

REVIEW

Intracellular Signaling Mechanisms of Acetaminophen-Induced Liver Cell Death

Hartmut Jaeschke¹ and Mary Lynn Bajt

Liver Research Institute, University of Arizona, Tucson, Arizona 85737

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Acetaminophen hepatotoxicity is the leading cause of drug-induced liver failure. Despite substantial efforts in the past, the mechanisms of acetaminophen-induced liver cell injury are still incompletely understood. Recent advances suggest that reactive metabolite formation, glutathione depletion, and alkylation of proteins, especially mitochondrial proteins, are critical initiating events for the toxicity. Bcl-2 family members Bax and Bid then form pores in the outer mitochondrial membrane and release intermembrane proteins, e.g., apoptosis-inducing factor (AIF) and endonuclease G, which then translocate to the nucleus and initiate chromatin condensation and DNA fragmentation, respectively. Mitochondrial dysfunction, due to covalent binding, leads to formation of reactive oxygen and peroxynitrite, which trigger the membrane permeability transition and the collapse of the mitochondrial membrane potential. In addition to the diminishing capacity to synthesize ATP, endonuclease G and AIF are further released. Endonuclease G, together with an activated nuclear Ca^{2+} , Mg^{2+} -dependent endonuclease, cause DNA degradation, thereby preventing cell recovery and regeneration. Disruption of the Ca^{2+} homeostasis also leads to activation of intracellular proteases, e.g., calpains, which can proteolytically cleave structural proteins. Thus, multiple events including massive mitochondrial dysfunction and ATP depletion, extensive DNA fragmentation, and modification of intracellular proteins contribute to the development of oncotic necrotic cell death in the liver after acetaminophen overdose. Based on the recognition of the temporal sequence and interdependency of these mechanisms, it appears most promising to therapeutically target either the initiating event (metabolic activation) or the central propagating event (mitochondrial dysfunction and peroxynitrite formation) to prevent acetaminophen-induced liver cell death.

Key Words: acetaminophen hepatotoxicity; oncotic necrosis; apoptosis; endonucleases; DNA fragmentation; oxidant stress; peroxynitrite; covalent binding; reactive metabolites.

Acetaminophen (AAP) is a safe and effective analgesic/antipyretic drug when used at therapeutic levels (Rumack, 2004). However, an acute or cumulative overdose can cause severe liver injury with the potential to progress to liver failure (Lee, 2004). In fact, AAP overdose is the most frequent cause of drug-induced liver failure in the United States and in Great Britain (Lee, 2004). In addition to its clinical relevance, AAP is extensively used as a model toxin to evaluate novel hepatoprotective agents. Based on the mechanistic insight gained from early preclinical studies (Jollow *et al.*, 1973; Mitchell *et al.*, 1973a,b), *N*-acetylcysteine was introduced in the 1970s and still is the only clinical antidote against AAP-induced liver injury (Polson and Lee, 2005). Despite substantial efforts to investigate AAP-induced liver cell death during the last 30 years, there are many details of the mechanism that are still unknown. This review will focus on more recent advances in our understanding of intracellular signaling events after AAP overdose. As discussed elsewhere (Jaeschke, 2005), additional aspects of the pathophysiology, e.g., inflammatory mediators and leukocytes, are also important for the overall outcome. However, since the inflammatory response is dependent on the initial injury to hepatocytes, intracellular events may offer the most promising and effective targets for therapeutic interventions.

INITIATION OF AAP-INDUCED CELL DEATH

Among the most extensively studied and least controversial issues is the metabolic activation of AAP. A large portion of a therapeutic dose of AAP is directly conjugated with glucuronic acid or sulfate through glucuronyltransferases or sulfotransferases, respectively (Nelson, 1990). The AAP-glucuronide is excreted into bile through the canalicular multidrug resistance-associated protein 2 (Mrp2, *Abcc2*) and into blood through Mrp3 (*Abcc3*) (Xiong *et al.*, 2000). The biliary elimination of AAP-sulfate is mediated mainly by Mrp2 and to a lesser degree by breast cancer resistance protein (BCRP, *ABCG2*) (Zamek-Gliszczynski *et al.*, 2005). On the

¹ To whom correspondence should be addressed at Liver Research Institute, University of Arizona, College of Medicine, 1501 N. Campbell Ave, Room 6309, Tucson, AZ 85724. Fax: (520) 626-5975. E-mail: jaeschke@email.arizona.edu.

other hand, AAP-sulfate transport into the blood appears not to be mediated by Mrp3 but by other, not fully identified organic anion transporters (Zamek-Gliszczynski *et al.*, 2005). The remaining part of the dose, which is not directly conjugated with hydrophilic groups, is metabolized by the P450 system to a reactive metabolite (Mitchell *et al.*, 1973a), presumably *N*-acetyl-*p*-benzoquinone imine (NAPQI) (Nelson, 1990). Although a number of P450 enzymes can metabolize AAP, the most relevant isoenzyme is CYP2E1, especially in humans (Gonzalez, 2005; Raucy *et al.*, 1989). NAPQI reacts with glutathione (GSH) spontaneously or catalyzed by glutathione-S-transferases to form a GSH-adduct, which is mainly excreted into bile through Mrp2 (Chen *et al.*, 2003). Thus, the earliest effect of AAP metabolism is a profound depletion of hepatocellular glutathione (Mitchell *et al.*, 1973b), which affects both the cytosolic and the mitochondrial compartments. Once GSH is exhausted, any remaining NAPQI formed will react with alternative targets, in particular cellular proteins (Jollow *et al.*, 1973). The covalent modification of cellular proteins was originally thought to cause necrotic cell death (Jollow *et al.*, 1973; Mitchell *et al.*, 1973a,b). This hypothesis was supported by the fact that protein binding preceded cell death and occurred in cells, which became later necrotic (Roberts *et al.*, 1991). In addition, any intervention that prevented covalent binding also prevented cell death (Jollow *et al.*, 1973; Mitchell *et al.*, 1973a,b). A major criticism of the protein-binding hypothesis was an apparent lack of dose responsiveness. Part of this effect could be explained by the influx of protein into the liver due to severe hemorrhage, which diluted the covalently modified protein pool (Corcoran *et al.*, 1985). Overall, there is no AAP hepatotoxicity without protein binding. However, there are examples of AAP-mediated protein binding without hepatotoxicity. One explanation for this observation could be that the total binding to cellular proteins may be less relevant than selective modifications of specific critical targets. Therefore, a considerable effort was made to identify covalently modified proteins (Cohen *et al.*, 1997; Qiu *et al.*, 1998). However, as one would expect from a spontaneous chemical reaction of a reactive metabolite with numerous potential intracellular targets, the activity of most modified enzymes was only modestly affected (Halmes *et al.*, 1996; Pumford *et al.*, 1997). The exceptions are glutamine synthetase and carbamyl phosphate synthetase-1, which are inhibited by more than 50% after AAP treatment (Gupta *et al.*, 1997). The significant loss of the function of these enzymes may be responsible for the hyperammonemia observed after AAP overdose but is less likely to cause acute cell death. Thus, covalent binding to cellular proteins in general or the selective modification of one or more critical enzymes does not appear to be the direct cause of AAP-induced liver cell necrosis.

The regioisomer of AAP, 3'-hydroxyacetanilide, can cause glutathione depletion and a similar degree of covalent binding to cellular proteins as AAP, but does not cause liver injury (Tirmenstein and Nelson, 1989). Despite the same overall

protein binding, AAP induces more prominent covalent modifications of mitochondrial proteins than 3'-hydroxyacetanilide (Qiu *et al.*, 2001; Tirmenstein and Nelson, 1989). Since the binding of NAPQI to mitochondrial proteins correlates with the potential to cause liver injury, these findings suggest that metabolic activation of AAP and protein binding of the reactive metabolite is a critical initiating event in the toxicity, which needs to be amplified and propagated in order to cause cell death (Jaeschke *et al.*, 2003). This concept is further supported by many pharmacological interventions that protected but did not affect reactive metabolite formation and protein binding (e.g., Birge *et al.*, 1988; Corcoran *et al.*, 1985; Jaeschke, 1990; James *et al.*, 2003c; Salminen *et al.*, 1998; Slitt *et al.*, 2004).

MITOCHONDRIAL DYSFUNCTION AND PROPAGATION OF AAP-INDUCED CELL INJURY

AAP overdose triggers mitochondrial dysfunction as indicated by inhibition of mitochondrial respiration (Burcham and Harman, 1991; Meyers *et al.*, 1988; Ramsay *et al.*, 1989) and declining ATP levels (Jaeschke, 1990; Tirmenstein and Nelson, 1990). Since the mitochondrial effects of AAP *in vivo* occur immediately after GSH depletion (Donnelly *et al.*, 1994) and can be directly reproduced by NAPQI in isolated mitochondria (Burcham and Harman, 1991; Ramsay *et al.*, 1989), covalent binding to mitochondrial proteins may be to a significant degree responsible for the initial mitochondrial dysfunction. However, other contributing factors cannot be excluded. In addition to the inhibition of the mitochondrial respiration, it was recognized that when hepatic GSH levels are beginning to recover after the initial depletion by NAPQI, hepatic concentrations of glutathione disulfide (GSSG), a marker of intracellular reactive oxygen formation, increase substantially above baseline (Jaeschke, 1990; Tirmenstein and Nelson, 1990). Interestingly, no release of GSSG into bile was detected (Jaeschke, 1990). This finding suggested that GSSG was formed in an intracellular compartment, which does not release GSSG, i.e., mitochondria. In fact, when mitochondria were isolated from AAP-treated animals, GSSG levels were extremely high and could account for most if not all GSSG within the cell (Jaeschke, 1990; James *et al.*, 2003b; Knight *et al.*, 2001). These data support the conclusion that AAP induces a mitochondrial oxidant stress. Although it was hypothesized that this oxidant stress may be a consequence rather than the cause of cell injury (Rogers *et al.*, 2000), detailed time-course experiments of the oxidation of 2',7'-dichlorodihydrofluorescein, another marker of cellular oxidant stress, showed that reactive oxygen formation starts immediately after GSH depletion and precedes cell death by several hours (Bajt *et al.*, 2004). Although these results are consistent with an important role of reactive oxygen in the pathophysiology, GSH peroxidase-1-deficient (GPx1^{-/-}) mice were not more susceptible to AAP-induced liver injury than wildtype

animals (Knight *et al.*, 2002). However, GPx1^{-/-} mice showed more extensive liver injury in response to oxidant stress induced by neutrophils (Jaeschke *et al.*, 1999, 2002) or diquat (Fu *et al.*, 1999). In addition, AAP treatment did not cause a relevant increase in lipid peroxidation, and loading cells with vitamin E did not protect against AAP overdose (Knight *et al.*, 2003). Taken together, these data indicate that reactive oxygen species such as hydrogen peroxide and hydroxyl radicals are of limited relevance for the mechanism of AAP-induced cell death.

Superoxide can react with nitric oxide (NO) to form peroxynitrite, a potent oxidant and nitrating species (Denicola and Radi, 2005). This reaction is diffusion limited and is actually several times faster even than the superoxide dismutase-catalyzed dismutation reaction (Denicola and Radi, 2005). Immunohistochemical staining for nitrotyrosine protein adducts in cells undergoing necrosis provided evidence for peroxynitrite formation after AAP overdose (Hinson *et al.*, 1998; Knight *et al.*, 2001). Subcellular fractionation showed that peroxynitrite is predominantly generated in mitochondria (Cover *et al.*, 2005b) (Fig. 1), which is consistent with the increased formation of superoxide in these cell organelles (Jaeschke, 1990; Tirmenstein and Nelson, 1990). The source(s) of NO after AAP treatment remain somewhat unclear. On the one hand, nitrotyrosine staining is reduced in inducible nitric oxide synthase-deficient (iNOS^{-/-}) mice (Gardner *et al.*, 2002; Michael *et al.*, 2001), which would suggest that iNOS is a major source of NO for peroxynitrite formation after AAP treatment. In addition, animals lacking the anti-inflammatory gene interleukin-10 (IL-10) responded to an AAP overdose with higher iNOS induction and increased liver injury compared to wildtype animals (Bourdi *et al.*, 2002). However, nitrotyrosine protein adducts were detected as early as 0.5 to 1 h after AAP exposure in the absence of iNOS induction (Cover *et al.*, 2005b; Knight *et al.*, 2001). These results indicate that iNOS is an important, but not the only, possible source for NO in AAP hepatotoxicity.

Although there is extensive evidence for peroxynitrite formation before tissue injury, the important question remains if this reactive nitrogen species is a relevant mediator for the overall cell death. Experiments with iNOS^{-/-} mice (Gardner *et al.*, 2002; Michael *et al.*, 2001) or iNOS inhibitors (Gardner *et al.*, 1998; Hinson *et al.*, 2002; Kamanaka *et al.*, 2003) yielded conflicting results. Interventions that enhanced iNOS induction increased AAP-induced liver injury (Bourdi *et al.*, 2002; Tinel *et al.*, 2004). We used a different approach to address this critical question. Since GSH is a potent scavenger of peroxynitrite (Kirsch *et al.*, 2001), we hypothesized that delayed treatment with GSH after AAP may accelerate the recovery of cellular and, in particular, mitochondrial GSH levels, which then may be able to scavenge peroxynitrite *in vivo* and may attenuate cell injury (Knight *et al.*, 2002). Indeed, intravenous administration of GSH, which is rapidly degraded in the kidney and provides amino acids for the resynthesis of

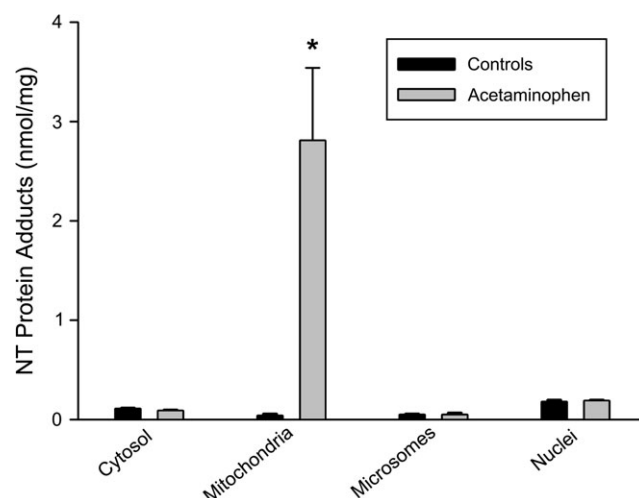


FIG. 1. Assessment of nitrotyrosine (NT) protein adducts by ELISA in subcellular fractions isolated from livers of untreated C3Heb/FeJ mice (controls) and animals treated with 300 mg/kg acetaminophen for 4 h. Data represent means \pm SE of $n = 4$ animals per group. * $p < 0.05$ (compared to controls) (data adapted from Cover *et al.*, 2005b).

GSH in the liver (Wendel and Jaeschke, 1982), effectively prevented nitrotyrosine staining, strongly attenuated AAP-induced liver injury, and promoted cell cycle activation and regeneration (Bajt *et al.*, 2003; Knight *et al.*, 2002). Since this treatment did not prevent mitochondrial dysfunction and the mitochondrial oxidant stress in wildtype as well as in GPx1^{-/-} mice, these results strongly supported the hypothesis that peroxynitrite is a relevant mediator of AAP hepatotoxicity (Knight *et al.*, 2002). These findings were later confirmed by using *N*-acetylcysteine (James *et al.*, 2003c).

CONSEQUENCES OF MITOCHONDRIAL OXIDANT STRESS AND PEROXYNITRITE FORMATION

Oxidant stress caused by reactive oxygen and nitrogen species and increased Ca²⁺ levels are well-known inducers of the mitochondrial membrane permeability transition (MPT) in many cell types including hepatocytes (Kim *et al.*, 2003). The MPT is characterized by mitochondrial swelling, uncoupling of the oxidative phosphorylation, and formation of pores in the inner mitochondrial membrane. Opening of these pores allows the passage of solutes of ≤ 1500 Dalton molecular weight (Kim *et al.*, 2003). A consequence of the MPT is the breakdown of the mitochondrial membrane potential ($\Delta\Psi_m$), the inability to synthesize ATP, and finally, necrotic cell death (Kim *et al.*, 2003). If the insult is milder and only a limited number of mitochondria undergo the MPT, the resulting release of cytochrome c can promote apoptotic cell death (Kim *et al.*, 2003). In general, the MPT is dependent on the presence of Ca²⁺ and can be inhibited by cyclosporin A (CsA). However, a more severe insult may trigger a Ca²⁺-independent, CsA-insensitive MPT (He and Lemasters, 2002).

Since AAP overdose depletes mitochondrial GSH levels, induces formation of peroxynitrite in mitochondria (Cover *et al.*, 2005b) (Fig. 1), and causes cellular Ca^{2+} accumulation (Corcoran *et al.*, 1988) through inhibition of the Ca^{2+} - Mg^{2+} -ATPase (Tsokos-Kuhn *et al.*, 1988), it is not surprising that AAP induced the mitochondrial MPT in primary cultured mouse hepatocytes 4–6 h after exposure to AAP (Kon *et al.*, 2004a). The MPT occurred after GSH depletion and after initiation of reactive oxygen formation, but preceded necrotic cell death (Bajt *et al.*, 2004; Kon *et al.*, 2004a). The AAP-induced MPT and cell necrosis could be substantially delayed with CsA but not prevented at later time points (Kon *et al.*, 2004a). These data suggest that AAP initially triggers a CsA-sensitive, regulated MPT, which is followed by a CsA-insensitive, unregulated MPT (Kon *et al.*, 2004a). CsA treatment also protected against AAP hepatotoxicity *in vivo* (Haouzi *et al.*, 2002; Masubuchi *et al.*, 2005). More recent observations indicate that ferrous iron mobilized from lysosomes and oxidant stress appears to be involved in triggering the MPT after AAP (Kon *et al.*, 2004b). This mechanism explains the previously reported protective effect of iron chelation against AAP toxicity *in vitro* (Adamson and Harman, 1993). In addition, *N*-acetylcysteine treatment 2 h after AAP attenuated the oxidant stress, the loss of the mitochondrial membrane potential, and cell injury (Bajt *et al.*, 2004; Reid *et al.*, 2005). Taken together, these findings support the hypothesis that reactive oxygen and Fenton-type reaction products may be important in the induction of the MPT and cell necrosis *in vitro*. Consistent with this conclusion, antioxidants such as vitamin E have been shown to protect against AAP-induced cell injury in culture (Nagai *et al.*, 2002). However, in contrast to the findings *in vitro*, the iron chelator desferoxamine is either not at all protective (Smith *et al.*, 1986) or only delays toxicity *in vivo* (Schnellmann *et al.*, 1999). Treatment with α - or γ -tocopherol, which clearly prevented lipid peroxidation and cell injury after iron/allyl alcohol administration, did not protect against AAP-induced liver injury (Knight *et al.*, 2003). Thus, together these observations indicate that there might be a higher emphasis on oxidant stress mechanisms *in vitro* compared to the *in vivo* situation where peroxynitrite appears to dominate (Knight *et al.*, 2002). The reason for this effect could be an artificially enhanced mitochondrial oxidant stress due to the generally used hyperoxic cell culture conditions (Halliwell, 2003).

In addition to the MPT, peroxynitrite may cause a number of other adverse effects in the mitochondria. Recently, we showed a decline of mitochondrial DNA (mtDNA) during AAP hepatotoxicity as assessed by a slot blot hybridization assay (Cover *et al.*, 2005b). When nitrotyrosine formation in mitochondria was eliminated by treatment with GSH, which accelerated the recovery of mitochondrial GSH levels, the loss of mtDNA was only partially prevented (Cover *et al.*, 2005b). This suggests that peroxynitrite is only one of several causes of mtDNA modifications (Rogers *et al.*, 1997). Although the effect of mtDNA

loss on acute cell toxicity may be minimal, if the cell survives the initial insult, long-term survival may be jeopardized.

Mitochondrial release of Ca^{2+} , together with the inhibition of the Ca^{2+} - Mg^{2+} -ATPase in the plasma membrane (Tsokos-Kuhn *et al.*, 1988), may lead to an increase of cytosolic Ca^{2+} levels sufficient to activate Ca^{2+} -dependent intracellular proteases such as calpains. These enzymes can proteolytically cleave structural proteins within the cell and contribute to oncotic necrosis (Liu *et al.*, 2004). In addition, release of calpains from necrotic cells can affect neighboring cells and expand the injury within the liver (Limaye *et al.*, 2003). Another consequence of the disrupted intracellular Ca^{2+} homeostasis is the accumulation of Ca^{2+} in the nucleus and activation of a Ca^{2+} -dependent endonuclease (Ray *et al.*, 1990).

MITOCHONDRIAL DYSFUNCTION AND NUCLEAR DNA FRAGMENTATION

AAP overdose causes fragmentation of nuclear DNA and karyolysis both *in vivo* and in primary cultured hepatocytes, as first recognized by Corcoran and coworkers (Ray *et al.*, 1990; Shen *et al.*, 1991). A characteristic DNA ladder on agarose gel provided evidence for internucleosomal DNA cleavage (Ray *et al.*, 1990; Shen *et al.*, 1991). DNA fragmentation was later confirmed through other assays such as the antihistone ELISA (Lawson *et al.*, 1999), which measures the presence of small DNA fragments in the cytosol, and the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (Lawson *et al.*, 1999), which indicates DNA strand-breaks. Together these assays clearly demonstrate nuclear DNA damage, which occurs in parallel to AAP-induced liver cell injury (Lawson *et al.*, 1999; Ray *et al.*, 1990). The fact that DNA damage and cell injury was prevented by a general endonuclease inhibitor (Shen *et al.*, 1992) suggested that endonuclease-mediated DNA fragmentation is a critical event in the pathophysiology. The finding that no nitrotyrosine protein adducts could be detected in the nucleus supports the hypothesis that peroxynitrite was not directly responsible for the DNA damage after AAP overdose (Cover *et al.*, 2005b) (Fig. 1). However, delayed treatment with GSH effectively scavenged mitochondrial peroxynitrite and prevented nuclear DNA fragmentation (Cover *et al.*, 2005b). Thus, there appears to be a connection between mitochondrial dysfunction and nuclear DNA damage.

The most studied endonuclease is the DNA fragmentation factor (DFF40) or caspase-activated DNase (CAD), which is activated through cleavage of its inhibitor (DFF45/ICAD) by caspase-3 (Nagata *et al.*, 2003). In the liver, caspase-3 is mostly activated through cytochrome c release from mitochondria and caspase-9 activation by the apoptosome (Hill *et al.*, 2003). Although mitochondrial cytochrome c release has been shown during AAP hepatotoxicity (Adams *et al.*, 2001; El-Hassan

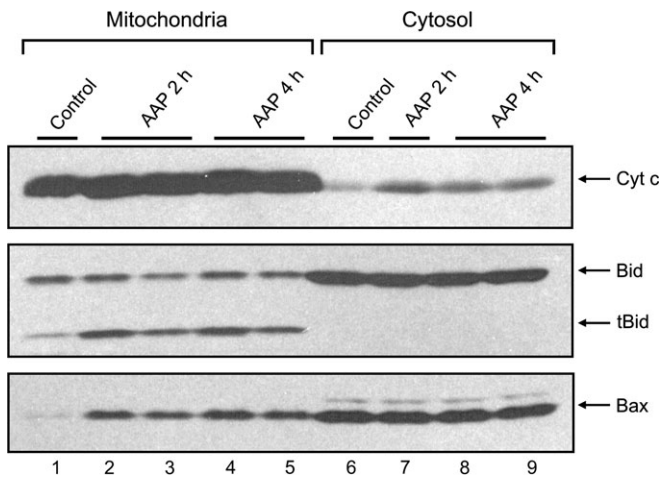


FIG. 2. Western blot analysis of cytosolic and mitochondrial cytochrome c, Bax, Bid, and its truncated form (tBid) in livers of untreated C3Heb/FeJ mice and in animals treated with 300 mg/kg acetaminophen for 2 or 4 h. Each lane represents samples from an individual animal.

et al., 2003; Knight and Jaeschke, 2002) (Fig. 2), a number of laboratories consistently reported either no or at best a minor activation of caspases (Adams *et al.*, 2001; El-Hassan *et al.*, 2003; Ferret *et al.*, 2001; Gujral *et al.*, 2002; Lawson *et al.*, 1999; Nagai *et al.*, 2002; Tinel *et al.*, 2004). In addition, the staining pattern of the TUNEL assay and the amount of low-molecular-weight DNA fragments in the cytosol or plasma are clearly different between AAP-induced cell injury and death receptor-mediated apoptosis, where activation of DFF/CAD is undisputed (Cover *et al.*, 2005b; Gujral *et al.*, 2002; Jahr *et al.*, 2001; Lawson *et al.*, 1999). Thus, despite DNA ladders undistinguishable from apoptosis, there is no evidence for a relevant activation of DFF/CAD during AAP hepatotoxicity. Nevertheless, until the responsible endonucleases are definitively identified, a contribution of DFF/CAD to nuclear DNA fragmentation cannot be completely ruled out.

During apoptosis, DFF/CAD is thought to cleave DNA first into 50 kb fragments (Nagata *et al.*, 2003). These large fragments are then degraded to smaller internucleosomal repeats by a $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonuclease (DNAS1L3) located in the nucleus (Yakovlev *et al.*, 1999). Indirect support for a role of DNAS1L3 comes from earlier work by Corcoran's group showing a correlation between DNA ladders and nuclear Ca^{2+} accumulation (Ray *et al.*, 1990). In addition, treatment with Ca^{2+} channel blockers inhibited nuclear Ca^{2+} accumulation, attenuated DNA fragmentation and liver injury (Ray *et al.*, 1993). Interestingly, DNAS1L3 is inhibited by poly(ADP-ribose) polymerase (PARP) (Yakovlev *et al.*, 2000). During apoptosis, this inhibitory effect is generally overcome by caspase-3 activation, which inactivates PARP (Yakovlev *et al.*, 2000). However, PARP activation occurs predominantly after major DNA fragmentation during AAP-induced liver injury (Cover *et al.*, 2005a), and although there is no caspase

activation during AAP hepatotoxicity, there is still PARP cleavage (Zhang *et al.*, 2000), presumably through other intracellular proteases. Thus, activation of DNAS1L3 appears to be involved in AAP-induced DNA fragmentation. However, it remains unclear what endonuclease replaces DFF/CAD.

The pro-apoptotic Bcl-2 family member Bax resides in the cytosol but can translocate to mitochondria and can form pores in the outer mitochondrial membrane alone or in combination with other Bcl-2 proteins, e.g., Bad and the truncated form of Bid (Chao and Korsmeyer, 1998). Formation of these pores together with formation of the MPT pores in the inner membrane can release proteins from the intermembrane space of mitochondria (Jaeschke and Lemasters, 2003; Scorrano and Korsmeyer, 2003). Apoptosis-inducing factor (AIF) (Susin *et al.*, 2000) and endonuclease G (van Loo *et al.*, 2001) are proteins that have been implicated in chromatin condensation and nuclear DNA fragmentation, respectively. Therefore, we assessed the release of endonuclease G and AIF from mitochondria and their translocation to the nucleus in response to AAP treatment. In primary cultured mouse hepatocytes, nuclear translocation of endonuclease G and AIF was observed between 3 and 6 h after AAP exposure, i.e., after GSH depletion but before cell death (Bajt *et al.*, unpublished observation). Nuclear translocation of endonuclease G and AIF and DNA fragmentation was inhibited by treatment with *N*-acetylcysteine (Bajt *et al.*, unpublished observation), which also attenuated cell death (Bajt *et al.*, 2004). *In vivo*, mitochondrial translocation of Bax and truncated Bid (tBid) precedes nuclear DNA fragmentation and mitochondrial oxidant stress (Adams *et al.*, 2001; Bajt *et al.*, 2005; El-Hassan *et al.*, 2003) (Fig. 2). However, in Bax-deficient ($\text{Bax}^{-/-}$) mice, only DNA fragmentation and cell injury but not peroxynitrite formation was delayed (Bajt *et al.*, 2005). On the other hand, DNA damage and injury was similar in $\text{Bax}^{-/-}$ and in wild-type mice at later time points (≥ 12 h) (Bajt *et al.*, 2005; Bajt *et al.*, unpublished results). These findings indicate that AAP-induced Bax translocation to the mitochondria may induce the early release of intermembrane proteins, e.g., endonuclease G, AIF, the second mitochondria-derived activator of caspases (Smac), and cytochrome c. Endonuclease G and AIF translocate to the nucleus and cause DNA damage, which correlates with cellular necrosis. Therefore, endonuclease G and AIF may be the link between Bax-mediated pore formation in the outer mitochondrial membrane and nuclear DNA fragmentation. Furthermore, endonuclease G may be the DNase that generates the large DNA fragments for the activation of DNAS1L3. However, since the oxidant stress and peroxynitrite formation occur independent of Bax, the resulting MPT will eventually cause endonuclease G and AIF release, which leads to further DNA fragmentation and cell injury. Thus, the Bax-mediated signaling mechanism is overridden by peroxynitrite and the mitochondrial MPT at later time points.

How DNA damage contributes to cell necrosis remains unclear. One possible link could be the activation of PARP,

which is critical for DNA repair (Meyer-Ficca *et al.*, 2005). However, excessive activation of PARP, which depletes cellular levels of NAD^+ and ATP, leads to an energy crisis within the cell, causing cell necrosis (Ha and Snyder, 1999). Although there is clear evidence for PARP activation after AAP overdose, the activation of this enzyme occurs after the onset of DNA fragmentation and cell injury (Cover *et al.*, 2005a). In addition, no protective effect was observed in PARP-deficient ($\text{PARP}^{-/-}$) mice or with specific PARP inhibitors (Cover *et al.*, 2005a). In fact, $\text{PARP}^{-/-}$ mice showed a slightly increased injury (Cover *et al.*, 2005a). This finding is similar to a previous *in vitro* study using a PARP inhibitor (Shen *et al.*, 1992). In contrast, it was reported that high doses of the PARP inhibitor 3-aminobenzamide protected against AAP hepatotoxicity *in vivo* (Ray *et al.*, 2001). However, this effect appears to be independent of PARP, because the chemical was as effective in wildtype as in $\text{PARP}^{-/-}$ mice (Cover *et al.*, 2005a). Together, these findings suggest that PARP activation is a response to DNA damage after AAP, but is not actively involved in the process of cell injury. On the other hand, regeneration was suppressed in $\text{PARP}^{-/-}$ mice at later time points after AAP overdose (Bajt *et al.*, unpublished observations).

AAP HEPATOTOXICITY AND REGENERATION

The liver is a unique organ in the sense that a significant loss of liver cells due to drug toxicity or other insults can be overcome by regeneration (Mehendale, 2005). The replacement of necrotic cells occurs predominantly through proliferation of hepatocytes, which need to exit the quiescent state (G_0) and enter the cell cycle (Michalopoulos and DeFrances, 2005). Although the entire process is highly complex and has been extensively studied, only a few key aspects will be addressed. The first step involves the priming of hepatocytes by $\text{TNF-}\alpha$ (Akerman *et al.*, 1992) and interleukin-6 (IL-6) (Cressman *et al.*, 1996), which makes the cells more responsive to growth factors (Fausto, 2000). Various growth factors then induce expression of cell cycle proteins including cyclins, cyclin-dependent kinases, and cell cycle inhibitors. The simultaneous expression of activators and inhibitors of the cell cycle ensures a sensitive regulation of the process (Fausto, 2000). One of the markers of cell cycle activation is the expression of cyclin D_1 , which signals the commitment of the cell to DNA synthesis (Fausto, 2000). Consistent with the general concept of regeneration, cell proliferation after AAP overdose involves IL-6 (James *et al.*, 2003a) and $\text{TNF-}\alpha$ (Chiu *et al.*, 2003), as indicated by the impaired regenerative response in $\text{IL-6}^{-/-}$ and $\text{TNF receptor type 1 (p55)}^{-/-}$ mice. Moreover, scavenging of mitochondrial peroxynitrite with GSH attenuates AAP-induced liver injury and causes expression of cyclin D_1 and proliferating cell nuclear antigen (PCNA) (Bajt *et al.*, 2003). The most extensively stained cells are located in the

transition zone between necrotic and healthy cells (Bajt *et al.*, 2003). Interestingly, neither pretreatment with growth factors (Francavilla *et al.*, 1993) nor IL-6 (Bajt *et al.*, 2003) prevented AAP-induced liver injury. On the hand, forcing hepatocytes into cell division with a low dose of thioacetamide attenuated AAP hepatotoxicity (Chanda *et al.*, 1995). These data suggest that cell cycle activation and regeneration is a critical event in the pathophysiology of AAP-induced liver failure. A more detailed knowledge of the events may reveal important new therapeutic strategies.

APOPTOTIC SIGNALING PATHWAYS AND AAP-INDUCED ONCOTIC NECROSIS

As discussed, there is a significant overlap between events normally associated with apoptosis and AAP-induced cell death. The first observation of this kind was DNA fragmentation as indicated by DNA ladders after AAP overdose (Ray *et al.*, 1990). This initial finding lead to the conclusion that about 40% of hepatocytes actually die by apoptosis (Ray *et al.*, 1996). However, a detailed morphological study of AAP-induced cell death in comparison with TNF -induced apoptosis clearly demonstrated that >95% of injured hepatocytes die through oncotoc necrosis *in vivo* (Gujral *et al.*, 2002). Apoptosis is quantitatively at best a minor issue. Similarly, necrosis is the dominant cell death in primary hepatocytes (Bajt *et al.*, 2004; Kon *et al.*, 2004a; Nagai *et al.*, 2002). Nevertheless, the increasing overlap in signaling events, e.g., Bax and tBid translocation to mitochondria (Fig. 2), cytochrome c release from mitochondria (Fig. 2), PARP cleavage (Zhang *et al.*, 2000), internucleosomal DNA cleavage (Ray *et al.*, 1990), between apoptosis and AAP-mediated cell events continuously provides fuel for new speculation regarding the mode of AAP-induced cell death. These observations, together with a recent report showing that the pancaspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) protected against AAP-induced hepatotoxicity, lead to the hypothesis that AAP induces hepatocellular apoptosis, which is rapidly aborted due to declining ATP levels (El-Hassan *et al.*, 2003). This study agrees with our previous data that there is no relevant caspase activation during AAP hepatotoxicity (Lawson *et al.*, 1999). In fact, the mitochondrial dysfunction induced by AAP overdose is so severe that it effectively prevents Fas receptor-mediated caspase activation and apoptosis (Knight and Jaeschke, 2002). In addition, treatment with 10 mg/kg Z-VAD-fmk 1.5 and 3 h after AAP administration was ineffective in preventing AAP-induced cell death (Lawson *et al.*, 1999). The same dose is highly effective in eliminating Fas- or TNF -receptor mediated apoptosis *in vivo* (Bajt *et al.*, 2000; Jaeschke *et al.*, 1998, 2000; Lawson *et al.*, 1999). However, it remained unclear why pretreatment with a suicide substrate of caspases (10 mg/kg Z-VAD-fmk) can prevent AAP hepatotoxicity even in the absence of any caspase activation (El-Hassan *et al.*, 2003). More

recent findings provide an explanation for this unexpected effect. Even a small amount of the solvent dimethyl sulfoxide, which is necessary to get these inhibitors in solution, is sufficient to inhibit metabolic activation of AAP and prevent hepatotoxicity (Jaeschke *et al.*, 2005). This explains why only treatment with caspase inhibitors before but not after AAP administration protects (Jaeschke *et al.*, 2005). Thus, there is no reliable evidence *in vivo* or in primary hepatocytes that caspase inhibitors are able to protect against AAP hepatotoxicity (Jaeschke *et al.*, 2005; Lawson *et al.*, 1999; Nagai *et al.*, 2002). Other arguments, which do not support the hypothesis that AAP-induced cell death is an apoptotic process rapidly deteriorating into secondary necrosis, include the fact that, during secondary necrosis, apoptotic features (e.g., massive caspase activation, morphological characteristics) are still clearly detectable (Bajt *et al.*, 2000; Gujral *et al.*, 2004; Jaeschke *et al.*, 2004). None of these more specific parameters of apoptosis are detectable in quantities relevant to explain the extent of cell death seen during AAP hepatotoxicity.

Currently, there are only two experimental conditions where AAP unequivocally causes apoptotic cell death. First, exposure of hepatoma cell lines to high concentrations of AAP in cell culture will induce classical caspase-dependent apoptosis (Boulares *et al.*, 2002; Macanas-Pirard *et al.*, 2005). Since these hepatoma cell lines lack the capacity to metabolically activate a relevant amount of AAP and therefore do not properly mimic the initiating events of AAP hepatotoxicity, the relevance of these mechanisms for the pathophysiology *in vivo* has to be questioned. Second, AAP induces apoptosis in primary hepatocytes when the necrotic cell death is prevented by fructose and glycine treatment (Kon *et al.*, 2004a). However, these findings in cell culture remain to be confirmed *in vivo*. In general, inhibition of AAP-induced cell necrosis *in vivo* results in a permanent protection and does not induce apoptosis.

GENOMICS AND PROTEOMICS APPROACHES

In the recent past, livers from AAP-treated mice were subjected to genomics and proteomics analysis. When mRNA expression of livers from AAP-treated animals (300 mg/kg; 6 h) was compared to controls, numerous genes encoding stress proteins, cell cycle and growth inhibitors, adhesion molecules and structural proteins, inflammatory mediators, and cell signaling proteins were upregulated, and many genes involved in cell metabolism were downregulated (Reilly *et al.*, 2001). While the relevance for the pathophysiology of AAP-induced liver injury of some of the identified genes is known (e.g., heat shock proteins, heme oxygenase-1), the importance of most of the more than 300 up- or downregulated genes is still unclear. In a related study, which compared toxic and nontoxic doses of AAP at different time points, the authors demonstrated major changes in mitochondrial protein expression consistent with

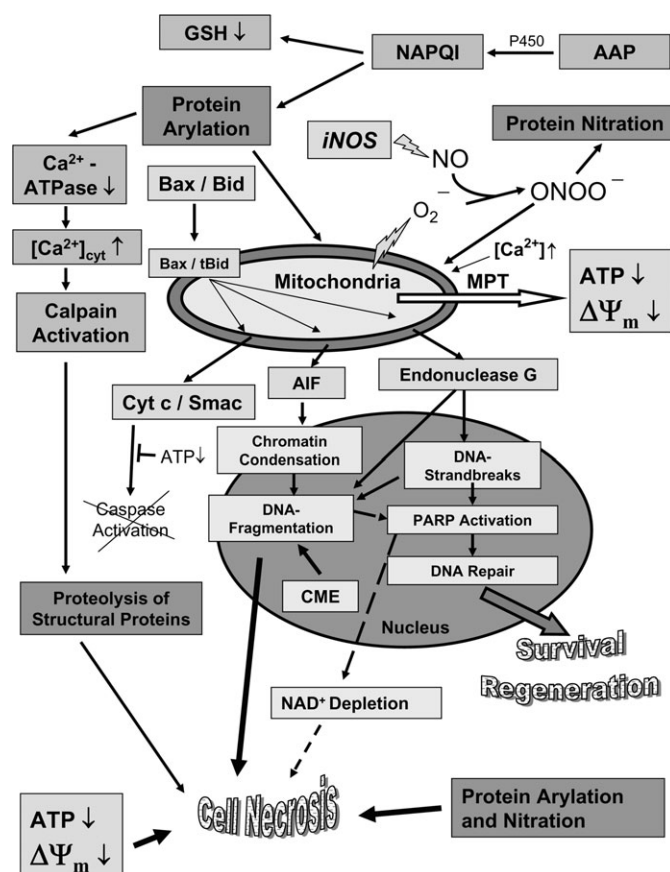


FIG. 3. Proposed sequence of events leading to acetaminophen (AAP)-induced hepatotoxicity (see text for details). Abbreviations: AIF, apoptosis-inducing factor; CME, nuclear Ca²⁺/Mg²⁺-dependent endonuclease (DNAS1L3); cyt, cytosolic; cyt c, cytochrome c; ΔΨ_m, mitochondrial membrane potential; GSH, reduced glutathione; iNOS, inducible NO synthase; MPT, mitochondrial membrane permeability transition; NAPQI, *N*-acetyl-*p*-benzoquinone imine; NO, nitric oxide; O₂⁻, superoxide; ONOO⁻, peroxynitrite; PARP, poly(ADP-ribose) polymerase; Smac, second mitochondria-derived activator of caspases; tBid, truncated form of Bid.

the hypothesis that mitochondria are a critical target for the toxicity (Ruepp *et al.*, 2002). However, most gene expression alterations occurred after the protein changes, suggesting that genomic changes may modulate more downstream events of the toxicity rather than any early events. A more recent proteomic analysis of livers of two strains of mice, one with a higher susceptibility for AAP hepatotoxicity than the other, showed a more pronounced loss of several mitochondrial proteins in the susceptible strain (Welch *et al.*, 2005). In addition, the resistant strain had a higher expression of a number of proteins related to stress response, antioxidant enzymes, and cell proliferation (Welch *et al.*, 2005). Thus, genomic and proteomic analysis confirmed the importance of mitochondria as a target for AAP hepatotoxicity. The pathophysiological importance of many of the altered genes and proteins after AAP overdose remains to be investigated in more detail.

SUMMARY

A fraction of the dose of AAP is metabolically activated to a reactive metabolite (NAPQI), which first depletes cellular glutathione and subsequently covalently binds to cellular proteins (Fig. 3). These initiating events lead to disturbances of the cellular Ca^{2+} homeostasis, with increase of the cytosolic Ca^{2+} levels, Bax and Bid translocation to the mitochondria, and a mitochondrial oxidant stress and peroxynitrite formation. The Bcl-2 family members form pores in the outer mitochondrial membrane and release cytochrome c, Smac, AIF, and endonuclease G from the mitochondrial intermembrane space. Reactive oxygen species and peroxynitrite induce the membrane permeability transition, which causes the collapse of the mitochondrial membrane potential, eliminates ATP synthesis, and causes further release of mitochondrial proteins. The declining ATP levels appear to prevent caspase activation by the release of cytochrome c and Smac. AIF and endonuclease G translocate to the nucleus and induce DNA fragmentation, which is further aggravated by the nuclear $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonuclease DNase1L3. The massive nuclear DNA damage and the rapid elimination of functional mitochondria, together with activation of intracellular proteases (calpains), lead to cell membrane failure and oncotic necrosis of the hepatocytes. The postulated intracellular signaling events after AAP overdose can explain the massive cell death and liver failure. However, many aspects are still unclear and require further investigation. In addition, it has to be kept in mind that AAP-induced cell death *in vivo* can be modulated by changes in the expression levels of P450 and phase II detoxification enzymes, variation in the GSH and antioxidant levels (nutritional status), and preexisting conditions affecting the susceptibility of hepatocytes (steatosis, mitochondrial abnormalities, inflammation). Therefore, to most effectively protect against AAP overdose, it is important to focus on central mechanisms of the pathophysiology. At the present time, this appears to be the metabolic activation as initiating event in the toxicity and mitochondrial dysfunction as the key cellular event that controls the propagation of the injury.

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