Pharm Res (2013) 30:2174–2187
DOI 10.1007/s11095-013-1007-6

EXPERT REVIEW

Metabolism and Disposition of Acetaminophen: Recent Advances in Relation to Hepatotoxicity and Diagnosis

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Received: 19 December 2012 / Accepted: 6 February 2013 / Published online: 6 March 2013

ABSTRACT Acetaminophen (APAP) is one of the most widely used drugs. Though safe at therapeutic doses, overdose causes mitochondrial dysfunction and centrilobular necrosis in the liver. The first studies of APAP metabolism and activation were published more than 40 years ago. Most of the drug is eliminated by glucuronidation and sulfation. These reactions are catalyzed by UDP-glucuronosyltransferases (UGT1A1 and 1A6) and sulfotransferases (SULT1A1, 1A3/4, and 1E1), respectively. However, some is converted by CYP2E1 and other cytochrome P450 enzymes to a reactive intermediate that can bind to sulfhydryl groups. The metabolite can deplete liver glutathione (GSH) and modify cellular proteins. GSH binding occurs spontaneously, but may also involve GSH-S-transferases. Protein binding leads to oxidative stress and mitochondrial damage. The glucuronide, sulfate, and GSH conjugates are excreted by transporters in the canalicular (Mrp2 and Bcrp) and basolateral (Mrp3 and Mrp4) hepatocyte membranes. Conditions that interfere with metabolism and metabolic activation can alter the hepatotoxicity of the drug. Recent data providing novel insights into these processes, particularly in humans, are reviewed in the context of earlier work, and the effects of altered metabolism and reactive metabolite formation are discussed. Recent advances in the diagnostic use of serum adducts are covered.

KEY WORDS acetaminophen · drug metabolism · drug transporters · hepatotoxicity · nuclear receptors

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INTRODUCTION

At therapeutic doses, acetaminophen (APAP) is a safe and effective analgesic and fever reducer. In fact, it is the most commonly used drug in the United States (1). In 2008 alone, more than 24.6 billion doses were sold (2). However, overdose of APAP can cause severe liver injury. The first cases of APAP hepatotoxicity were reported in 1966 (3). It is now the principal cause of acute liver failure in many Western countries (4–8). In the U.S., APAP overdose is responsible for 50–80,000 emergency department visits each year (9,10), as well as 26,000 hospitalizations and nearly 500 deaths (9). Fortunately, early mechanistic studies in mice led to the discovery that N-acetyl-cysteine (NAC) is a very effective antidote for APAP overdose when administered early, during APAP metabolism. The primary therapeutic effect of NAC is replenishment of glutathione (GSH), which can scavenge the reactive metabolite of APAP (11,12). However, later effects include the scavenging of reactive oxygen in mitochondria and support of mitochondrial energy metabolism (13,14).

The year 2013 marks the 40th anniversary of the publication of a series of seminal papers that played a major part in the clinical development of NAC (15–18). Recent research has focused heavily on the later events in the mechanism of APAP toxicity (Fig. 1). It is now believed that protein binding and mitochondrial damage are central in the toxicity of APAP. In mice, liver mitochondria display altered morphology (19), have reduced respiration (20), and have signs of oxidative stress after APAP treatment (21–23). The initial reactive oxygen species (ROS) formation leads to activation of the mitogen-activated protein kinase (MAPK) c-Jun N-terminal kinase (JNK) through mixed lineage kinase 3 (MLK3) (24) and apoptosis-signal regulating kinase 1 (ASK1) (25), and translocation of active JNK into

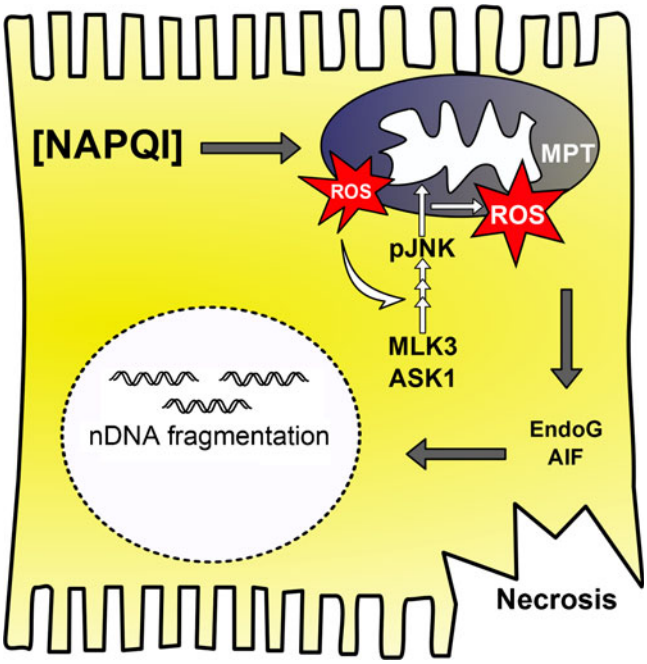

Fig. 1 Late mechanisms in APAP hepatotoxicity. Protein binding, particularly to mitochondrial proteins, leads to oxidative stress and mitochondrial dysfunction. The initial oxidative stress activates the mitogen-activated protein kinases MLK3 and ASK1, which activate JNK in turn. JNK translocates to into the mitochondria, amplifying the oxidative stress and injury. Occurrence of the mitochondrial permeability transition (MPT) and rupture of the outer membrane result in release of the endonucleases apoptosis-inducing factor (AIF) and endonuclease G (EndoG), which enter the nucleus and degrade nuclear DNA.

mitochondria exacerbates the oxidative stress and cell injury (26–30) (Fig. 1). The occurrence of the mitochondrial membrane permeability transition (MPT) results in collapse of mitochondrial membrane potential (31,32) and is the likely cause of the reduced respiration. Also, translocation of Bax to the mitochondria and loss of that organelle's membrane integrity lead to release of mitochondrial proteins, including endonucleases that can translocate to the nucleus and cleave nuclear DNA (33–35). The result of these events is hepatocyte necrosis (36). Although it has been occasionally suggested that apoptosis may be an important mode of cell death during APAP hepatotoxicity, the preponderance of data argue against this (30,36). Importantly, while most of work described above has been done in rodents, the same mechanisms appear to be relevant in humans (37–39). Together, these studies have provided great insight into the mechanisms of APAP hepatotoxicity. However, it is important to remember that the later mechanisms in APAP hepatotoxicity critically depend upon the metabolism of APAP that occurs upstream. Unfortunately, despite decades of progress, questions remain regarding the effect of metabolism and metabolic activation in APAP hepatotoxicity. The trigger of the initial oxidative stress in mitochondria and specific targets of the reactive metabolite of APAP that can explain the cell injury have yet to be identified (reviewed in 30,40).

The purpose of this review is to summarize and discuss the cumulative knowledge regarding the metabolism and hepatotoxicity of APAP, including the latest advances in these fields, and how they are related. Phase I, II, and III metabolism will be included (beginning with phase II, which is first in the timeline for APAP). Importantly, recent work toward an understanding of the specific targets of APAP-protein binding and new developments in the possible clinical use of measurements of acetaminophen-protein binding will be reviewed.

ACETAMINOPHEN METABOLISM

Absorption and Phase II Metabolism

Absorption and Glucuronidation

APAP is a weak acid with pKa≈9.5. Thus, at physiological pH it is almost entirely neutral and is therefore rapidly absorbed from the duodenum. Because of this, measurement of plasma APAP levels following ingestion is a convenient way to assess gastric emptying rates in clinical studies (41,42) and has been used for decades for this purpose. In humans, the half-life of APAP in blood after a therapeutic dose is 1.5–3 h (43,44), but increases after toxic doses and with liver injury (45). Elimination occurs in the liver, where the majority of the drug is either glucuronidated or sulfated and then excreted in the urine. APAP-glucuronide accounts for 50–70% of the administered drug after a therapeutic dose in humans. Glucuronidation is catalyzed by UDP-glucuronosyl transferases (UGT) (Fig. 2). These enzymes transfer the glucuronosyl group of uridine 5′-diphosphoglucuronic acid (UDP-glucuronic acid) to target molecules, making them more water-soluble. A number of UGTs have been described in humans and rodents, belonging to four families (UGT1, UGT2, UGT3, and UGT8) (46). It was shown in the 1980s that Gunn rats, which are known to be poor bilirubin glucuronidators, were more susceptible to APAP hepatotoxicity than other strains, which were completely resistant (47). The increase in injury was probably due to increased formation of the downstream reactive metabolite (47). These findings were extended to humans in a clinical study comparing APAP glucuronidation and bioactivation in UGT-deficient Gilbert's syndrome patients and normal volunteers (48). It was later found that the primary defect in Gilbert's syndrome is in the promoter for UGT1A1 (49,50). There is now evidence for involvement of several UGTs in APAP glucuronidation from both *in vitro* and *in vivo* experiments (48,51,52). The strongest evidence from humans suggests that 1A1 and 1A6 are

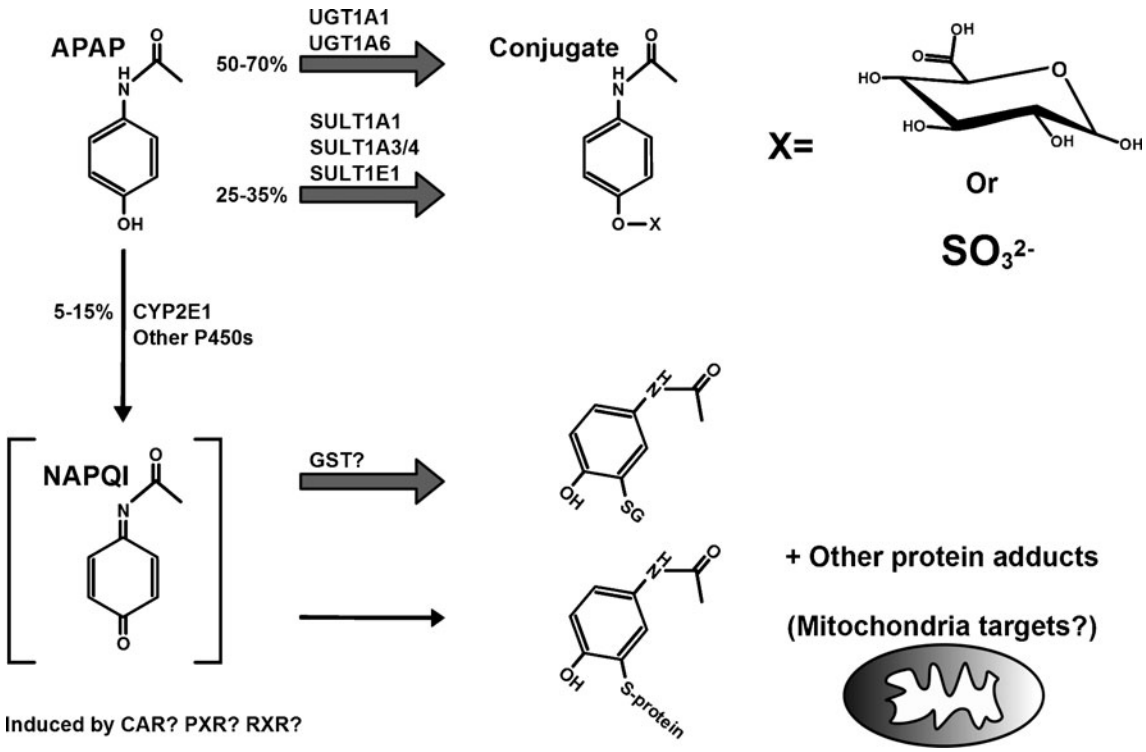

Fig. 2 Metabolism and metabolic activation of APAP. Most of the drug is glucuronidated or sulfated before excretion, catalyzed by UDP-glucuronosyltransferases (UGT) and sulfotransferases (SULT), respectively. A small percentage is converted to a reactive metabolite (NAPQI) by cytochromes P450 (primarily CYP2E1). This may be regulated in part by nuclear receptors such as CAR, PXR, and RXR. The metabolite can be detoxified by conjugation with glutathione (GSH). Alternatively, it can react with protein thiols. There is evidence that mitochondrial proteins in particular are targeted by NAPQI.

critical. The role of 1A1 has been questioned on the basis of conflicting data from other work with individuals with Gilbert's syndrome (53,54). However, some of the discrepancy may be due to differences in experimental design, including patient selection criteria and normalization of the dose of APAP (48). It has also been suggested that concurrent mutations in other UGTs associated with 1A1 through linkage disequilibrium in some Gilbert's syndrome patients could account for the differences (54). Interestingly, it was recently shown that obese mice with steatosis have higher expression of UGTs than wildtype controls, and samples from these animals had higher concentrations of APAP-glucuronide (55,56). The mechanism by which obesity leads to increased glucuronidation in mice is not yet known. A trend toward increased expression of certain UGTs has also been observed in humans with nonalcoholic fatty liver disease (57). However, this trend did not achieve significance for any isoform and there was no difference in APAP-glucuronidation activity compared with controls (57).

Sulfation

Relatively less work has been done to understand APAP sulfation. It is known that 25–35% of a therapeutic dose of APAP is recovered as APAP-sulfate (Fig. 2). Interestingly, it has been shown that mice lacking NaS1, a kidney transporter that is involved in reabsorption of inorganic sulfate (SO_4^{2-}), are more susceptible to APAP hepatotoxicity, and NaS1 polymorphisms are known to occur in humans (58). Sulfation is catalyzed by sulfotransferase (SULT) enzymes. Generally, these enzymes transfer a sulfo group from 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to an acceptor, like APAP. PAPS is synthesized from sulfate derived from diet. At least thirteen SULT isoforms are known in humans and are organized into four families (59). Sulfation of xenobiotics, in particular, is usually catalyzed by cytosolic SULTs (the other major group, Golgi membrane-associated SULTs, act on larger substrates, including proteins) (59). Using platelet preparations as surrogates for xenobiotic metabolism in the liver, it was shown that human SULT1A1 and 1A3/4 (thermostable and thermolabile sulfotransferases, respectively) can catalyze APAP sulfation (60). These findings were recently confirmed through *in vitro* assays using fetal human liver samples, and expanded to include SULT1E1 (61). Moreover, increased protein levels of SULT1A1 have been observed in pregnant mice with a corresponding increase in APAP-sulfation activity in liver fractions (62). Studies of APAP pharmacokinetics in humans with polymorphisms in these SULTs would be helpful to

determine which isoforms are clinically relevant. Interestingly, new data have shown that SULT1A1 protein is significantly increased in liver from humans with steatosis, and microsomal fractions from these samples had higher APAP-sulfation activity (57).

Before moving on, it is worth noting that most of the above work was done in humans and human models. Differences in metabolism are known to exist between humans, mice, and rats. Much of our knowledge concerning specific enzymes and processes involved in phase I APAP metabolism has come from rodent studies, with limited corroboration in human models. Thus, interpretation of these data and extrapolation to humans must be carried out with caution.

Phase I Metabolism

Cytochrome P450-Mediated Metabolic Activation

After a therapeutic dose of APAP, about 5–15% is excreted in urine as a mercapturic acid or cysteine conjugate (Fig. 2). This is due to conversion of APAP to a reactive intermediate which can bind to the cysteine thiol of GSH. While the glucuronide and sulfate conjugates of APAP are directly excreted in urine, APAP-GSH is initially excreted in bile, degraded in other organs including the kidney (63,64), and the degradation products are ultimately excreted in urine (65).

The metabolic activation of APAP is principally catalyzed by cytochrome P450 enzymes (17) and the reactive metabolite of greatest relevance for hepatotoxicity is generally believed to be N-acetyl-p-benzoquinone imine (NAPQI) (66). NAPQI is a soft electrophile that reacts readily with nucleophilic sulfhydryl groups. Overdose of APAP results in formation of excess NAPQI, which can deplete GSH levels and bind to proteins (16). Evidence for GSH depletion and protein binding is not limited to rodents. In patients given bromosulphthalein, the plasma concentration of the GSH conjugate of this drug was decreased after APAP overdose (67). More convincingly, increasing therapeutic doses of APAP were found to increase the turnover of GSH in volunteer subjects (68) and APAP-protein adducts can be measured in samples from APAP overdose patients (69). Cysteine residues are the major targets for covalent modification by the reactive intermediate of APAP (70), though binding to lysine has also been reported and may contribute to mitochondrial damage during APAP toxicity (71) and it is possible that other amino acids react with NAPQI under certain conditions. The observation that alcohol and isoniazid could affect APAP-induced liver injury led to the hypothesis that CYP2E1 is the major P450 responsible for conversion of APAP to NAPQI (72–77). Accordingly, Cyp2e1 knockout mice were found to be less susceptible to APAP-induced liver injury (78). In addition, beta-catenin gene-deficient mice show almost complete elimination of Cyp2e1 and Cyp1a2 protein levels, which correlated with resistance to APAP hepatotoxicity (79). CYP1A2, 2D6, and 3A4 have also been shown to activate APAP in various model systems (80–82). However, Cyp1a2 −/− mice were not protected against APAP toxicity (83). Moreover, the finding that Cyp2e1 −/− mice were resistant to the hepatotoxicity caused by high doses of APAP, while the same knockout mice transgenically expressing human CYP2E1 were susceptible (84) indicates that CYP2E1 is indeed the main P450 enzyme involved in APAP activation. Data from humans support this conclusion (85,86). One flaw in the latter studies is reliance upon pharmacological CYP inducers and inhibitors which may or may not be specific. More importantly, only low doses of APAP could be given in the human experiments and there is evidence from mice that other P450s, including CYP1A2, become important with increasing exposure (87). Consistent with this, APAP-protein adducts and toxicity have been measured in APAP-treated human HepaRG cells (37), which express relatively low levels of CYP2E1 (88). Some studies with human liver microsomes also suggest that CYP3A4 are more important than CYP2E1 (89). Together, the data suggest that CYP2E1 is the primary enzyme responsible for conversion of APAP to its reactive intermediate, but a role for P450s other than CYP2E1 (particularly 1A2 and 3A4) cannot be ruled out.

Induction of some cytochrome P450 enzymes is known to occur after APAP treatment. Limited evidence suggests that the constitutive androstane receptor (CAR), the pregnane X receptor (PXR), and the retinoid X receptor alpha (RXRα) are activated during APAP toxicity and can potentiate APAP hepatotoxicity through upregulation of P450 enzymes or altered GSH homeostasis (90–93). However, the results of these studies should be interpreted with caution as CAR and PXR increase expression of P450 enzymes other than Cyp2e1. As mentioned, these other P450 isoforms likely have only a minor contribution to NAPQI formation. In fact, CAR activation was found to modestly decrease Cyp2e1 (90). Thus, the proposed mechanisms in the above papers may not be correct (94). In contrast to these nuclear receptors, activation of the peroxisome proliferator-activated receptor alpha (PPARα) reduces APAP-induced injury, though this may not involve changes in metabolism (95,96). Similarly, farnesoid X receptor (FXR) and liver X receptor (LXR) activation appear to protect by decreasing expression of phase I enzymes and/or increasing expression of protective and detoxifying enzymes (97,98). However, these data are limited at best.

Recent reports indicated that deficiency of natural killer T cell (NKT) in mice (CD1d−/− and Jα18−/− mice) increased Cyp2e1 protein expression and enzyme activities, which enhanced metabolic activation of APAP, increased

protein binding, and aggravated hepatotoxicity (99), It was concluded that increased ketone body formation during starvation in NKT-deficient mice was responsible for the induction of Cyp2e1 (99). Consequently, mice fed *ad libitum* showed no difference in injury between wild type and NKT-deficient mice (99). However, another paper using the same mice (Jα18−/− mice) without starvation reported protection of NKT cell deficient mice against APAP toxicity (100). The authors concluded that increased hepatic GSH levels in these mice were the cause of protection (100). These publications are representative of many immunological studies in which conflicting results have been reported using the same animals and where differences in experimental design (fed *vs* starved) have a profound impact on the results (101).

Glutathione-S-transferases

Though glutathionylation is a phase II reaction, in the context of APAP it only occurs after phase I metabolism (Fig. 2). As mentioned, the reactive metabolite of APAP can bind to the cysteine thiol of GSH and this is a critical mechanism of detoxification. The reaction of NAPQI with GSH has been shown to occur both spontaneously and enzymatically (102). Enzymatic GSH conjugation is catalyzed by a group of enzymes called the glutathione-S-transferases (GST). It was thought that GST-Pi was most likely responsible for the enzymatic conjugation of APAP and GSH (102). However, Gst-Pi knockout mice actually had reduced injury after APAP treatment (103). It was later found that this effect may have been due to upregulation of cytoprotective genes as a result of constitutive JNK activation in the knockout mice (104). GSTM-null mice were also found to be resistant to APAP (105). While a recent study showed that altered function of GSTT and GSTM are associated with idiosyncratic hepatotoxicity (106), another group failed to identify a similar association between GST isoforms and prothrombin time or outcome in APAP overdose patients (107). Unfortunately, the latter study relied on a very small cohort with few negative outcomes. More work is needed to understand the role of GSTs in APAP hepatotoxicity, including those roles other than scavenging of reactive metabolites.

Glutathione Depletion, Protein Binding, and Mitochondria

A paradigm in the field of APAP hepatotoxicity is that depletion of approximately 70% of liver GSH is necessary for protein binding to occur (18). However, several observations have challenged this idea. First, the non-hepatotoxic meta isomer of APAP, 3′-hydroxyacetanilide or AMAP, binds to proteins despite having a less severe effect on hepatic GSH levels (108,109). Second, protein binding is detectable in human HepaRG cells within 1 h of APAP treatment, before GSH depletion (37). Finally, APAP-protein adducts can be measured in human serum after only therapeutic doses (110). Despite this, there is a clear inverse relationship between APAP metabolic activation and GSH levels (18), and measuring the early hepatic GSH depletion kinetics remains one of the best ways to assess NAPQI formation (21,111–113)

The discovery that the reactive metabolite of APAP could become covalently bound to proteins led to the hypothesis that protein binding was the cause of injury. Unfortunately, the subsequent search for a specific protein that could explain the toxicity did not yield many promising candidates (114,115). Most of the adducted proteins discovered were enzymes, the activities of which were only minimally affected (116,117), although there are exceptions (118). Moreover, none of them appeared to be proteins with vital functions. However, a comparison of doses of APAP and the non-hepatotoxic isomer AMAP that caused similar total liver protein binding revealed that the reactive intermediate of APAP binds more to mitochondrial proteins than the reactive metabolite(s) of AMAP (119). It was also shown that mitochondrial protein binding occurs before the onset of injury (120). It had previously been reported that APAP could inhibit mitochondrial respiration in mice (20). Around the same time, it was observed that APAP caused an increase in mitochondrial oxidative stress (21). More recently, an analysis of adducted proteins in mouse liver using 2D gel electrophoresis and mass spectrometry identified several specific mitochondrial targets (121). Also, expression of CYP2E1 in mitochondria only (not in the endoplasmic reticulum) was shown to be sufficient to cause cell injury after APAP treatment (122). Together, these data strongly suggest that binding to mitochondrial proteins results in decreased mitochondrial respiration and increased oxidative stress. However, some concerns exist regarding this hypothesis. It is important to note that all of these data are correlative. There is no direct evidence that selectively preventing mitochondrial protein binding can eliminate injury after APAP overdose. Furthermore, recent data have shown that AMAP can have toxic effects in rat and human precision cut liver slices (123). While mitochondrial protein adducts were not measured in this study, the results do challenge the earlier data comparing APAP and AMAP. Despite this, data from rats and mice revealed that rats are less susceptible to APAP-induced liver injury and have lower mitochondrial protein binding than mice (124). Comparing APAP with itself in two different species is more direct than comparing APAP and AMAP, different drugs with markedly different effects. Overall, the data are consistent with a critical role for mitochondrial protein binding in the mechanism of APAP-induced liver injury.

New insight into the mechanism of APAP-induced liver injury came from the discovery that JNK is activated during

APAP hepatotoxicity in mice and translocates to mitochondria, and a JNK inhibitor can protect against injury (26,27,29). However, it appears that activation of JNK may require an initial oxidative stress, which is then amplified by mitochondrial translocation and leads to the MPT (24,29,30). The source of this initial oxidative stress is not yet known. It was once thought that P450-mediated metabolism of APAP could generate ROS that could play a role in toxicity. However, while this may be the case during metabolism of ethanol (125), there is no evidence for oxidative stress at early time points after APAP treatment, when metabolism is taking place (126–128). Additional work is needed to fully understand the molecular events leading to APAP hepatotoxicity during and after metabolism.

APAP-Protein Adducts in Serum

Shortly after the development of the first method to specifically measure APAP-cysteine (APAP-CYS) in the liver (129), it was found that cysteine adducts on proteins can also be detected in serum during APAP hepatotoxicity (120). In addition, protein adducts appear to be reduced in necrotic areas of the liver at later time points (130). Because these adducts could only be measured when ALT was elevated, it was believed that they were released into serum as a result of necrosis and cell contents release. The recent discovery that serum APAP-protein adducts can be detected in humans after only therapeutic doses of APAP casts doubt on this (110,131). In any case, with the advent of more sensitive and accurate techniques, it has been suggested that serum APAP-CYS can be used as a diagnostic marker for APAP overdose in cases of liver injury in which the cause is unknown or uncertain (69). This is an intriguing possibility. Prior to this, definitive diagnosis depended on the measurement of the parent drug in serum, along with an accurate patient history. The short serum half-life of APAP made this problematic. Confident use of this parameter required that the patient presented soon after ingestion of the drug and the approximate time of ingestion could be established. APAP- protein adducts persist much longer in serum, making this a much better option. The half-life was found to be 1–2 days after an overdose for both children and adults (131).

Accurate diagnosis of the cause of liver injury can affect how patients are treated. For example, early decisions regarding the necessity of liver transplant may be based in part on etiology (132). Furthermore, when intentional overdose is suspected, the clinician can ensure that the patient receives proper psychiatric treatment. Thus, the measurement of serum APAP-CYS could be a major step forward in patient care, if it is adopted clinically. This could become another example of a significant clinical advance made through basic research in the APAP hepatotoxicity field. However, because these adducts can be detected in serum after therapeutic doses, selection of a sensitive but specific threshold concentration is critical. A combination of ≥1.1 μM APAP-CYS peak concentration and >1,000 U/L ALT has been proposed (110,133), but it is not yet known how polypharmacy and various co-morbidities can affect this parameter.

Phase III Metabolism

Metabolite Transport

The conjugates of APAP that result from phase I and II metabolism must be eliminated. However, unlike the parent drug, movement of these metabolites requires transporters (Fig. 3). Using transporter-deficient rodent models and canonical inducers, it was found that biliary excretion of both APAP-glucuronide and APAP-sulfate is largely dependent on Mrp2 and Bcrp in the canalicular hepatocyte membrane (134–138) while basolateral excretion of APAP-glucuronide involves Mrp3 (134,135,139,140). The basolateral transporters involved in APAP-sulfate excretion are less clear, but Mrp3 and Mrp4 both appear to play a role (139). Additional work confirmed these findings and revealed that the biliary excretion of APAP-GSH also requires Mrp2 (141). Despite significant species differences, it is interesting that induction of basolateral and canalicular efflux transporters has been shown in both APAP-treated rodents (142,143) and APAP overdose patients (144). MRP2, BCRP, MRP4 and MRP5 protein levels were shown to be increased in samples from APAP overdose patients (144,145). Unfortunately, information regarding the time post-ingestion was not available in this study. Apparently, the samples were obtained from patients who required liver transplant (144), and this is likely when most of the samples were taken. Thus, the time points measured were probably several days after APAP overdose and onset of injury. It is possible that some early changes in transporter expression were missed, while changes occurring secondary to injury were detected. Therefore, the results, while interesting, must be interpreted carefully. In any case, altered transporter expression after APAP treatment seems to involve transcription factors and nuclear receptors. Some changes in transporter expression in mice (Mrp3 and Mrp4) after APAP are dependent on Nrf2 (146), and may be influenced by Kupffer cell-derived cytokines (147). There is also evidence that nuclear receptors that have been shown to play a role in APAP toxicity can induce expression of several of these transporters under certain conditions (145) (Fig. 3).

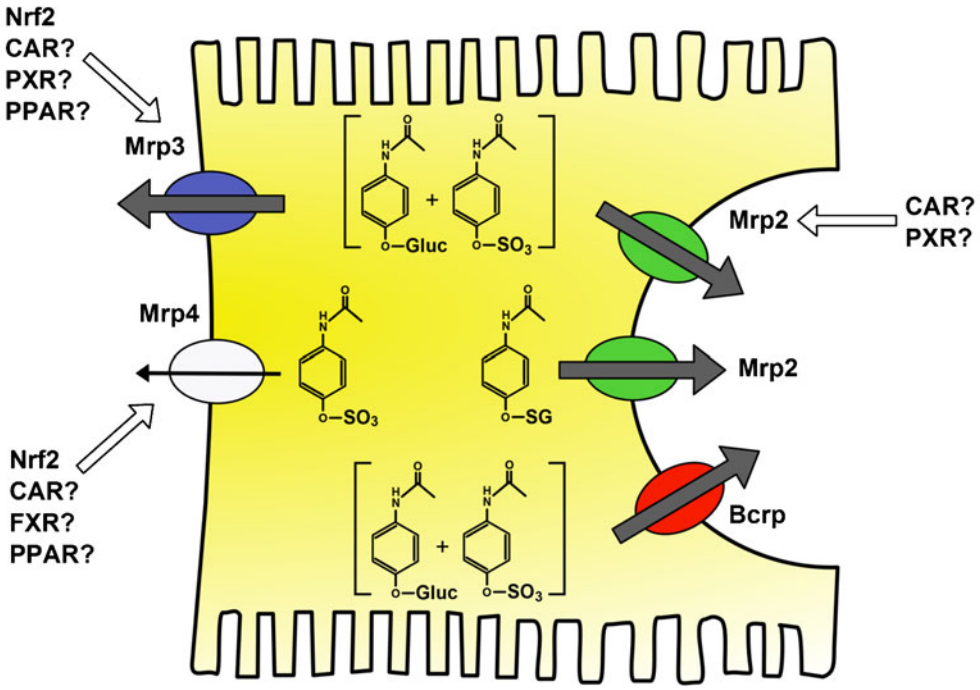

Fig. 3 The glucuronide, sulfate, and glutathione conjugates of APAP are excreted into blood and bile by transporters in the basolateral and canalicular membranes, respectively. Expression of these transporters may be regulated in part by several nuclear receptors, including Nrf2, CAR, PXR, FXR, and PPAR (see text for details).

INTERVENTIONS AFFECTING APAP METABOLISM

Because metabolic activation is necessary for the downstream toxic effects, inhibition of P450 enzymes is a very effective way to prevent APAP-induced liver injury. Ethanol has been shown to be a competitive inhibitor of P450-mediated APAP metabolism when co-administered in some rodent models (74,148,149). There is also limited evidence for inhibition of metabolism and reduced toxicity as a result of alcohol co-ingestion in humans (110,150,151), although this is controversial (149,152). While acute ethanol exposure may reduce APAP-induced liver injury, chronic alcohol exposure can increase the metabolic activation and toxicity of APAP in rodents (72,73). The same effects may also occur in humans (77,152,153), though this too is controversial (154). The effect of chronic alcohol treatment on APAP in rodents is likely due to induction of P450 enzymes, especially cyp2e1. Although ethanol may enhance APAP toxicity after an overdose, there is no evidence that it can cause toxicity after therapeutic doses of APAP (155,156). Similar to ethanol, isoniazid has been shown to both inhibit and enhance APAP metabolism, depending upon whether the drug was co-ingested or administered chronically before withdrawal (75). Some other drugs, including the histamine receptor antagonist cimetidine (157), can interfere with APAP metabolism in both rodents and humans.

A number of natural products and herbal therapeutics thought to protect against APAP hepatotoxicity by acting as antioxidants or cell death signaling disruptors actually interfere with APAP metabolic activation (111–113,158). Unfortunately, such mistakes are not limited to work with natural products. An experimentally important inhibitor of P450-mediated APAP metabolism is the commonly used drug vehicle dimethyl sulfoxide (DMSO) (159–161). Ignoring the ability of DMSO, which is used as a solvent for caspase inhibitors, to inhibit metabolic activation has led to a number of controversies regarding the mode of cell death (apoptosis or necrosis) after APAP treatment (30,161). In addition, failure to properly assess the metabolic effects of pharmacological interventions in models of APAP hepatotoxicity has led to unjustified conclusions regarding the therapeutic efficacy and mechanism of action of some compounds (162). It is very important to ensure reactive metabolite formation is not affected in studies of APAP-induced liver injury. Metabolic activation can be assessed by measuring hepatic GSH or APAP-protein adducts at early time points following APAP treatment (21,112,158). The resistance of rats to APAP toxicity also appears to be partially dependent upon reduced formation of the reactive intermediate of APAP (124). As a result of this and other effects downstream of the metabolic activation, the rat is generally not a good model for the study of APAP hepatotoxicity (124), though it has been somewhat useful in the study of APAP metabolism in general.

Aside from inhibition or induction of P450s, any compounds that can deplete GSH or enhance its resynthesis after APAP could affect APAP-induced liver injury through either reduced or increased scavenging of the reactive metabolite, respectively. Classical GSH depleting agents like diethyl maleate and buthionine sulfoxide (BSO) enhance the toxicity of APAP (18). Importantly, opioids can reduce levels of hepatic GSH and may potentiate APAP-induced liver injury in mice and even in humans (163,164). A more recent

study has shown that fenbendazole, an anthelmintic drug often administered to laboratory rodents in their chow, can prolong GSH depletion after APAP and enhance injury (165). Interestingly, chronic ethanol exposure can selectively deplete mitochondrial GSH in hepatocytes (166). It is possible that this could increase APAP toxicity as well. Other compounds that have been shown to alter the metabolism of APAP include tyrosine kinase inhibitors and oral contraceptives, both of which may affect glucuronidation (167,168).

CONSIDERATIONS FOR CELL CULTURE EXPERIMENTS

P450-mediated activation is critical for the study of APAP toxicity in cell culture as well. The formation of APAP-protein adducts in isolated hepatocytes from different species correlates with cell death and enzyme release (169) and inhibition of P450 enzymes can prevent this injury (170). Currently, primary hepatocytes are considered the gold standard for studies of drug metabolism and toxicity *in vitro*. Cultured cells are convenient to work with and facilitate the investigation of toxic mechanisms at a level of detail often not possible *in vivo*. However, the expression and activity of drug metabolizing enzymes in cultured rodent hepatocytes decrease significantly beyond 24 h (171,172) and although expression of some cytochromes P450 is maintained longer in human hepatocytes, eventually these cells also lose their drug-metabolizing capabilities (173). In addition, variation in drug metabolism across donors and the difficulty in obtaining tissue can make the use of primary human hepatocytes difficult or impractical. A number of strategies to prevent changes in expression of drug metabolizing enzymes in long term cultures of primary hepatocytes exist. Various supplements, including dexamethasone, dimethyl sulfoxide (DMSO), and P450 inducers (e.g. Phenobarbital), can be added to the culture medium (171,174,175). Growth and maintenance of the cells on matrigel or in three dimensional culture vessels may also help to prevent or delay dedifferentiation (175). However, the best results are still achieved with freshly isolated primary hepatocytes.

Cell lines are widely available, relatively inexpensive, and often easier to work with than primary cells. Unfortunately, most hepatoma cell lines express P450s at very low levels compared to freshly isolated hepatocytes or to intact liver (176) and may not be representative of the *in vivo* situation. CYP2E1-transfected HepG2 cells have been used to study APAP toxicity (177), but the relevance of this model is unclear. HepaRG cells are a relatively new human liver cell line. These cells were isolated and grown from tissue of a female patient with hepatocellular carcinoma subsequent to chronic hepatitis C virus infection (178,179). The initial popularity of the HepaRG cell line was due to its unique complaisance to hepatitis B infection (178) and because it has the unusual property of bipotency, capable of differentiating into both hepatocytes and biliary epithelial cells (179). A wealth of literature now attests to the drug metabolizing capabilities of HepaRG cells (180). Importantly, the mechanisms of APAP toxicity in HepaRG cells appear to mimic those in mice (37). However, very high concentrations of the drug are required. It is interesting that the long time course of toxicity in HepaRG cells after exposure to APAP more closely resembles humans (37). However, the reason for the delay in injury is not yet clear and it is important to remember that this is still a hepatoma line. Differences in cell signaling and cell death pathways may exist between HepaRG cells and freshly isolated hepatocytes. Also, DMSO is required to maintain differentiation of these cells. As mentioned, DMSO is also a potent inhibitor of P450 enzymes (159–161). The differentiation medium must be removed from these cultures and replaced with DMSO-free medium prior to treatment with APAP.

Oxygen concentration is also an important consideration when conducting drug studies using cell culture models. There is evidence that P450 expression in some cell lines is optimal at low, near-physiological oxygen levels for the centrilobular region of the liver (about 3–5%) (181). On the other hand, hypoxia has been shown to reduce the expression of drug-metabolizing enzymes in HepaRG cells (182). When thinking about oxygen levels in cell culture experiments, it is important to remember the effect of medium volume (181), as gas must diffuse through the medium. Thus, cells kept under atmospheric (21%) oxygen are not actually exposed to this level. In order to make comparisons across multiple studies, the volume and depth of the culture medium should be consistent. Even with this in mind, 95% oxygen is probably not appropriate for any cell culture studies of drug metabolism or toxicity. In the case of APAP, oxygen concentration can also affect the mechanisms of toxicity downstream of metabolism. Not surprisingly, lowering oxygen levels can reduce and even prevent APAP-induced oxidative stress and protect against toxicity *in vitro* (183).

SUMMARY AND CONCLUSIONS

It has been 40 years since the first studies were performed that led to the establishment of the mouse model of APAP hepatotoxicity and the introduction of NAC as an antidote for APAP poisoning. We know that elimination of APAP involves phase I, II, and III metabolism. Phase II metabolism removes most of the drug prior to phase I and involves UGT1A1 and 1A6, as well as SULT1A1, 1A3/4, and possibly 1E1. Phase I metabolism is predominantly

mediated by CYP2E1 and produces a reactive metabolite. This toxic intermediate can bind to sulfhydryl groups, spontaneously reacting with GSH. It can also bind to hepatic proteins. Protein binding is the critical initiating event in the cell death observed during APAP-induced liver injury. Though new data have challenged old ideas concerning the relationship between GSH depletion and protein binding, the covalent modification of proteins in the liver remains central in the mechanism of toxicity and any intervention that affects this will alter the downstream mitochondrial dysfunction, oxidative stress, and injury. Unfortunately, efforts to identify a specific target of the APAP metabolite failed to find any that could account for the massive hepatic necrosis following an APAP overdose, though there is evidence that binding to mitochondrial proteins is important. While recent work has strengthened this hypothesis, a better mechanistic understanding is still needed. Importantly, protein adducts can also be detected in serum and measurement of these serum adducts may become an important addition to the clinician's diagnostic toolbox in the near future. Finally, in rodents the excretion of APAP-glucuronide, APAP-sulfate, and APAP-GSH is mediated by Mrp2, Mrp3, and Bcrp. Inhibition of any of these steps, especially P450-mediated metabolic activation, can affect the injury.

ACKNOWLEDGMENTS AND DISCLOSURES

Work in the authors' laboratory was supported in part by National Institutes of Health Grants AA12916 and DK070195 and by grants from the National Center for Research Resources (5P20RR021940-07) and the National Institute of General Medical Sciences (8 P20 GM103549-07) from the National Institutes of Health. M.R. McGill was supported by the "Training Program in Environmental Toxicology" (T32 ES007079-26A2) from the National Institute of Environmental Health Sciences. The authors declare that there are no conflicts of interest.

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