 212-220.

Knockaert, L., Descatoire, V., et al. (2011). "Mitochondrial CYP2E1 is sufficient to mediate oxidative stress and cytotoxicity induced by ethanol and acetaminophen." Toxicology in Vitro **25**(2): 475-484.

Kon, K., Kim, J. S., et al. (2004). "Mitochondrial permeability transition in acetaminophen-induced necrosis and apoptosis of cultured mouse hepatocytes." Hepatology **40**(5): 1170-1179.

Kon, K., Kim, J. S., et al. (2010). "Lysosomal iron mobilization and induction of the mitochondrial permeability transition in acetaminophen-induced toxicity to mouse hepatocytes." Toxicol Sci **117**(1): 101-108.

Larson, A. M. (2007). "Acetaminophen hepatotoxicity." Clin Liver Dis **11**(3): 525-548, vi.

Latchoumycandane, C., Goh, C. W., et al. (2007). "Mitochondrial protection by the JNK inhibitor leflunomide rescues mice from acetaminophen-induced liver injury." Hepatology **45**(2): 412-421.

Latchoumycandane, C., Seah, Q. M., et al. (2006). "Leflunomide or A77 1726 protect from acetaminophen-induced cell injury through inhibition of JNK-mediated mitochondrial permeability transition in immortalized human hepatocytes." Toxicol Appl Pharmacol **217**(1): 125-133.

Lauterburg, B. H., Corcoran, G. B., et al. (1983). "Mechanism of action of N-acetylcysteine in the protection against the hepatotoxicity of acetaminophen in rats in vivo." J Clin Invest **71**(4): 980-991.

Lawson, J. A., Fisher, M. A., et al. (1999). "Inhibition of Fas receptor (CD95)-induced hepatic caspase activation and apoptosis by acetaminophen in mice." Toxicol Appl Pharmacol **156**(3): 179-186.

Lee, H. S., Hwang, C. Y., et al. (2014). "MLK3 Is Part of a Feedback Mechanism That Regulates Different Cellular Responses to Reactive Oxygen Species." Science Signaling **7**(328).

Lee, S. S., Buters, J. T., et al. (1996). "Role of CYP2E1 in the hepatotoxicity of acetaminophen." J Biol Chem **271**(20): 12063-12067.

Lee, W. M. (2004). "Acetaminophen and the U.S. Acute Liver Failure Study Group: lowering the risks of hepatic failure." Hepatology **40**(1): 6-9.

Lee, W. M. (2012). "Acute liver failure." Semin Respir Crit Care Med **33**(1): 36-45.

Li, J. X., Feng, J. M., et al. (2014). "The B-Raf(V600E) inhibitor dabrafenib selectively inhibits RIP3 and alleviates acetaminophen-induced liver injury." Cell Death Dis **5**: e1278.

Lindsay, J., Wang, L. L., et al. (2008). "Structure, function and polymorphism of human cytosolic sulfotransferases." Curr Drug Metab **9**(2): 99-105.

Liu, H., Nishitoh, H., et al. (2000). "Activation of apoptosis signal-regulating kinase 1 (ASK1) by tumor necrosis factor receptor-associated factor 2 requires prior dissociation of the ASK1 inhibitor thioredoxin." Molecular and Cellular Biology **20**(6): 2198-2208.

LoGuidice, A. and Boelsterli, U. A. (2011). "Acetaminophen Overdose-Induced Liver Injury in Mice is Mediated by Peroxynitrite Independently of the Cyclophilin D-Regulated Permeability Transition." Hepatology **54**(3): 969-978.

Lotharius, J., Falsig, J., et al. (2005). "Progressive degeneration of human mesencephalic neuron-derived cells triggered by dopamine-dependent oxidative stress is dependent on the mixed-lineage kinase pathway." J Neurosci **25**(27): 6329-6342.

Masubuchi, Y., Suda, C., et al. (2005). "Involvement of mitochondrial permeability transition in acetaminophen-induced liver injury in mice." J Hepatol **42**(1): 110-116.

Matsuzawa, A., Saegusa, K., et al. (2005). "ROS-dependent activation of the TRAF6-ASK1-p38 pathway is selectively required for TLR4-mediated innate immunity." Nature Immunology **6**(6): 587-592.

McGill, M. R., Cao, M. D., et al. (2014a). "Argininosuccinate synthetase as a plasma biomarker of liver injury after acetaminophen overdose in rodents and humans." Biomarkers **19**(3): 222-230.

McGill, M. R. and Jaeschke, H. (2014). "Mechanistic biomarkers in acetaminophen-induced hepatotoxicity and acute liver failure: from preclinical models to patients." Expert Opin Drug Metab Toxicol **10**(7): 1005-1017.

McGill, M. R., Lebofsky, M., et al. (2013). "Plasma and liver acetaminophen-protein adduct levels in mice after acetaminophen treatment: dose-response, mechanisms, and clinical implications." Toxicol Appl Pharmacol **269**(3): 240-249.

McGill, M. R., Li, F., et al. (2014b). "Circulating acylcarnitines as biomarkers of mitochondrial dysfunction after acetaminophen overdose in mice and humans." Archives of Toxicology **88**(2): 391-401.

McGill, M. R., Sharpe, M. R., et al. (2012a). "The mechanism underlying acetaminophen-induced hepatotoxicity in humans and mice involves mitochondrial damage and nuclear DNA fragmentation." J Clin Invest **122**(4): 1574-1583.

McGill, M. R., Staggs, V. S., et al. (2014c). "Serum mitochondrial biomarkers and damage-associated molecular patterns are higher in acetaminophen overdose patients with poor outcome." Hepatology **60**(4): 1336-1345.

McGill, M. R., Williams, C. D., et al. (2012b). "Acetaminophen-induced liver injury in rats and mice: Comparison of protein adducts, mitochondrial dysfunction, and oxidative stress in the mechanism of toxicity." Toxicol Appl Pharmacol **264**(3): 387-394.

McGill, M. R., Yan, H. M., et al. (2011). "HepaRG cells: a human model to study mechanisms of acetaminophen hepatotoxicity." Hepatology **53**(3): 974-982.

Meyers, L. L., Beierschmitt, W. P., et al. (1988). "Acetaminophen-induced inhibition of hepatic mitochondrial respiration in mice." Toxicol Appl Pharmacol **93**(3): 378-387.

Mizushima, N. and Komatsu, M. (2011). "Autophagy: renovation of cells and tissues." Cell **147**(4): 728-741.

Muldrew, K. L., James, L. P., et al. (2002). "Determination of acetaminophen-protein adducts in mouse liver and serum and human serum after hepatotoxic doses of

acetaminophen using high-performance liquid chromatography with electrochemical detection." Drug Metab Dispos **30**(4): 446-451.

Myers, T. G., Dietz, E. C., et al. (1995). "A comparative study of mouse liver proteins arylated by reactive metabolites of acetaminophen and its nonhepatotoxic regioisomer, 3'-hydroxyacetanilide." Chem Res Toxicol **8**(3): 403-413.

Nakagawa, H., Maeda, S., et al. (2008). "Deletion of apoptosis signal-regulating kinase 1 attenuates acetaminophen-induced liver injury by inhibiting c-Jun N-terminal kinase activation." Gastroenterology **135**(4): 1311-1321.

Navarro, S. L., Chen, Y., et al. (2011). "UGT1A6 and UGT2B15 polymorphisms and acetaminophen conjugation in response to a randomized, controlled diet of select fruits and vegetables." Drug Metab Dispos **39**(9): 1650-1657.

Nelson, E. B. (1980). "The pharmacology and toxicology of meta-substituted acetanilide I: acute toxicity of 3-hydroxyacetanilide in mice." Res Commun Chem Pathol Pharmacol **28**(3): 447-456.

Ni, H. M., Bockus, A., et al. (2012a). "Activation of autophagy protects against acetaminophen-induced hepatotoxicity." Hepatology **55**(1): 222-232.

Ni, H. M., Jaeschke, H., et al. (2012b). "Targeting autophagy for drug-induced hepatotoxicity." Autophagy **8**(4): 709-710.

Ni, H. M., Williams, J. A., et al. (2013). "Zonated induction of autophagy and mitochondrial spheroids limits acetaminophen-induced necrosis in the liver." Redox Biol **1**: 427-432.

Noguchi, T., Takeda, K., et al. (2005). "Recruitment of tumor necrosis factor receptor-associated factor family proteins to apoptosis signal-regulating kinase 1

signalosome is essential for oxidative stress-induced cell death." J Biol Chem **280**(44): 37033-37040.

Nourjah, P., Ahmad, S. R., et al. (2006). "Estimates of acetaminophen (paracetamol)-associated overdoses in the United States." Pharmacoepidemiology and Drug Safety **15**(6): 398-405.

Parent, R., Marion, M. J., et al. (2004). "Origin and characterization of a human bipotent liver progenitor cell line." Gastroenterology **126**(4): 1147-1156.

Pierce, R. H., Franklin, C. C., et al. (2002). "Cell culture model for acetaminophen-induced hepatocyte death in vivo." Biochem Pharmacol **64**(3): 413-424.

Placke, M. E., Ginsberg, G. L., et al. (1987). "Ultrastructural changes during acute acetaminophen-induced hepatotoxicity in the mouse: a time and dose study." Toxicol Pathol **15**(4): 431-438.

Polson, J. and Lee, W. M. (2005). "AASLD position paper: the management of acute liver failure." Hepatology **41**(5): 1179-1197.

Prescott, L. F. (1980). "Kinetics and metabolism of paracetamol and phenacetin." Br J Clin Pharmacol **10 Suppl 2**: 291S-298S.

Prescott, L. F. (1996). "Paracetamol (Acetaminophen). A Critical Bibliographic Review."

Prescott, L. F., Ballantyne, A., et al. (1977). "Treatment of Paracetamol (Acetaminophen) Poisoning with N-Acetylcysteine." Lancet **2**(8035): 432-434.

Pumford, N. R., Hinson, J. A., et al. (1989). "Immunochemical quantitation of 3-(cystein-S-yl)acetaminophen adducts in serum and liver proteins of acetaminophen-treated mice." J Pharmacol Exp Ther **248**(1): 190-196.

Qiu, Y., Benet, L. Z., et al. (2001). "Identification of hepatic protein targets of the reactive metabolites of the non-hepatotoxic regioisomer of acetaminophen, 3'-hydroxyacetanilide, in the mouse in vivo using two-dimensional gel electrophoresis and mass spectrometry." Adv Exp Med Biol **500**: 663-673.

Ramachandran, A., Lebofsky, M., et al. (2011a). "Cyclophilin D deficiency protects against acetaminophen-induced oxidant stress and liver injury." Free Radic Res **45**(2): 156-164.

Ramachandran, A., Lebofsky, M., et al. (2011b). "The impact of partial manganese superoxide dismutase (SOD2)-deficiency on mitochondrial oxidant stress, DNA fragmentation and liver injury during acetaminophen hepatotoxicity." Toxicol Appl Pharmacol 251(3): 226-233.

Ramachandran, A., McGill, M. R., et al. (2013). "Receptor Interacting Protein Kinase 3 Is a Critical Early Mediator of Acetaminophen-induced Hepatocyte Necrosis in Mice." Hepatology **58**(6): 2099-2108.

Remien, C. H., Adler, F. R., et al. (2012). "Mathematical modeling of liver injury and dysfunction after acetaminophen overdose: early discrimination between survival and death." Hepatology **56**(2): 727-734.

Roberts, S. A., Price, V. F., et al. (1990). "Acetaminophen structure-toxicity studies: in vivo covalent binding of a nonhepatotoxic analog, 3-hydroxyacetanilide." Toxicol Appl Pharmacol **105**(2): 195-208.

Rodriguez-Antona, C., Donato, M. T., et al. (2002). "Cytochrome P450 expression in human hepatocytes and hepatoma cell lines: molecular mechanisms that determine lower expression in cultured cells." Xenobiotica **32**(6): 505-520.

Rumack, B. H. and Matthew, H. (1975). "Acetaminophen poisoning and toxicity." Pediatrics **55**(6): 871-876.

Rumack, B. H., Peterson, R. C., et al. (1981). "Acetaminophen overdose. 662 cases with evaluation of oral acetylcysteine treatment." Archives of Internal Medicine **141**(3 Spec No): 380-385.

Saberi, B., Ybanez, M. D., et al. (2014). "Protein kinase C (PKC) participates in acetaminophen hepatotoxicity through c-jun-N-terminal kinase (JNK)-dependent and -independent signaling pathways." Hepatology **59**(4): 1543-1554.

Saito, C., Lemasters, J. J., et al. (2010a). "c-Jun N-terminal kinase modulates oxidant stress and peroxynitrite formation independent of inducible nitric oxide synthase in acetaminophen hepatotoxicity." Toxicol Appl Pharmacol **246**(1-2): 8-17.

Saito, C., Yan, H. M., et al. (2010b). "Mechanism of protection by metallothionein against acetaminophen hepatotoxicity." Toxicol Appl Pharmacol **242**(2): 182-190.

Saito, C., Zwingmann, C., et al. (2010c). "Novel mechanisms of protection against acetaminophen hepatotoxicity in mice by glutathione and N-acetylcysteine." Hepatology **51**(1): 246-254.

Saitoh, M., Nishitoh, H., et al. (1998). "Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1." EMBO J **17**(9): 2596-2606.

Sato, C. and Lieber, C. S. (1981). "Mechanism of the preventive effect of ethanol on acetaminophen-induced hepatoxicity." J Pharmacol Exp Ther **218**(3): 811-815.

Schiodt, F. V., Atillasoy, E., et al. (1999). "Etiology and outcome for 295 patients with acute liver failure in the United States." Liver Transpl Surg **5**(1): 29-34.

Schiodt, F. V., Rochling, F. A., et al. (1997). "Acetaminophen toxicity in an urban county hospital." N Engl J Med **337**(16): 1112-1117.

Schmidt, L. E. and Dalhoff, K. (2005). "Alpha-fetoprotein is a predictor of outcome in acetaminophen-induced liver injury." Hepatology **41**(1): 26-31.

Schyschka, L., Sanchez, J. J., et al. (2013). "Hepatic 3D cultures but not 2D cultures preserve specific transporter activity for acetaminophen-induced hepatotoxicity." Archives of Toxicology **87**(8): 1581-1593.

Seglen, P. O. (1976). "Preparation of isolated rat liver cells." Methods Cell Biol **13**: 29-83.

Shakil, A. O., Kramer, D., et al. (2000). "Acute liver failure: clinical features, outcome analysis, and applicability of prognostic criteria." Liver Transpl **6**(2): 163-169.

Sharma, M., Gadang, V., et al. (2012). "Critical role for mixed-lineage kinase 3 in acetaminophen-induced hepatotoxicity." Mol Pharmacol **82**(5): 1001-1007.

Shinohara, M., Ybanez, M. D., et al. (2010). "Silencing glycogen synthase kinase-3beta inhibits acetaminophen hepatotoxicity and attenuates JNK activation and loss of glutamate cysteine ligase and myeloid cell leukemia sequence 1." J Biol Chem **285**(11): 8244-8255.

Singer, A. J., Carracio, T. R., et al. (1995). "The temporal profile of increased transaminase levels in patients with acetaminophen-induced liver dysfunction." Ann Emerg Med **26**(1): 49-53.

Streeter, A. J., Dahlin, D. C., et al. (1984). "The covalent binding of acetaminophen to protein. Evidence for cysteine residues as major sites of arylation in vitro." Chem Biol Interact **48**(3): 349-366.

Smith, C. V. and Jaeschke, H. (1989). "Effect of acetaminophen on hepatic content and biliary efflux of glutathione disulfide in mice." Chem Biol Interact **70**(3-4): 241-248.

Streeter, A. J., Dahlin, D. C., et al. (1984). "The covalent binding of acetaminophen to protein. Evidence for cysteine residues as major sites of arylation in vitro." Chem Biol Interact **48**(3): 349-366.

Strom, S. C., Jirtle, R. L., et al. (1982). "Isolation, culture, and transplantation of human hepatocytes." J Natl Cancer Inst **68**(5): 771-778.

Tee, L. B. G., Boobis, A. R., et al. (1986). "Reversal of Acetaminophen Toxicity in Isolated Hamster Hepatocytes by Dithiothreitol." Toxicol Appl Pharmacol **83**(2): 294-314.

Thulin, P., Nordahl, G., et al. (2013). "Keratin-18 and microRNA-122 complement alanine aminotransferase as novel safety biomarkers for drug-induced liver injury in two human cohorts." Liver Int.

Thummel, K. E., Slattery, J. T., et al. (2000). "Ethanol and production of the hepatotoxic metabolite of acetaminophen in healthy adults." Clin Pharmacol Ther **67**(6): 591-599.

Tirmenstein, M. A. and Nelson, S. D. (1989). "Subcellular binding and effects on calcium homeostasis produced by acetaminophen and a nonhepatotoxic regioisomer, 3'-hydroxyacetanilide, in mouse liver." J Biol Chem **264**(17): 9814-9819.

Ullrich, D., Sieg, A., et al. (1987). "Normal pathways for glucuronidation, sulphation and oxidation of paracetamol in Gilbert's syndrome." Eur J Clin Invest **17**(3): 237-240.

Vale, J. A., Meredith, T. J., et al. (1981). "Treatment of Acetaminophen Poisoning - the Use of Oral Methionine." Archives of Internal Medicine **141**: 394-396.

Vliegenthart, A. D., Shaffer, J. M., et al. (2015). "Comprehensive microRNA profiling in acetaminophen toxicity identifies novel circulating biomarkers for human liver and kidney injury." Sci Rep **5**: 15501.

Wancket, L. M., Meng, X., et al. (2012). "Mitogen-activated protein kinase phosphatase (Mkp)-1 protects mice against acetaminophen-induced hepatic injury." Toxicol Pathol **40**(8): 1095-1105.

Ward, J., Kanchagar, C., et al. (2014). "Circulating microRNA profiles in human patients with acetaminophen hepatotoxicity or ischemic hepatitis." Proc Natl Acad Sci U S A **111**(33): 12169-12174.

Webster, P. A., Roberts, D. W., et al. (1996). "Acetaminophen toxicity in children: Diagnostic confirmation using a specific antigenic biomarker." Journal of Clinical Pharmacology **36**(5): 397-402.

Weerasinghe, S. V., Jang, Y. J., et al. (2014). "Carbamoyl phosphate synthetase-1 is a rapid turnover biomarker in mouse and human acute liver injury." Am J Physiol Gastrointest Liver Physiol **307**(3): G355-364.

Wilkening, S., Stahl, F., et al. (2003). "Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties." Drug Metab Dispos **31**(8): 1035-1042.

Williams, C. D., Antoine, D. J., et al. (2011a). "Role of the Nalp3 inflammasome in acetaminophen-induced sterile inflammation and liver injury." Toxicol Appl Pharmacol **252**(3): 289-297.

Williams, C. D., Bajt, M. L., et al. (2010). "Acetaminophen-induced hepatic neutrophil accumulation and inflammatory liver injury in CD18-deficient mice." Liver Int **30**(9): 1280-1292.

Williams, C. D., Bajt, M. L., et al. (2014). "Neutrophil activation during acetaminophen hepatotoxicity and repair in mice and humans." Toxicol Appl Pharmacol **275**(2): 122-133.

Williams, C. D., Farhood, A., et al. (2010). "Role of caspase-1 and interleukin-1beta in acetaminophen-induced hepatic inflammation and liver injury." Toxicol Appl Pharmacol **247**(3): 169-178.

Williams, C. D., Koerner, M. R., et al. (2011b). "Mouse strain-dependent caspase activation during acetaminophen hepatotoxicity does not result in apoptosis or modulation of inflammation." Toxicol Appl Pharmacol **257**(3): 449-458.

Williams, J. A., Ni, H. M., et al. (2015a). "Chronic Deletion and Acute Knockdown of Parkin Have Differential Responses to Acetaminophen-induced Mitophagy and Liver Injury in Mice." Journal of Biological Chemistry **290**(17): 10934-10946.

Williams, J. A., Wen-Xing Ding (2015b). "Targeting Pink1-Parkin-mediated mitophagy for treating liver injury." Pharmacological Research **102**(Dec): 264-269.

Win, S., Than, T. A., et al. (2011). "c-Jun N-terminal kinase (JNK)-dependent acute liver injury from acetaminophen or tumor necrosis factor (TNF) requires mitochondrial Sab protein expression in mice." J Biol Chem **286**(40): 35071-35078.

Win, S., Than, T. A., et al. (2016). "JNK mediates mouse liver injury through a novel Sab (SH3BP5) dependent pathway leading to inactivation of intramitochondrial Src." Hepatology.

Woolbright, B. L., McGill, M. R., et al. (2014). "Glycodeoxycholic Acid Levels as Prognostic Biomarker in Acetaminophen-Induced Acute Liver Failure Patients." Toxicological Sciences **142**(2): 436-444.

Xie, Y., McGill, M. R., et al. (2015a). "Time course of acetaminophen-protein adducts and acetaminophen metabolites in circulation of overdose patients and in HepaRG cells." Xenobiotica **45**(10): 921-929.

Xie, Y., McGill, M. R., et al. (2014). "Mechanisms of acetaminophen-induced cell death in primary human hepatocytes." Toxicol Appl Pharmacol **279**(3): 266-274.

Xie, Y., McGill, M. R., et al. (2015b). "Mitochondrial protein adducts formation and mitochondrial dysfunction during N-acetyl-m-aminophenol (AMAP)-induced hepatotoxicity in primary human hepatocytes." Toxicol Appl Pharmacol.

Xie, Y., Ramachandran, A., et al. (2015c). "Inhibitor of apoptosis signal-regulating kinase 1 protects against acetaminophen-induced liver injury." Toxicol Appl Pharmacol **286**(1): 1-9.

Xie, Y., Williams, C. D., et al. (2013). "Purinergic receptor antagonist A438079 protects against acetaminophen-induced liver injury by inhibiting p450 isoenzymes, not by inflammasome activation." Toxicol Sci **131**(1): 325-335.

Xie, Y. W., B.L.; Kos M.; McGill M.R.; Dorko K.; Kumer S.C.; Schimitt T.M.; Jaeschke H (2015d). "Lack of Direct Cytotoxicity of Extracellular ATP against

Hepatocytes: Role in the Mechanism of Acetaminophen Hepatotoxicity." Journal of Clinical and Translationa Research **2015**(2): 100-106.

Zand, R., Nelson, S. D., et al. (1993). "Inhibition and induction of cytochrome P4502E1-catalyzed oxidation by isoniazid in humans." Clin Pharmacol Ther **54**(2): 142-149.

Zhang, Y. F., He, W., et al. (2014). "Role of receptor interacting protein (RIP)1 on apoptosis-inducing factor-mediated necroptosis during acetaminophen-evoked acute liver failure in mice." Toxicol Lett **225**(3): 445-453.