9.21 Mechanisms of Acetaminophen Hepatotoxicity

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Abbreviations

AIF	apoptosis-inducing factor
ALT	alanine aminotransferase
AP-1	activator protein-1
APAP	acetaminophen
Bcrp1	breast cancer resistance protein 1
cyp	cytochrome P450
DMSO	dimethyl sulfoxide
EndoG	endonuclease G
eNOS	endothelial nitric oxide synthase
GSH	glutathione
GSSG	glutathione disulfide
GSTPi	glutathione *S*-transferase Pi
HMGB1	high-mobility group box 1 protein
HO-1	hemeoxygenase-1
ICAM-1	intercellular adhesion molecule-1
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
MCP-1	monocyte chemoattractant protein 1
MMP	matrix metalloproteinase
MPT	mitochondrial permeability transition
mrp	multidrug resistance-associated protein
NAC	*N*-acetylcysteine
NAPQI	*N*-acetyl-*p*-benzoquinone imine

NK	natural killer	**SEC**	sinusoidal endothelial cell
NKT	natural killer T	**Smac**	second mitochondria-derived activator of caspase
NO	nitric oxide		
O_2^-	superoxide	**SULT**	sulfotransferase
$ONOO^-$	peroxynitrite	**TNF**	tumor necrosis factor
PAR-1	protease-activated receptor-1	**UGT**	UDP-glucuronosyltransferase
PARP-1	poly-(ADP-ribose) polymerase-1	**$\Delta\Psi m$**	mitochondrial membrane potential
RNS	reactive nitrogen species	**VEGF**	vascular endothelial growth factor
ROS	reactive oxygen species	**Z-VAD-fmk**	Z-Val-Ala-Asp-fluoromethylketone

9.21.1 Introduction

Acetaminophen (*N*-acetyl-*p*-aminophenol, APAP, 4-hydroxyacetanilide, paracetamol) is a widely used analgesic and antipyretic. At therapeutic levels, APAP is considered an efficacious and safe drug even for susceptible individuals, for example, alcoholics (Dart and Bailey 2007; Dart *et al.* 2000; Kuffner *et al.* 2007; Prescott 2000). However, an acute or cumulative overdose can cause centrilobular necrosis and even induce liver failure. APAP hepatotoxicity is currently not only the most frequent cause of drug-induced liver failure in the United States and other industrialized nations but also the most frequent cause of acute liver failure of any etiology (Larson *et al.* 2005). Although APAP overdose can also cause nephrotoxicity, the incidence is low compared to the hepatotoxic effects (Blakely and McDonald 1995).

The first mechanistic investigations into APAP-induced liver injury in mice were published more than 35 years ago (Mitchell *et al.* 1973a). These original studies, which identified the critical role of metabolic activation, covalent binding, and the beneficial effects of glutathione in APAP hepatotoxicity (Jollow *et al.* 1973; Mitchell *et al.* 1973a,b; Potter *et al.* 1973), led to the introduction of *N*-acetylcysteine (NAC) as a clinical antidote (Mitchell *et al.* 1974; Smilkstein *et al.* 1988). However, it was only recently that the complex intracellular signaling mechanisms became more apparent. Although the events at the level of hepatocytes are sufficient to cause cell death, these mechanisms can be modulated *in vivo* by inflammatory mediators and immune mechanisms (Jaeschke 2005, 2006a, 2008; Laskin and Laskin 2001; Liu and Kaplowitz 2006).

9.21.2 Drug Metabolism and Initiation of Cell Injury

The metabolism of APAP has been extensively studied and several excellent comprehensive reviews are available (Nelson 1990; Nelson and Bruschi 2001). APAP can either directly undergo phase II metabolism or is first metabolized by the P450 system and then is conjugated with glutathione (**Figure 1**).

9.21.2.1 Phase II Metabolism

APAP is mainly conjugated by sulfation and glucuronidation at its phenolic group. At therapeutic doses, about 50% of APAP is conjugated with glucuronic acid by UDP-glucuronosyltransferases (UGTs), especially UGT1A6 (Bock and Köhle 2005), and about 30% is sulfated by phenol sulfotransferases, for example, SULT1 (Lindsay *et al.* 2008). The glucuronic acid conjugates are rapidly removed from hepatocytes by excretion mainly (>75%) into bile (Slitt *et al.* 2003). The biliary excretion is almost entirely dependent on the canalicular transporter multidrug resistance-associated protein 2 (mrp2) (Xiong *et al.* 2000). A smaller fraction (<25%) of the APAP-glucuronide is excreted into the plasma through the basolateral transporter mrp3 (Zamek-Gliszczynski *et al.* 2006). The predominantly biliary excretion of the glucuronide can be shifted to the plasma by chemical-induced induction of mrp3 (Ghanem *et al.* 2005; Slitt *et al.* 2003). Alternatively, the impaired basolateral excretion of APAP-glucuronide in mrp3-deficient mice results in lower plasma levels, higher accumulation in hepatocytes, and a 10-fold higher biliary excretion of the metabolite compared to wild-type animals (Manautou *et al.* 2005).

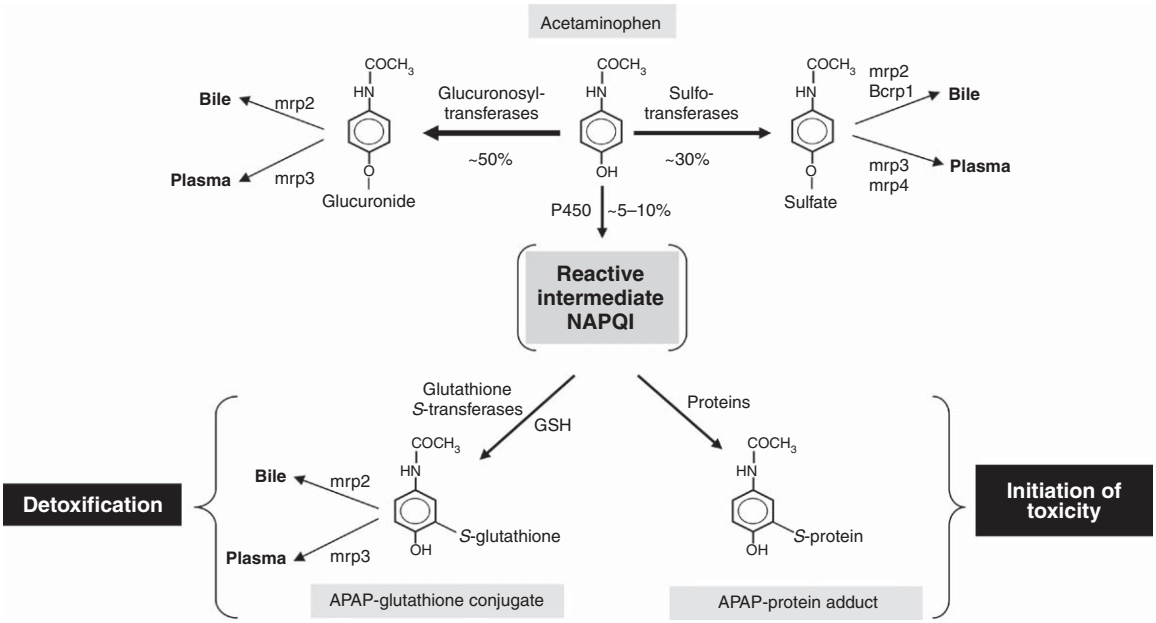

Figure 1 Basic metabolism of acetaminophen (APAP) in hepatocytes and initiation of toxicity (see text for details). Bcrp1, breast cancer resistance protein 1; GSH, glutathione; mrp, multidrug resistance-associated protein; NAPQI, *N*-acetyl-*p*-benzoquinone imine.

The APAP-sulfate conjugate is excreted into bile via mrp2 and to a lesser degree by human breast cancer resistance protein 1 (Bcrp1) (Zamek-Gliszczynski *et al.* 2005). The basolateral excretion of APAP-sulfates involves mrp3 and mrp4 (Zamek-Gliszczynski *et al.* 2006).

The pathophysiological importance of these conjugation pathways has been demonstrated. Gunn rats, which are deficient in UGTs, show increased hepatotoxicity due to impaired glucuronidation, which results in increased bioactivation (de Morais *et al.* 1992a). A similar deficiency in humans (Gilbert's syndrome) also causes decreased glucuronidation and increased bioactivation of APAP, which increases the risk of liver injury after APAP overdose (de Morais *et al.* 1992b; Esteban and Pérez-Mateo 1999).

Furthermore, a recent study with mice lacking the NaS1 sulfate transporter showed reduced sulfation reactions and increased APAP hepatotoxicity due to enhanced bioactivation (Lee *et al.* 2006). Overall, these findings indicate that a substantial impairment of either conjugation pathway may lead to enhanced bioactivation of APAP and therefore increases the risk of liver injury. However, minor modifications in either of these pathways can be compensated by the other. This may be the reason why toxicologically relevant interferences in the phase II metabolism of APAP by other drugs are rare (Nelson and Bruschi 2001).

9.21.2.2 P450-Dependent Metabolism

A relatively small fraction (5–10%) of a therapeutic dose of APAP is metabolized by the cytochrome P450 (cyp) system to form *N*-acetyl-*p*-benzoquinone imine (NAPQI), the principal toxic metabolite of APAP (Dahlin *et al.* 1984; Jollow *et al.* 1973; Nelson 1990). A number of cyp isoforms can metabolize APAP *in vitro*, but the relevance of each isoform for liver toxicity can vary considerably (Patten *et al.* 1993). Cyp1A2 is an effective catalyst for NAPQI formation *in vitro*, but cyp1A2 knockout mice are not more susceptible to APAP-induced liver injury than wild-type mice (Tonge *et al.* 1998). Cyp3A4 is most effective in catalyzing NAPQI formation (Thummel *et al.* 1993). Although cyp3A4 represents 30% of the total cyp activity in human livers, the low K_m for APAP limits its impact *in vivo*. Based on inhibitor studies *in vitro* and inducer studies with therapeutic doses *in vivo*, it was estimated that only approximately 10% of NAPQI in humans is formed by cyp3A4 (Manyike *et al.* 2000; Thummel *et al.* 1993). Thus, due to its low K_m, cyp3A4 is of limited

relevance for the toxicity after an overdose. On the other hand, cyp2E1 is generally considered the most important cyp isoform for NAPQI formation *in vivo* (Chen *et al.* 1998; Manyike *et al.* 2000; Patten *et al.* 1993; Raucy *et al.* 1989). Several important drug–drug interactions are known. Isoniazid and alcohol are substrates for cyp2E1 and inhibit APAP metabolism while these drugs are present (Sato and Lieber 1981; Zand *et al.* 1993). However, both compounds are inducers of cyp2E1 and shortly after isoniazid and alcohol are eliminated there is an increased risk of APAP toxicity (Sato *et al.* 1981; Thummel *et al.* 2000; Zand *et al.* 1993). This effect is considered a reason for the increased hepatotoxicity in alcoholics after an APAP overdose. However, since NAPQI formation after acute alcohol ingestion is increased by only <40% in healthy volunteers (Thummel *et al.* 2000) and was found to be not significantly increased in alcoholics (Lauterburg and Velez 1988), other risk factors need to be considered. Ethanol can enhance the translocation of endotoxin from the gastrointestinal tract leading to Kupffer cell activation and enhanced inflammatory cytokine formation (Enomoto *et al.* 2001), which may promote APAP toxicity. Poor nutrition in alcoholics can lead to reduced hepatic GSH (glutathione) levels, which limits the capacity to detoxify NAPQI (Lauterburg and Velez 1988). In addition, chronic alcohol consumption in rats reduced mitochondrial GSH levels and enhanced susceptibility to cell death (Fernández-Checa *et al.* 1993; Hirano *et al.* 1992; Zhao *et al.* 2002). As discussed later, mitochondrial GSH is critical for detoxification of peroxynitrite formed in mitochondria after APAP overdose (Knight *et al.* 2002). Thus, the increased risk of liver injury after APAP overdose in alcoholics is due to multiple factors related to the formation and detoxification of NAPQI and oxidant stress.

9.21.2.3 Consequences of NAPQI Formation

NAPQI is both a strong electrophile and an oxidant. As such, it has the capacity to react with numerous targets within the cell. Because of the abundance of GSH within hepatocytes (5–10 mmol l^{-1}), NAPQI reacts extensively with GSH (**Figure 1**). This reaction is catalyzed by glutathione *S*-transferases, especially GSTPi, but can also occur spontaneously (Coles *et al.* 1988). After a therapeutic dose, GSH levels are moderately reduced by the limited NAPQI formation. If an appropriate interval is observed before the next dose, there is no risk of cell injury and the drug can be used chronically. In support of this conclusion, none of the osteoarthritis patients treated daily with maximal therapeutic doses of APAP for 1 year showed any evidence of liver dysfunction or injury (Temple *et al.* 2006). However, if an overdose is consumed, GSH is depleted and the excess NAPQI can react with alternative targets within the cell. The most relevant reaction is covalent binding to cellular proteins (Hart *et al.* 1995; Jollow *et al.* 1973; Roberts *et al.* 1991). Adducts are formed predominantly with cysteine residues on proteins (Streeter *et al.* 1984). Other adducts appear to be of limited relevance for the toxicity (Matthews *et al.* 1996). Protein binding of NAPQI occurs mainly in centrilobular hepatocytes, which eventually undergo necrosis (Roberts *et al.* 1991). The localization of APAP protein adducts and the fact that no toxicity is observed without adduct formation led to the conclusion that protein binding is a critical event in APAP-induced cell death (Jollow *et al.* 1973; Mitchell *et al.* 1981; Roberts *et al.* 1991). However, the protein-binding hypothesis has been challenged. The main concern was a lack of correlation between the degree of injury and the overall protein binding of NAPQI (reviewed by Smith *et al.* 1985). Some of these issues have been resolved (Corcoran *et al.* 1985). However, the fact remains that the overall protein binding is relatively low. It was therefore hypothesized that it is not the degree of overall protein binding but the binding to essential target proteins within the cell that is most critical. This hypothesis led to the search for and ultimately identification of a substantial number of covalent protein adducts of APAP (Cohen *et al.* 1997; Pumford and Halmes 1997; Qiu *et al.* 1998). However, no clear target was identified that could explain the rapid cell death after APAP overdose. In fact, the enzyme activity of most of these proteins changed only moderately (Pumford and Halmes 1997; Pumford *et al.* 1997). In addition, the nonhepatotoxic regioisomer of APAP, 3′-hydroxyacetanilide, caused a similar degree of overall protein binding as APAP itself (Rashed *et al.* 1990; Tirmenstein and Nelson 1989). However, one important difference emerged. Metabolic activation of APAP affects more mitochondrial proteins than 3′-hydroxyacetanilide (Qiu *et al.*, 2001; Tirmenstein and Nelson 1989). This finding led to the concept that protein binding of NAPQI is a critical initiating stress event, which requires amplification and propagation within the cells. As discussed later, mitochondrial dysfunction is central to this process (**Figure 2**).

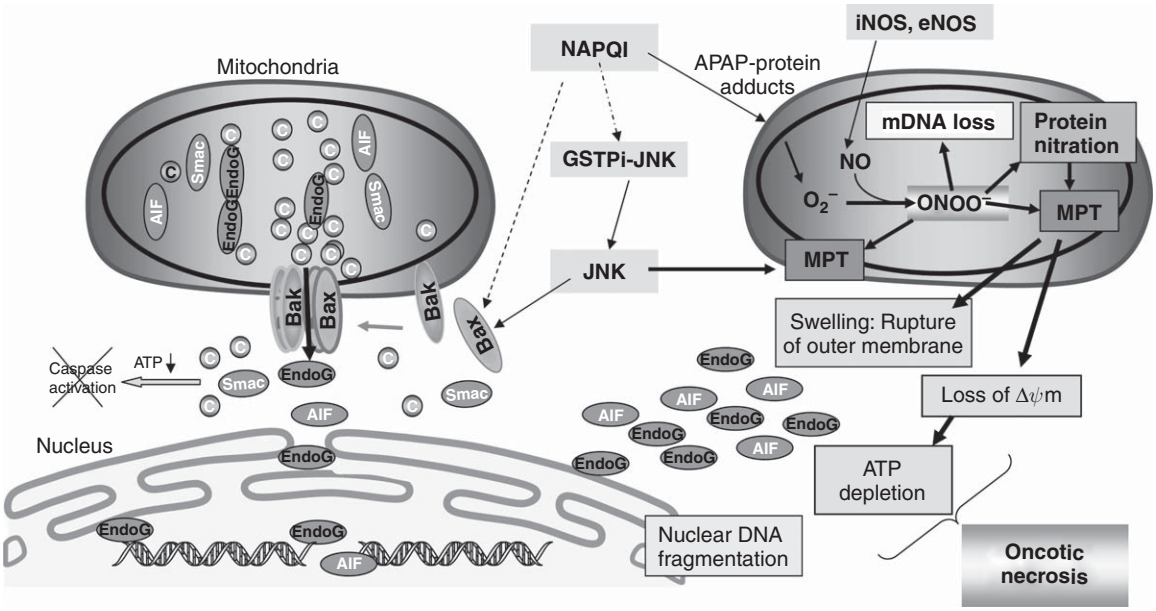

Figure 2 Propagation of cell injury after formation of the reactive metabolite NAPQI with the central role of mitochondrial dysfunction and nuclear DNA fragmentation (see text for details). AIF, apoptosis-inducing factor; C, cytochrome *c*; EndoG, endonuclease G; eNOS, endothelial nitric oxide synthase; GSTPi, glutathione *S*-transferase Pi; iNOS, inducible nitric oxide synthase; mDNA, mitochondrial DNA; MPT, mitochondrial permeability transition; NAPQI, *N*-acetyl-*p*-benzoquinone imine; NO, nitric oxide; O_2^-, superoxide; ONOO$^-$, peroxynitrite; Smac, second mitochondria-derived activator of caspases; $\Delta\Psi$m, mitochondrial membrane potential.

Additional consequences of NAPQI formation include formation of xanthine oxidase (Jaeschke 1990; Tirmenstein and Nelson 1990), which could be a source of reactive oxygen formation. Although the xanthine oxidase inhibitor allopurinol inhibits APAP-induced liver injury (Jaeschke 1990; Tirmenstein and Nelson 1990), a detailed dose–response evaluation indicated that much higher doses of allopurinol are necessary for the effect on APAP hepatotoxicity than to inhibit xanthine oxidase activity (Jaeschke 1990). Allopurinol does not affect NAPQI formation but effectively prevents APAP-induced mitochondrial dysfunction and oxidant stress (Jaeschke 1990; Knight *et al.* 2001). Thus, despite the conversion of xanthine dehydrogenase to oxidase after APAP overdose, this enzyme is unlikely to contribute to APAP-induced cell death.

Another consequence of NAPQI formation is the inhibition of the Ca^{2+}-ATPase activity in the plasma membrane (Tsokos-Kuhn *et al.* 1988), which is thought to be responsible for the increase in cytosolic Ca^{2+} concentrations (Corcoran *et al.* 1988; Moore *et al.* 1985; Tsokos-Kuhn 1989). Disturbances of the intracellular Ca^{2+} homeostasis can induce cell death by activation of proteases, phospholipases, and endonucleases and by triggering mitochondrial dysfunction. However, time-course experiments with direct measurement of cytosolic Ca^{2+} levels in cultured hepatocytes indicated that the rise in Ca^{2+} concentrations occurred after the initiation of cell injury but shortly before the loss of cell viability (Harman *et al.* 1992). This led to the conclusion that Ca^{2+} does not play a central role in the initiation of cell death (Harman and Maxwell 1995; Harman *et al.* 1992). On the other hand, Ca^{2+} chelators and Ca^{2+} channel blockers prevented APAP-induced nuclear Ca^{2+} accumulation and DNA fragmentation and attenuated cell injury *in vitro* and *in vivo* (Ray *et al.* 1990, 1993; Salas and Corcoran 1997; Shen *et al.* 1991). These data suggest that disturbances of the intracellular Ca^{2+} homeostasis occur during the propagation of cell injury.

9.21.3 Mitochondrial Dysfunction and Oxidant Stress

9.21.3.1 Cause of Mitochondrial Dysfunction

Morphological changes in mitochondria can be detected during the first hours after treatment with APAP, that is, well before any cell death occurs

(Placke *et al.* 1987; Ruepp *et al.* 2002; Walker *et al.* 1980). Hepatotoxic doses of APAP also caused functional changes in mitochondria including a decrease in state 3 (ADP-stimulated) respiration and an increase in state 4 (resting) respiration, which led to a decrease in the respiratory control ratio (Donnelly *et al.* 1994; Meyers *et al.* 1988). Respiration stimulated by succinate (site 2) appears to be more sensitive than that supported by NADH-linked substrates (site 1) to inhibition by APAP in cultured hepatocytes (Burcham and Harman 1991). Direct exposure of mitochondria to NAPQI can reproduce these respiratory changes (Meyers *et al.* 1988). In addition, mitochondrial dysfunction *in vivo* is dependent on the metabolism of APAP and can be prevented by treatment with NAC, which facilitates scavenging of NAPQI by GSH (Donnelly *et al.* 1994; Meyers *et al.* 1988). These observations suggest that covalent binding of NAPQI to mitochondrial proteins may be responsible for inhibition of mitochondrial respiration. In direct support of this hypothesis, APAP protein adducts in mitochondria were identified (Jollow *et al.* 1973; Qiu *et al.* 2001; Tirmenstein and Nelson 1989). The critical role of mitochondrial protein adducts in the toxicity of APAP was emphasized by the fact that the regioisomer 3′-hydroxyacetanilide, which also forms a reactive metabolite that binds to proteins, did not cause mitochondrial protein binding or liver injury (Qiu *et al.* 2001; Tirmenstein and Nelson 1989). On the other hand, the reactive metabolites of both APAP and 3′-hydroxyacetanilide bind to proteins in isolated mitochondria (Ramsay *et al.* 1989). The reason for the differential toxicity of APAP and 3′-hydroxyacetanilide *in vivo* may be that only NAPQI, the reactive metabolite of APAP, has the reactivity and long-enough half-life to reach mitochondria (Ramsay *et al.* 1989). Together these findings indicate that the binding of NAPQI to mitochondrial proteins is overall more damaging than forming adducts with proteins of other cell organelles.

9.21.3.2 Mitochondrial Oxidant Stress and Peroxynitrite Formation

A consequence of the mitochondrial dysfunction induced by covalent binding is the enhanced formation of reactive oxygen species (ROS) as indicated by elevated levels of glutathione disulfide (GSSG) in mitochondria after APAP exposure. This suggests that mitochondria are the main source of intracellular oxidant stress (Jaeschke 1990; Knight *et al.* 2001; Tirmenstein and Nelson 1990) (**Figure 2**). The fact that GSSG levels did not increase during the early metabolism phase after APAP treatment indicated that reactive oxygen was not released by cyp especially cyp2E1, during NAPQI formation (Lauterburg *et al.* 1984; Smith and Jaeschke 1989). However, the late formation of GSSG raised the concern that the oxidant stress was a consequence of cell death. Using a fluorescent probe to detect ROS, it was shown that ROS formation occurs after GSH depletion, correlates with the onset of mitochondrial dysfunction, and precedes cell death by several hours (Bajt *et al.* 2004). The mechanism of cell death involving ROS formation initially focused mainly on Fenton chemistry and lipid peroxidation (Wendel and Feuerstein 1981). However, lipid peroxidation severe enough to be directly responsible for cell killing is observed only in vitamin E-deficient mice and not in animals on a regular diet (Jaeschke *et al.* 2003; Knight *et al.* 2003). More recent evidence also suggests that *in vivo* there is formation of peroxynitrite from superoxide and nitric oxide (NO) after APAP overdose (Hinson *et al.* 1998; Knight *et al.* 2001). Peroxynitrite is generated after GSH depletion mainly inside mitochondria (Cover *et al.* 2005b). The reason for the intramitochondrial location is related to the intramitochondrial generation of superoxide anions, which are unable to cross the mitochondrial membrane (Cover *et al.* 2005b). On the other hand, NO is freely membrane permeable and may travel longer distances. It has been suggested that the NO for peroxynitrite formation after APAP overdose was generated by the inducible NO synthase (iNOS) (Gardner *et al.* 1998, 2002; Michael *et al.* 2001; Salhanick *et al.* 2006) or the endothelial NOS (eNOS) (Salhanick *et al.* 2006). It was reported that knockout mice of both iNOS and eNOS are less susceptible to APAP-induced liver injury compared to the respective wild-type animals (Gardner *et al.* 2002; Michael *et al.* 2001; Salhanick *et al.* 2006); in addition, iNOS inhibitors were shown to be protective (Gardner *et al.* 1998; Kamanaka *et al.* 2003). However, these data are not without controversy (Hinson *et al.* 2002; Michael *et al.* 2001). Therefore, a different approach was used to test if peroxynitrite is a critical mediator. By intravenous administration of GSH, which provides amino acids for the resynthesis of GSH in the liver, the recovery of mitochondrial GSH levels was substantially accelerated (Knight *et al.* 2002). The presence of GSH in the mitochondria led to the scavenging of peroxynitrite and protected against liver injury, improved survival, and facilitated

regeneration (Bajt *et al.* 2003; Knight *et al.* 2002). Similar results were also obtained with NAC (James *et al.* 2003c). Since GSH treatment was also effective in mice deficient in glutathione peroxidase-1, these data suggest that the spontaneous scavenging of peroxynitrite, not the enzymatic detoxification of hydrogen peroxide, is the main reason for the protection. Thus, peroxynitrite is a critical mediator of APAP-induced liver injury (Knight *et al.* 2002).

9.21.3.3 Consequences of Mitochondrial Oxidant Stress

Although there is now solid experimental support for the presence and pathophysiological relevance of ROS and especially peroxynitrite, potential consequences of this oxidant stress are less clear (**Figure 2**). Lipid peroxidation (LPO), although still sometimes reported, does not play a significant role. Quantitatively, there is not enough LPO to account for the extensive cell damage and various forms of vitamin E, a chain-breaking antioxidant, are ineffective in normal animals (Knight *et al.* 2003). Massive loss of protein sulfhydryl groups has also been postulated as a cause of cell injury (Moore *et al.* 1985). However, there is no relevant change in the sulfhydryl content of cellular or mitochondrial proteins after APAP overdose (Andringa *et al.* 2008; Tirmenstein and Nelson 1990). A proteomics approach identified one protein, 3-hydroxy-3-methylglutaryl coenzyme A synthase 2, which selectively lost some of its sulfhydryl groups (Andringa *et al.* 2008). This observation correlated with a moderate loss of protein function (Andringa *et al.* 2008). However, the pathophysiological relevance of the relatively minor change in this enzyme activity remains unclear.

Since oxidant stress can induce opening of the mitochondrial permeability transition (MPT) pores with consequent collapse of the membrane potential and depletion of ATP, this mechanism was investigated by confocal microscopy in primary cultured hepatocytes (Kon *et al.* 2004). The data indicate that the MPT occurs about 4–5 h after APAP exposure (Kon *et al.* 2004), that is, after GSH depletion and after the onset of the mitochondrial oxidant stress (Bajt *et al.* 2004) but before the loss of plasma membrane integrity (cell necrosis) (Kon *et al.* 2004). The APAP-induced MPT could be inhibited by cyclosporin A, which binds to cyclophilin D, one of the MPT pore-forming proteins. However, the effect of cyclosporin A was transient, that is, there was a delay in cell death but not a complete prevention *in vitro* (Kon *et al.* 2004). This suggests that initially the MPT is regulated but appears to become unregulated once the insult is overwhelming (He and Lemasters 2002; Kon *et al.* 2004). The relevance of the MPT in APAP-induced cell death was confirmed *in vivo* (Haouzi *et al.* 2002; Masubuchi *et al.* 2005). However, inhibition of the MPT *in vivo* appears to have a permanent rather than a transient effect. Nevertheless, the evidence is quite strong that the MPT, most likely triggered by mitochondrial oxidant stress and peroxynitrite formation, is a critical event in APAP-induced liver cell death (**Figure 2**).

9.21.3.4 Activation of c-Jun N-Terminal Kinase

Activation of c-Jun N-terminal kinase (JNK) was suggested to be another consequence of the mitochondrial oxidant stress (Hanawa *et al.* 2008). Because it requires the diffusion of the intramitochondrial ROS into the cytosol, the most relevant oxidant would be hydrogen peroxide. However, there is no evidence of GSSG formation in the cytosol or GSSG excretion into the bile at either early or later time points after APAP (Jaeschke 1990; Knight *et al.* 2001; Smith and Jaeschke 1989). Although intracellular oxidant stress can activate JNK *in vivo* (Hong *et al.* 2009), the lack of GSSG formation in the cytosol raises a question as to whether mitochondrial hydrogen peroxide was indeed responsible for JNK activation after APAP overdose. Independent of the activation mechanism, different approaches to prevent JNK activation resulted in a protective effect against APAP-induced liver injury (Gunawan *et al.* 2006; Henderson *et al.* 2007; Latchoumycandane *et al.* 2007). The proposed JNK involvement in the injury mechanisms included direct promotion of the MPT (Hanawa *et al.* 2008), induction of iNOS (Latchoumycandane *et al.* 2007), and promotion of mitochondrial bax translocation (Gunawan *et al.* 2006) (**Figure 2**). Regarding bax translocation, this effect is critical for the early phase of injury (Bajt *et al.* 2008a). However, elimination of bax does not lead to a permanent protection because the effects of bax (release of intermembrane proteins) can also be triggered by the MPT and swelling of the mitochondria (Bajt *et al.* 2008a). A concern with most JNK experiments is the administration of very high doses of APAP in these experiments, which is necessary because of the use of solvents such as dimethyl

sulfoxide (DMSO) (Gunawan *et al.* 2006; Hanawa *et al.* 2008). DMSO is a potent inhibitor of the metabolism of APAP and several misinterpretations of injury mechanisms have resulted by its effect (Jaeschke *et al.* 2006). The relevance of other JNK-related mechanisms, for example, MPT pore opening and iNOS induction, needs to be confirmed.

It has been shown that the monomer of glutathione *S*-transferase Pi (GSTPi) can inhibit JNK signaling by direct protein–protein interactions (Adler *et al.* 1999). During cellular stress, for example, formation of electrophiles, GSTPi dimerizes to form the active enzyme thereby releasing JNK from the JNK–GSTPi complex (Adler *et al.* 1999). Recent findings suggest that NAPQI can bind to GSTPi and inactivate the enzyme (Jenkins *et al.* 2008). The resulting JNK activation leads to activation of the transcription factor activator protein-1 (AP-1), which results in induction of several genes including hemeoxygenase-1 (HO-1) and UGTs. These genes can protect against APAP-induced liver injury. Deficiency of GSTPi leads to a high basal activation of JNK with enhanced expression of HO-1 and UGT activities (Elsby *et al.* 2003). This mechanism is thought to be the reason for the reduced APAP-mediated liver injury in GSTPi gene knockout mice compared to wild-type animals (Henderson *et al.* 2000). In addition, JNK activation may be important for regeneration (Bourdi *et al.* 2008). Thus, JNK activation can either induce a beneficial stress response and protect against APAP hepatotoxicity or promote cell death and aggravate liver damage. It will be important to understand under what circumstances JNK activation is beneficial or detrimental before JNK can be proposed as a potential therapeutic target.

9.21.4 DNA Fragmentation

9.21.4.1 Nuclear DNA Fragmentation

Almost 20 years ago, Corcoran and coworkers first recognized that APAP toxicity correlated with nuclear DNA fragmentation *in vivo* and *in vitro* (Ray *et al.* 1990; Shen *et al.* 1991). The presence of DNA ladder patterns suggested that it was not a random damage by reactive intermediates but mediated by endonucleases (Ray *et al.* 1990). DNA damage was further confirmed by the detection of cytosolic DNA fragments, which were identified by their histone core (Lawson *et al.* 1999). In addition, the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay demonstrated the presence of DNA strand breaks in the nucleus (Lawson *et al.* 1999). However, in contrast to the characteristic apoptotic DNA fragmentation, the TUNEL assay also stained the cytosol indicating the generation of larger DNA fragments (Gujral *et al.* 2002; Jaeschke and Lemasters 2003; Lawson *et al.* 1999). This hypothesis was directly confirmed by analysis of DNA fragments in plasma; the DNA fragments after APAP-induced necrotic cell death were substantially larger than fragments during apoptosis (Jahr *et al.* 2001; Tran *et al.* 2008).

Initially it remained unclear which endonucleases were responsible for nuclear DNA fragmentation during APAP hepatotoxicity. The fact that a Ca^{2+} channel blocker prevented nuclear Ca^{2+} accumulation and DNA damage suggested that a Ca^{2+}-dependent endonuclease might be involved (Ray *et al.* 1993; Shen *et al.* 1992). The absence of caspase activation after APAP overdose and the ineffectiveness of caspase inhibitors to reduce DNA damage ruled out that the caspase-activated DNase could be responsible (Jaeschke *et al.* 2006; Lawson *et al.* 1999). However, it was recognized that scavenging of peroxynitrite in mitochondria, which effectively protected against cell death, also eliminated DNA damage (Cover *et al.* 2005b). These data indicated that nuclear DNA damage was related to mitochondrial dysfunction. Since mitochondria contain endonuclease G (EndoG) and apoptosis-inducing factor (AIF) in the intermembrane space, the potential importance of these mitochondrial proteins for nuclear DNA damage was investigated in APAP hepatotoxicity. In cultured mouse hepatocytes, nuclear translocation of EndoG and AIF was observed as soon as GSH was depleted and mitochondrial dysfunction was detectable (Bajt *et al.* 2006). Since preventing mitochondrial dysfunction with NAC prevented nuclear accumulation of EndoG and AIF together with DNA damage, the results indicate that these mitochondrial enzymes are mainly responsible for nuclear DNA damage (Bajt *et al.* 2006) (**Figure 2**). The release of these proteins was triggered by translocation of bax to the mitochondria and formation of pores in the outer membrane during the early time points (Bajt *et al.* 2008a). However, at later times, nuclear DNA fragmentation became independent of bax most likely due to the MPT, mitochondrial swelling, and subsequent rupture of the outer membrane, which released AIF and EndoG independent of the bax pores (Bajt *et al.* 2008a). In addition to nuclear translocation

of mitochondrial AIF and EndoG, nuclear translocation of lysosomal deoxyribonuclease 1 (Dnase1) has been shown (Jacob *et al.* 2007; Napirei *et al.* 2006). DNA fragmentation was substantially reduced in Dnase1-deficient mice. It was hypothesized that Dnase1 is released by necrotic cells and contributes to the expansion of liver injury (Napirei *et al.* 2006). However, an intracellular release of this endonuclease has not been explored.

Despite the extensive characterization of nuclear DNA damage and the identification of some of the endonucleases involved, it has not been conclusively demonstrated that this effect is critical for cell death. It might be just an epiphenomenon or a consequence of cell death. Some of the experimental data that support the hypothesis that these endonucleases contribute to cell death include the protective effect of a general endonuclease inhibitor *in vitro* (Shen *et al.* 1992) and the fact that all cells with DNA damage developed necrosis (Lawson *et al.* 1999). In addition, reduced liver damage in bax-deficient animals and in Dnase1-deficient mice correlates with the reduced DNA damage (Bajt *et al.* 2008a; Napirei *et al.* 2006).

9.21.4.2 Activation of Poly-(ADP-Ribose) Polymerase-1

One of the earliest events in the repair process after DNA damage is the activation of the nuclear enzyme poly-(ADP-ribose) polymerase-1 (PARP-1), which catalyzes poly-ADP-ribosylation of nuclear proteins (Szabó and Dawson 1998). However, if DNA damage is severe, excessive activation of PARP-1 can cause depletion of NAD^+ and ATP and trigger necrotic cell death (Chiarugi 2002; Ha and Snyder 1999). Early studies with cultured hepatocytes did not show protection with PARP-1 inhibitors but rather a late increase of cell injury after APAP exposure (Shen *et al.* 1992). On the other hand, high doses of the PARP-1 inhibitor 3-aminobenzamide and others protected against APAP hepatotoxicity *in vivo*, suggesting that excessive PARP-1 activation may contribute to cell injury after APAP (Ray *et al.* 2001). However, these findings could not be confirmed in PARP-1 gene knockout mice (Cover *et al.* 2005a). PARP-deficient mice had slightly higher injury than wild-type animals. Furthermore, detailed assessment of PARP activation indicated that DNA damage and necrosis occur in most hepatocytes before PARP activation (Cover *et al.* 2005a). These data suggest that PARP activation does not contribute to APAP-induced cell death. Interestingly, the PARP inhibitor 3-aminobenzamide protected against APAP hepatotoxicity in both wild-type and PARP-1 knockout mice (Cover *et al.* 2005a). These results indicate that high doses of these inhibitors may have additional effects independent of the inhibition of PARP-1.

9.21.4.3 Mitochondrial DNA Damage

Mitochondrial DNA damage has also been shown in APAP hepatotoxicity (Cover *et al.* 2005b). The nature of the damage remains unclear. Nitrotyrosine protein adducts can be found only in mitochondria, and scavenging of peroxynitrite with GSH is highly effective in preventing the loss of mitochondrial DNA (Cover *et al.* 2005b). Based on these observations, it is likely that mitochondrial DNA damage is caused by a direct effect of oxidants rather than endonucleases. Independent of the mechanism, extensive mitochondrial DNA damage would limit the chance of successful repair and enhance the likelihood of cell death.

9.21.5 Apoptosis Versus Oncotic Necrosis

9.21.5.1 Morphological Characteristics of Cell Death

Given the elucidation of new signaling mechanisms in APAP-induced cell death and their significant overlap with apoptosis, it is not surprising that the hypothesis surfaced that the mode of cell death may actually be apoptotic. Two ideas were proposed. First, during APAP hepatotoxicity cells die to the same degree by either apoptosis or oncotic necrosis (Ray *et al.* 1996). Second, all cell death is apoptotic, which deteriorates rapidly to secondary necrosis (El-Hassan *et al.* 2003). Both hypotheses have been disputed (Gujral *et al.* 2002; Jaeschke *et al.* 2004; Lawson *et al.* 1999). To understand the issues, it is important to recall that apoptotic cell death is defined morphologically. It involves cell shrinkage, chromatin condensation, and formation of apoptotic bodies, which contain intact cell organelles and have an intact cell membrane (Kerr *et al.* 1972). On the other hand, most biochemical characteristics, with the exception of caspase activation, are not specific for apoptosis and cannot be used to define the mode of cell death (Jaeschke and Lemasters 2003; Jaeschke *et al.* 2004). A detailed morphological study of the time course of APAP toxicity in mouse liver

indicated cell swelling, eosinophilia, and eventual cell necrosis in correlation with the early release of cell contents (alanine aminotransferase (ALT)) at high and low overdoses in starved and fed animals (Gujral *et al.* 2002). In addition, electron microscopic studies showed early mitochondrial swelling before ALT release (Ruepp *et al.* 2002). These data, which are in line with numerous previous studies *in vivo*, indicate that APAP does not cause morphological changes consistent with apoptotic cell death. The few additional apoptotic cells (<<1% of hepatocytes) observed after APAP overdose are irrelevant for the overall injury compared to the 40–60% necrotic cells (Gujral *et al.* 2002). In addition, most *in vitro* findings using primary, cultured hepatocytes also suggest that the principal mode of cell death is oncotic necrosis (Bajt *et al.* 2004; Kon *et al.* 2004; Nagai *et al.* 2002; Shen *et al.* 1991, 1992). The few studies *in vitro* that show clear evidence of apoptosis after APAP exposure use immortalized cell lines, which have no P450 enzymes expressed (Boulares *et al.* 2002). These cell lines are not able to generate NAPQI, the essential reactive metabolite that initiates toxicity. Thus, mechanistic studies in these cell lines are not relevant for events in primary hepatocytes in animals or in humans.

9.21.5.2 Caspase Activation and DNA Fragmentation

In addition to morphological changes, activation of the caspase cascade is generally considered a strong support for apoptotic cell death. During APAP-induced liver injury *in vivo*, there is consensus in the literature that caspases are not activated (Adams *et al.* 2001; El-Hassan *et al.* 2003; Gujral *et al.* 2002; Jaeschke *et al.* 2006; Lawson *et al.* 1999). Activation of the caspase cascade through the mitochondrial pathway requires ATP for the formation of apoptosome (Li *et al.* 1997). Thus, the reason for the absence of caspase activation despite mitochondrial cytochrome *c* and second mitochondria-derived activator of caspase (Smac) release is most likely declining ATP levels rather than binding of NAPQI to critical sulfhydryl groups of procaspases (Knight and Jaeschke 2002; Lawson *et al.* 1999) (**Figure 2**). This conclusion is supported by the fact that Fas receptor-mediated apoptosis is not inhibited during APAP metabolism. However, at later time points when APAP caused mitochondrial dysfunction, Fas receptor-induced apoptosis is blocked (Knight and Jaeschke 2002; Lawson *et al.* 1999). The reason for this effect is that activation of the caspase cascade in hepatocytes requires intact mitochondria for the amplification of the initial apoptotic signal (Bajt *et al.* 2000; Yin *et al.* 1999). Consistent with the absence of caspase activation is the observation that pancaspase inhibitors, for example, Z-VAD-fmk (Z-Val-Ala-Asp-fluoromethylketone), do not protect against APAP-induced liver injury (Lawson *et al.* 1999). A recent study supported the lack of caspase activation but showed protection with ZVAD-fmk when administered before APAP (El-Hassan *et al.* 2003). First, a suicide substrate cannot inactivate a proform (zymogen) of an enzyme; it requires an active enzyme that is able to bind a substrate. Second, the protective effect of ZVAD-fmk pretreatment is caused by the solvent DMSO, which inhibits the metabolic activation of APAP (Jaeschke *et al.* 2006). Thus, the vast majority of experimental evidence suggests that APAP-induced cell death in primary hepatocytes or in the intact liver *in vivo* does not involve activation of the caspase cascade and apoptosis.

9.21.6 Propagation Mechanisms of Cell Injury

Although APAP can trigger intracellular events in individual hepatocytes leading to oncotic necrosis, the concept emerged that necrotic cells facilitate cell death in neighboring hepatocytes. Mehendale and coworkers provided the first evidence that activated calpain is released from necrotic cells and contributes to the expansion of the area of injury even after the hepatotoxin is already eliminated (Limaye *et al.* 2003, 2006). Similarly, Dnase1 released from necrotic cells can enter stressed cells and contribute to cell death (Napirei *et al.* 2004). The importance of this effect for APAP hepatotoxicity was demonstrated by the reduced DNA fragmentation and liver injury in Dnase1 knockout mice (Jacob *et al.* 2007; Napirei *et al.* 2006). However, a potential intracellular effect of calpains or Dnase1 in the cell of origin was not excluded in either of these studies. Other factors released by necrotic cells that could enhance cell injury include danger signals such as high-mobility group box 1 protein (HMGB1). HMGB1 can bind to various receptors including the toll-like receptors 2 and 4 (Szabo *et al.* 2006). Stimulation of these receptors on macrophages and neutrophils can trigger an inflammatory response, which not only activates leukocytes but also stimulates iNOS induction leading to enhanced peroxynitrite formation, mitochondrial dysfunction, and cell death (Bourdi *et al.* 2002). Although these propagation loops

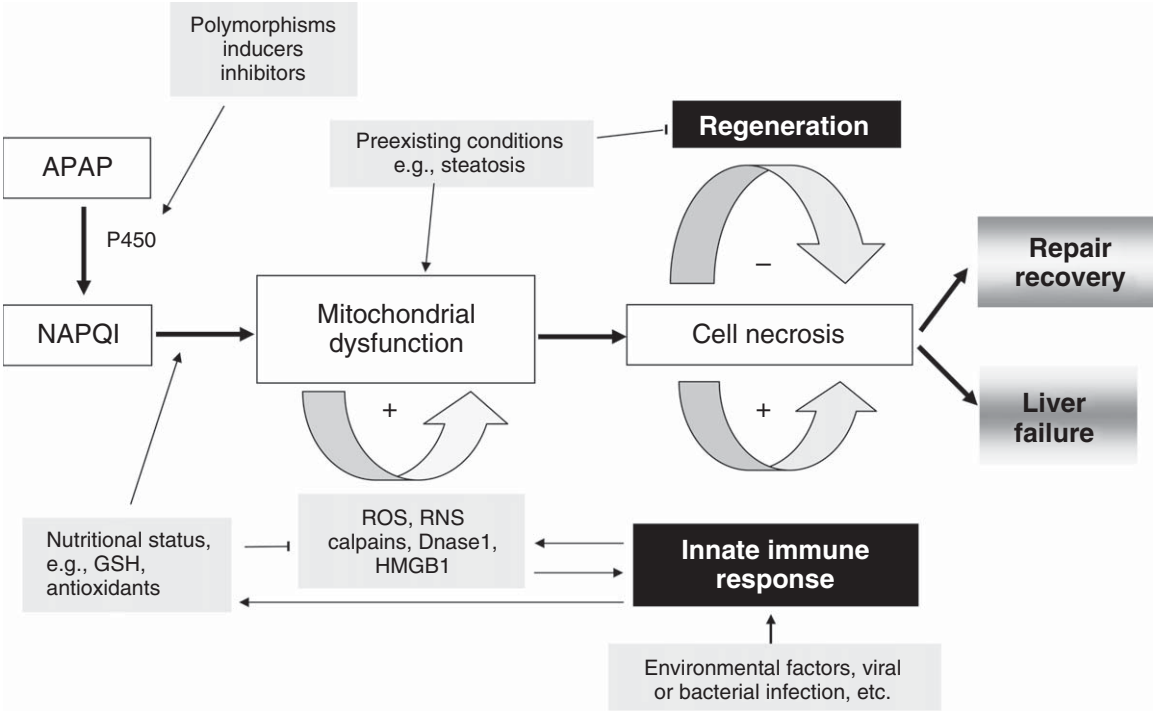

Figure 3 Factors that determine an individual's susceptibility to acetaminophen (APAP) overdose. Genetic background, diet, preexisting conditions, and many environmental factors can modulate the basic mechanisms of APAP-induced liver injury and ultimately determine whether an animal or person can repair the damage and survive or suffer acute liver failure. GSH, glutathione; HMGB1, high-mobility group box 1 protein; NAPQI, *N*-acetyl-*p*-benzoquinone imine; RNS, reactive nitrogen species; ROS, reactive oxygen species.

require further studies, the present information indicates that necrotic cells can release mediators that, directly or indirectly, promote cell injury in additional hepatocytes (**Figure 3**).

9.21.7 Innate Immune Response

Although the main focus of mechanistic investigations is intracellular signaling pathways leading to oncotic necrosis (Jaeschke and Bajt 2006), the pathophysiological role of innate immune cells including resident Kupffer cells and lymphocytes as well as infiltrating macrophages and neutrophils received more attention recently (Jaeschke 2005, 2006a, 2008; Laskin and Laskin 2001; Liu and Kaplowitz 2006) (**Figure 3**).

9.21.7.1 Role of Kupffer Cells and Tissue Macrophages in APAP Hepatotoxicity

Activation of Kupffer cells and recruitment of macrophages after APAP overdose were observed in rats (Laskin and Pilaro 1986; Laskin *et al.* 1995) and mice (Dambach *et al.* 2002; Holt *et al.* 2008; Michael *et al.* 1999). In addition, it was reported that functional inactivation of Kupffer cells with gadolinium chloride reduced liver injury and peroxynitrite formation (Laskin *et al.* 1995; Michael *et al.* 1999). However, other investigators observed only a modest protection with gadolinium chloride in mice (Ito *et al.* 2003; Ju *et al.* 2002; Knight and Jaeschke 2004) and found no evidence that Kupffer cells are a relevant source of reactive oxygen or peroxynitrite after APAP overdose (James *et al.* 2003b). Moreover, true elimination of Kupffer cells with liposomal clodronate or deficiency of the tumor necrosis factor (TNF) receptor type I actually enhanced APAP-induced injury most likely due to the elimination of anti-inflammatory mediators such as IL-10, which limits the TNF-induced upregulation of iNOS (Gardner *et al.* 2002, 2003; Ju *et al.* 2002). In addition, deficiency of the TNF receptor I reduced transcriptional activation of antioxidant genes, which protect against APAP-induced injury (Chiu *et al.* 2003a). Furthermore, Kupffer cell depletion and TNF receptor I deficiency delayed liver regeneration after APAP

overdose (Chiu *et al.* 2003b; Ju *et al.* 2002). These data suggest that Kupffer cell activation during the early phase of APAP-induced liver injury is actually beneficial by preventing an excessive inflammatory response, by promoting antioxidant defense mechanisms, and by promoting regeneration.

9.21.7.2 Role of NK and NKT Cells in APAP Hepatotoxicity

Depletion experiments identified natural killer (NK) cells and natural killer T (NKT) cells, a resident lymphocyte population, as important mediators of APAP hepatotoxicity in mice (Liu *et al.* 2004). The involvement of NK and NKT cells was attributed to the formation of interferon-γ, which enhanced chemokine formation, the recruitment of neutrophils into the liver, and the expression of Fas ligand on liver innate immune cells (Liu *et al.* 2004). The role of the Fas/Fas ligand system in APAP hepatotoxicity remains unclear. However, it is unlikely to induce cell death directly because there is no experimental evidence for apoptotic cell death after APAP overdose (Gujral *et al.* 2002). In addition, APAP even inhibits Fas receptor-mediated apoptosis by interfering with the mitochondrial signaling pathway (Knight and Jaeschke 2002). The relevance of NK and NKT cells for the pathophysiology of APAP-induced liver injury has been questioned recently (Masson *et al.* 2008). Pohl and coworkers (Masson *et al.* 2008) realized that the solvent DMSO, which was used by the previous authors to dissolve APAP, activated NK and NKT cells. Thus, depletion of NK cells protects only when APAP is given in DMSO but not when injected in saline (Masson *et al.* 2008). These new findings suggest that NK and NKT cells do not contribute to APAP-induced liver injury unless these cells are activated through independent stimuli before APAP administration. Since bacterial or viral infections may represent stimuli to activate NK and NKT cells, these findings may have some implication for the susceptibility of individuals to APAP toxicity.

9.21.7.3 Role of Neutrophils in APAP Hepatotoxicity

Neutrophils are activated and can contribute to liver injury in a variety of pathophysiological conditions (Jaeschke 2006b). Neutrophils accumulate in hepatic sinusoids in response to APAP-induced liver injury, but there is no systemic activation of neutrophils (Lawson *et al.* 2000). Consistent with these findings, recent data suggest that HMGB1, which is released from necrotic cells and can bind among others to toll-like receptors 2 and 4 on macrophages and neutrophils, participates in the recruitment of neutrophils into the liver after APAP overdose (Scaffidi *et al.* 2002). However, most neutrophils remain in sinusoids distant from the centrilobular areas of necrosis during the most active phase of injury (Cover *et al.* 2006). In addition, antibodies against HMGB1 or β_2 integrins, neutropenia antibodies, deficiency in intercellular adhesion molecule-1 (ICAM-1), or NADPH oxidase as well as various NADPH oxidase inhibitors did not protect against APAP-induced liver injury (Cover *et al.* 2006; James *et al.* 2003b; Lawson *et al.* 2000; Scaffidi *et al.* 2002). These data together strongly suggest no active participation of neutrophils in the injury process. However, in two recent investigations, the authors induced neutropenia 24 h prior to APAP administration and found protection against APAP toxicity (Ishida *et al.* 2006; Liu *et al.* 2006). The problem with this approach is that the antibody-tagged and functionally inactivated neutrophils, which disappear from the circulation, accumulate in the liver and are eliminated by Kupffer cells. This massive phagocytosis of cells or cell remnants triggers activation of Kupffer cells (Bautista *et al.* 1994) and release of mediators, which cause induction of acute-phase proteins, especially metallothionein (Jaeschke and Liu 2007). This preconditioning of the liver appears to be the main cause of protection by the neutropenia-inducing antibody rather than the absence of neutrophils (Jaeschke and Liu 2007). In support of this conclusion, the neutropenia-inducing antibody is not effective if administered after APAP injection but before liver injury (Cover *et al.* 2006). Taken together, the current available information suggests that cells of the innate immune system are activated in response to the massive injury after APAP overdose but do not seem to actively contribute to the injury. However, the mediators produced by innate immune cells can modulate the intracellular signaling mechanisms by inducing iNOS and antioxidant genes.

9.21.7.4 Hepatic Microcirculation

Endothelial cell injury and hemorrhage in the centrilobular area are well-established phenomena after APAP overdose (Ito *et al.* 2003; McCuskey 2008; Walker *et al.* 1985). APAP can deplete GSH levels and cause injury in cultured sinusoidal endothelial cells (SECs) (Deleve *et al.* 1997). In addition,

nitrotyrosine staining is observed in sinusoidal lining cells before nitrotyrosine adducts and injury are detectable in hepatocytes (Knight and Jaeschke 2004; Knight *et al.* 2001). SEC swelling, loss of scavenger function, reduced sinusoidal perfusion, and extravasation of red blood cells were also observed before hepatocellular injury (Ito *et al.* 2003). These microvascular effects were attenuated by an inhibitor of matrix metalloproteinases (MMPs) but not gadolinium chloride, suggesting that some of the early microcirculatory dysfunction was caused by MMPs and not by Kupffer cell activation (Ito *et al.* 2003, 2005; McCuskey 2008). Together, these observations indicate that SEC dysfunction and cell injury after APAP overdose are induced by MMP activity in combination with the direct cytotoxicity of APAP through mechanisms similar to what are seen in hepatocytes. As a result of this vascular damage and trapping of red blood cells in the space of Disse, there is impairment of sinusoidal perfusion and oxygen delivery. In addition, the vascular damage may contribute to the procoagulative state that is observed after APAP overdose in humans (Kerr *et al.* 2003; Payen *et al.* 2003) and in animals (Ganey *et al.* 2007). The activation of the coagulation system through tissue factor activation leads to thrombin generation, which can induce fibrin deposition and/or signaling through protease-activated receptor-1 (PAR-1). Mice deficient in either tissue factor or PAR-1 showed reduced fibrin deposition and experienced less liver injury during the early phase of APAP hepatotoxicity suggesting that thrombin contributes to APAP-induced cell injury (Ganey *et al.* 2007). Interestingly, the protective effect disappeared at 24 h (Ganey *et al.* 2007). Since PAR is predominantly expressed in SEC and Kupffer cells in the liver (Copple *et al.* 2003; Rullier *et al.* 2006), these results suggest that sinusoidal lining cells are involved in the early phase of injury but not at later time points. Another effect of thrombin is to promote fibrin deposition, which can cause microcirculatory problems and hypoxia. Fibrin deposition in the centrilobular areas correlates with the impairment of sinusoidal blood flow early after APAP overdose (Ganey *et al.* 2007; Ito *et al.* 2003). Although the fibrin deposition may be initially detrimental, it appears to be beneficial at later times (Bajt *et al.* 2008b). Mice deficient in plasminogen activator inhibitor show more liver injury and hemorrhage after 24 h suggesting that a too rapid removal of the fibrin deposits could lead to additional hemorrhage and ischemic necrosis (Bajt *et al.* 2008b). Thus, the coagulation system can have both detrimental and beneficial roles in the pathophysiology of APAP-induced liver injury.

9.21.8 Regeneration

9.21.8.1 Regenerative Response after APAP-Induced Liver Injury

In the liver, cell death triggers a regenerative response to replace lost hepatocytes and nonparenchymal cells. Under normal circumstances, the new parenchymal cells are derived from hepatocytes (Fausto 2000). Hepatic stem cells (oval cells) will contribute to liver regeneration only when hepatocyte replication is blocked (Dabeva and Shafritz 1993). Liver regeneration is a multistep process that involves cytokines such as TNF-α and interleukin-6 (IL-6), which prime hepatocytes to respond to growth factors (Fausto 2000). Cytokines and growth factors trigger the expression of cell cycle-regulating proteins, that is, cyclins, cyclin-dependent kinases, and their inhibitors (Taub 2004). The coordinated expression of these proteins moves cells from the G_0 phase into the active cell cycle.

TNF-α, IL-6, and vascular endothelial growth factor (VEGF) are important mediators of regeneration during APAP hepatotoxicity (Chiu *et al.* 2003b; Donahower *et al.* 2006; James *et al.* 2003a). However, regeneration can also be indirectly promoted through reduced liver injury by scavenging peroxynitrite (Bajt *et al.* 2003), preventing hemorrhage (Bajt *et al.* 2008b), blocking platelet-activating factor receptor (Grypioti *et al.* 2006), or activating protective genes (Masubuchi *et al.* 2003). In this respect, it is remarkable that activation of JNK2 promotes not only APAP-induced liver injury (Gunawan *et al.* 2006) but also regeneration (Bourdi *et al.* 2008). Together, these data strongly support the hypothesis that the ability to mount a timely regenerative response during APAP hepatotoxicity limits injury, facilitates hepatocyte proliferation, and improves the chances of survival in experimental animals and humans (Chanda *et al.* 1995; Horn *et al.* 1999; Mehendale 2005; Welch *et al.* 2005).

9.21.8.2 Innate Immune Cells and Regeneration after APAP-Induced Liver Injury

The main purpose of inflammatory cell recruitment into the liver after extensive cell necrosis is to remove dead cells. Necrotic hepatocytes are being

replaced by dividing hepatocytes closest to the area of necrosis (Bajt *et al.* 2003). Cell cycle activation and regeneration are dependent on Kupffer cell-derived TNF-α (Chiu *et al.* 2003b). However, excess injury and hemorrhage can strongly suppress the regenerative response (Bajt *et al.* 2003, 2008b). In addition to initiation of cell division in healthy hepatocytes, removal of necrotic cells is critical for regeneration to be successful. Thus, during the early regeneration phase, neutrophils and monocyte-derived macrophages migrate into the necrotic areas and eliminate it. The recruitment of macrophages into the liver is triggered mainly by the formation of monocyte chemoattractant protein 1 (MCP-1), which is generated by macrophages and hepatocytes in the area of injury (Dambach *et al.* 2002; Holt *et al.* 2008). The receptor for MCP-1, C-C chemokine receptor 2 (CCR2), is expressed on infiltrating macrophages (Dambach *et al.* 2002). Mice deficient in MCP-1 or CCR2 have similar injury during the first 24 h after APAP overdose (Dambach *et al.* 2002) but show a delayed regenerative response (Dambach *et al.* 2002; Holt *et al.* 2008). These data suggest that newly recruited macrophages and neutrophils are important for regeneration. Future studies are needed to elucidate whether these phagocytes are also involved in regulating cell cycle activation and the division of healthy hepatocytes around the area of necrosis.

9.21.9 Conclusions

During the last decade, significant progress has been made in our understanding of the mechanisms of APAP-induced liver cell injury. The metabolic activation of APAP with formation of NAPQI and covalent binding to proteins, especially mitochondrial proteins, is a critical initiating event (**Figure 1**). However, protein binding alone is not sufficient to cause cell death. Among other effects, this triggers reactive oxygen and peroxynitrite formation in mitochondria, bax translocation to mitochondria, and activation of JNK. Some of the consequences include mitochondrial DNA damage, MPT pore opening, ATP depletion, and release of intermembrane proteins, which accumulate in the nucleus and cause DNA degradation (**Figure 2**). Together, these events cause oncotic necrosis. The stress in living cells induces cell cycle activation and initiates regeneration. In addition to the intracellular events, pro- and anti-inflammatory mediators generated by macrophages and other innate immune cells can modulate the injury process by inducing iNOS and antioxidant genes and stimulating cell cycle activation (**Figure 3**). Even limited reduction of injury and promotion of regeneration can prevent liver failure and support the recovery of the liver. The newly discovered mechanisms of oncotic cell death after APAP overdose, innate immune mechanisms, and the process of regeneration provide promising targets for therapeutic interventions that could improve morbidity and mortality after drug overdose.

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