

Invited Review

Acetaminophen Toxicity: Novel Insights Into Mechanisms and Future Perspectives

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Acetaminophen (APAP) overdose is the most common cause of acute liver failure in the US, and decades of intense study of its pathogenesis resulted in the development of the antidote *N*-acetylcysteine, which facilitates scavenging of the reactive metabolite and is the only treatment in clinical use. However, the narrow therapeutic window of this intervention necessitates a better understanding of the intricacies of APAP-induced liver injury for the development of additional therapeutic approaches that can benefit late-presenting patients. More recent investigations into APAP hepatotoxicity have established the critical role of mitochondrial dysfunction in mediating liver injury as well as clarified mechanisms of APAP-induced hepatocyte cell death. Thus, it is now established that mitochondrial oxidative and nitrosative stress is a key mechanistic feature involved in downstream signaling after APAP overdose. The identification of specific mediators of necrotic cell death further establishes the regulated nature of APAP-induced hepatocyte cell death. In addition, the discovery of the role of mitochondrial dynamics and autophagy in APAP-induced liver injury provides additional insight into the elaborate cell signaling mechanisms involved in the pathogenesis of this important clinical problem. In spite of these new insights into the mechanisms of liver injury, significant controversy still exists on the role of innate immunity in APAP-induced hepatotoxicity.

Key words: Acetaminophen (APAP); Programmed necrosis; Mitochondria; Sterile inflammation; Neutrophils

INTRODUCTION

Acetaminophen (APAP) is one of the most common analgesic and antipyretic drugs in use globally¹. Though the drug is safe and effective at therapeutic doses, the therapeutic window is narrow, and an overdose is highly hepatotoxic. Because of the ubiquitous nature and broad availability of the drug, this has resulted in APAP hepatotoxicity being the most frequent cause of acute liver failure (ALF) in the US² and other Western countries³. While a number of APAP overdose cases are due to suicide attempts, the availability of combination products, where the presence of APAP may not be easily recognized, has led to an increase in unintentional and chronic APAP overdose, accounting for over 50% of cases of APAP-related ALF⁴. Thus, APAP hepatotoxicity contributes to around 70,000 hospitalizations each year in the US⁵. Overall, APAP overdose is responsible for 46% of all cases of ALF in the US and has now grown to be a

significant public health problem⁶. Decades of investigations into the mechanisms of APAP-induced liver injury have provided significant insight into the role of APAP metabolism and formation of a reactive metabolite in initiating the cascade of events ultimately leading to liver injury.

METABOLISM OF ACETAMINOPHEN

When consumed at therapeutic doses, the majority (80%–90%) of APAP is conjugated with glucuronic acid or sulfate and excreted through the kidneys⁷. A minor component is acted upon by cytochrome P450 enzymes such as Cyp2E1 and Cyp1A2 to form a reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI)⁸. Though highly reactive, NAPQI is rarely harmful after consumption of therapeutic doses because it is rapidly conjugated with abundant glutathione stores in the liver and excreted through the bile. However, this contrasts to the scenario

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after consumption of an overdose of APAP where the sulfation pathway is saturated⁷, and NAPQI generation is significantly elevated in spite of the high capacity of the glucuronidation pathway⁹. Excessive generation of NAPQI results in its robust reaction with hepatic glutathione stores and the subsequent rapid depletion of glutathione within the liver. This leaves free reactive NAPQI available for reaction with protein sulfhydryl groups to form APAP protein adducts⁷. Metabolism of APAP can be influenced by genotype differences, and variations in glucuronidation are seen in different populations due to polymorphisms in the UDP-glucuronosyltransferase (UGT) enzymes. It was recently shown that UGT2B15 *2/*2 genotype subjects showed higher APAP protein adduct concentrations than *1/*2 and *1/*1 individuals¹⁰. Formation of APAP protein adducts and their release into the circulation are now areas of intense study due to the clinical implications in the management of patients with APAP overdose, mainly since APAP protein adducts have been suggested to be biomarkers useful for diagnosing an APAP overdose¹¹. However, protein adducts are also detectable in the vast majority of subjects taking therapeutic doses of APAP¹², and protein-derived APAP–cysteine can be detected after repeated supratherapeutic ingestion of APAP in the absence of hepatotoxicity¹³. While the clinical utility of APAP protein adduct measurements in this context has been questioned¹⁴, a recently developed competitive immunoassay (AcetaSTAT) has been suggested to identify patients with APAP-induced acute liver injury or failure¹⁵. Because of these clinical implications, a better understanding of protein adduct formation and its relationship to hepatocyte necrosis is warranted. Mechanistically, it was considered earlier that glutathione levels need to be significantly depleted before NAPQI would react with proteins¹⁶. However, recent evidence suggests that this may not be the case, since APAP protein adducts were found to be generated even at therapeutic doses of APAP^{7,12,17,18}; in fact, protein adducts were formed before significant GSH depletion^{7,18,19}. Rather than assessing the overall protein adduct formation in the cell, which was initially considered to be critical for cell death²⁰, it now appears that protein adduct formation on mitochondrial proteins is most relevant for toxicity^{21–23}.

MITOCHONDRIA, PROTEIN ADDUCTS, AND APAP HEPATOTOXICITY

Mitochondria are essential organelles with the primary responsibility of cellular energy generation. However, mitochondria can also play significant roles in cellular signaling, for example, through generation of reactive oxygen species (ROS) (Fig. 1). This is facilitated, in part, by the translocation of cytosolic proteins to the mitochondria, a recurring paradigm in a number of cellular signaling contexts. Mitochondrial proteins are significant targets of

NAPQI, and mitochondrial protein adducts were unique to APAP treatment in contrast to the nontoxic regioisomer 3'-hydroxyacetanilide (AMAP) in mice^{22,24,25}. However, AMAP can be hepatotoxic in human hepatocytes^{23,26}, and this correlated with the formation of mitochondrial protein adducts and compromised mitochondrial function²³. Mitochondrial protein adducts also seem responsible for the APAP-induced mitochondrial dysfunction²³, though critical target proteins responsible for these effects are not well characterized yet. However, general proteomic techniques have identified a number of mitochondrial proteins with adducts of APAP, including ATP synthase and glutathione peroxidase²⁷, and a mitochondrial proteomic approach using blue native PAGE showed changes in proteins such as HMG CoA synthase, accompanied by inhibition of enzyme activity after APAP treatment²⁸. Additional mitochondrial proteins such as glycine amidinotransferase, the redox-sensitive chaperone PARK7, peroxiredoxin 6, and the voltage-gated ion channel VDAC2 were found to be modified by NAPQI in 3D cultures of human hepatocytes and nonparenchymal cells²⁹. In spite of the identification of these mitochondrial protein targets, a direct effect of their modification on compromising mitochondrial function is not evident, and some of these changes could be consequences rather than causes of mitochondrial dysfunction. Nonetheless, NAPQI binding to mitochondrial proteins correlates with APAP toxicity³⁰, and hence the effect could be a cumulative one, with mitochondrial function being affected once a threshold of mitochondrial protein modification is attained.

MITOCHONDRIAL REACTIVE OXYGEN AND REACTIVE NITROGEN SPECIES

The main characteristic of mitochondrial dysfunction induced by APAP adducts is increased generation of ROS such as superoxide³¹, as well as peroxynitrite³², which can modify proteins by nitration of their tyrosine residues³³ (Fig. 1). The importance of mitochondrial superoxide in mediating APAP hepatotoxicity is further illustrated by the significant exacerbation of liver injury in mice with a partial deficiency of manganese superoxide dismutase (SOD2)^{34,35}, which would usually scavenge superoxide within the mitochondria. Similarly, the mitochondria-targeted SOD mimetic mito-TEMPO effectively protected against APAP hepatotoxicity³⁶. The source of the mitochondrial superoxide production is likely the respiratory chain, since APAP has been shown to inhibit respiration through complex II by 47% in isolated mouse hepatocytes, while complex I activity was affected to a lesser extent^{37,38}. A more recent study also demonstrated that NAPQI directly inhibited complex II activity in a concentration-dependent manner, attaining >90% inhibition with concentrations in the μM range³⁹. In spite of these data, however, the quest for the exact source of superoxide within

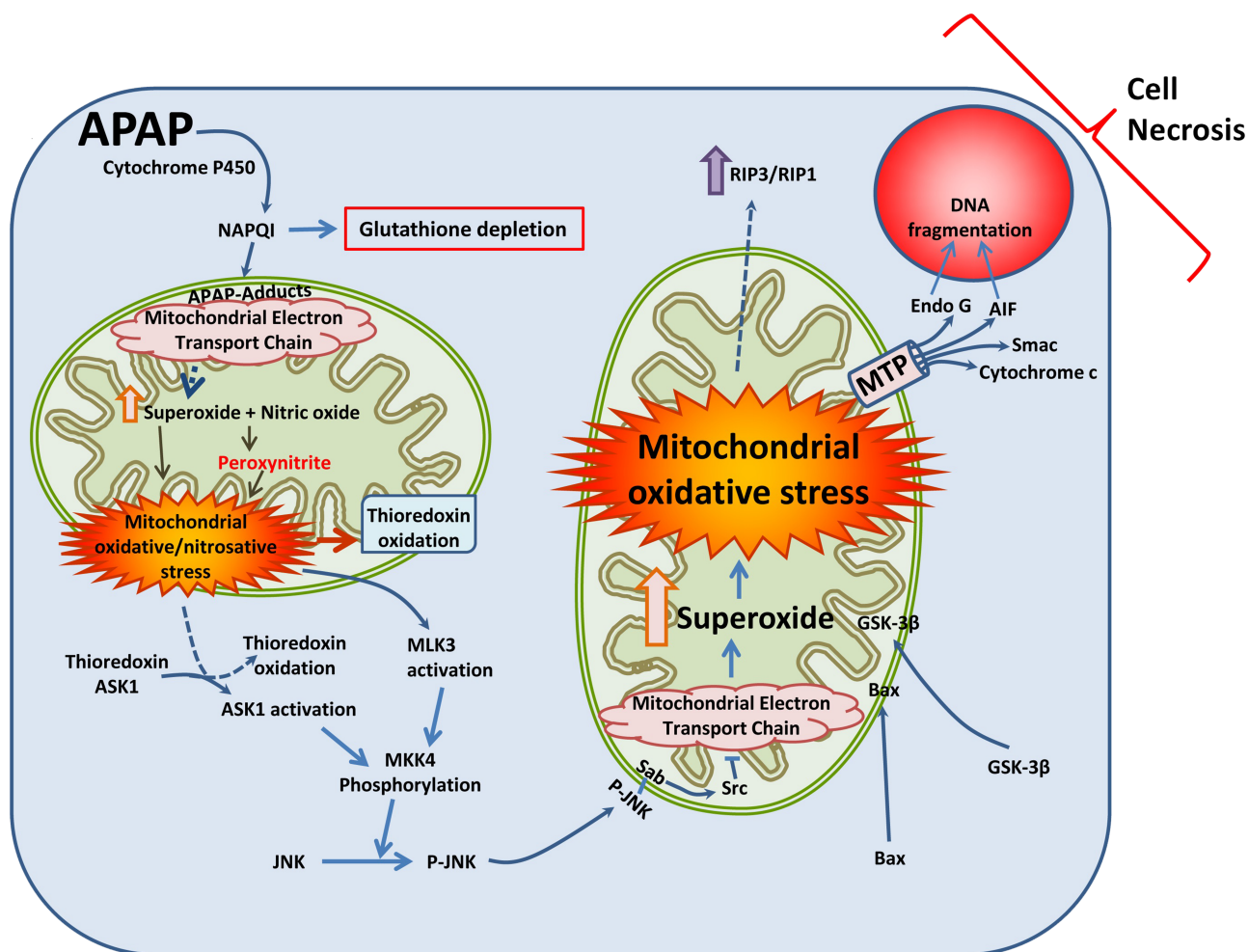


Figure 1. Mechanism of acetaminophen (APAP)-induced hepatocyte cell death. At high concentrations, APAP in hepatocytes is metabolized by components of the cytochrome P450 system to a reactive intermediate, *N*-acetyl-*p*-benzoquinone imine (NAPQI). High concentrations of NAPQI deplete cellular glutathione stores and subsequently form APAP protein adducts, especially on mitochondrial proteins. Components of the electron transport chain such as ATP synthase are affected, which compromises respiratory chain function and enhances generation of free radicals such as superoxide. This reacts with nitric oxide (NO) within the mitochondria to produce highly reactive peroxynitrite, which nitrates mitochondrial proteins such as manganese superoxide dismutase (MnSOD). This compromises mitochondrial antioxidant defenses, causing mitochondrial oxidant stress and oxidation of proteins such as mitochondrial thioredoxin. In the cytosol, oxidation of thioredoxin results in its detachment from its binding partner apoptosis signal-regulating kinase 1 (ASK1), which is then activated. ASK1, along with activated mixed-lineage kinase 3 (MLK3) then activate c-jun N-terminal kinase (JNK) to its phosphorylated form through MKK4 phosphorylation. Phosphorylated JNK translocates to the mitochondria and binds to Sab on the outer mitochondrial membrane, which, through a Src-mediated pathway, further inhibits mitochondrial electron transport. This amplifies mitochondrial oxidant stress, which is further exacerbated by translocation of Bax and glycogen synthase kinase-3 β (GSK-3 β) from the cytosol to the mitochondria. These events activate the mitochondrial permeability transition, which releases mitochondrial intermembrane proteins such as endonuclease G and apoptosis-inducing factor (AIF), along with cytochrome c and Smac. Translocation of AIF and endonuclease G to the nucleus then induces nuclear DNA fragmentation, which along with activation of receptor-interacting protein kinases 3/1 (RIP3/RIP1) finally induce programmed necrosis.

the electron transport chain is still ongoing. Nevertheless, as mentioned earlier, the reaction of superoxide with nitric oxide (NO) and the subsequent generation of peroxynitrite within the mitochondria are critical mediators of APAP-induced mitochondrial dysfunction^{32,40}. While all three nitric oxide synthases (NOSs) have been suggested to be potential sources of NO for this reaction⁴¹⁻⁴³,

current data point toward neuronal NOS (nNOS) as a source. Pharmacological inhibition of nNOS protected hepatocytes in culture against APAP-induced cell death⁴⁴, and mice deficient in nNOS showed less liver injury after an APAP overdose⁴¹. A mitochondrial source for NO has been postulated for a number of years⁴⁵, though the exact identity of this NOS has remained unresolved.

While earlier studies suggested that nNOS is unlikely to be the mitochondrial NOS (mtNOS)⁴⁶, subsequent studies suggest that mtNOS is nNOS or a spliced variant of it (likely nNOS α or nNOS μ), at least in the heart⁴⁷. Hence, it is possible that the source of mitochondrial NO to form peroxynitrite with superoxide from the respiratory chain could be nNOS. Whatever the source of NO, generation of peroxynitrite within the mitochondria results in modification of a number of proteins by nitration of tyrosine residues. When these targets are mitochondrial DNA³² or critical antioxidant enzymes such as superoxide dismutase, whose activity is compromised by nitration after APAP overdose⁴⁸, it can have further cascading consequences. The importance of peroxynitrite in mediating APAP-induced liver injury is also illustrated by the fact that direct scavenging of peroxynitrite by mitochondrial GSH^{49,50} or resveratrol⁵¹ also prevented protein nitration and protected against APAP-induced liver injury.

MITOCHONDRIA AS A SIGNAL INTEGRATION PLATFORM IN APAP HEPATOTOXICITY

In addition to being a source of free radicals which initiate signaling events, the mitochondria also act as signal-integrating platforms, where cytosolic proteins are translocated to amplify damage induced by the initial oxidative stress. This is probably enabling a threshold effect, such that hepatocytes exposed to varying levels of free radicals can respond differently to modulate the liver's functional response to the insult. This paradigm would enable necrosis to be limited to cells exposed to highest levels of APAP around the centrilobular area, sparing cells further away, which could stimulate recovery and regeneration and allow subsequent repopulation and recovery of liver function. Thus, the initial oxidative stress in mitochondria induced by APAP adducts subsequently results in oxidation of thioredoxin (Trx) within the mitochondria⁵², and NAPQI was also shown to modify and inhibit the activity of Trx 1 and 2⁵³ (Fig. 1). In addition, treatment with a recombinant human serum albumin–Trx 1 fusion protein (HSA–Trx) was also shown to protect against APAP-induced liver injury when administered up to 4 h after APAP⁵⁴. The oxidation of Trx 1 causes its detachment from its binding partner apoptosis signal-regulating kinase 1 (ASK1), resulting in ASK1 activation by phosphorylation⁵⁵. Mixed-lineage kinase 3 (MLK3) is another upstream MAPK3 that is activated by oxidant stress during APAP hepatotoxicity⁵⁶. Activated ASK1 and MLK3 phosphorylate MKK4⁵⁷, a MAPK2 kinase, which then phosphorylates c-jun N-terminal kinase (JNK) in the cytosol^{58,59}. The importance of ASK1 in this process is illustrated by the fact that ASK1 knockout mice are protected against APAP-induced activation of JNK⁵⁵. In addition, treatment of

mice with a pharmacological inhibitor of ASK1 also prevented APAP-induced liver injury⁶⁰.

Activation of JNK then initiates a cascading effect, with translocation of phosphorylated JNK to the mitochondrial outer membrane⁵⁸, where it binds with the Sab protein and initiates an Src-mediated inhibition of the electron transport and increases ROS production to amplify mitochondrial dysfunction⁶¹ and peroxynitrite formation⁵⁹. Disruption of the interaction between P-JNK and mitochondria has been shown to be protective against APAP-induced liver injury⁶², illustrating the importance of these steps in APAP-induced programmed necrosis of hepatocytes. In parallel with translocation of activated JNK to the mitochondria, cytosolic Bax also moves to the mitochondria^{63,64}, and this contributes to subsequent release of mitochondrial proteins to the cytosol detailed below, though this has no effect on peroxynitrite formation⁶³. However, Bax-deficient animals, though protected initially from hepatocyte necrosis, succumbed to liver injury at later time points due to the sustained mitochondrial oxidant stress⁶³, suggesting that in the absence of Bax, alternate mechanisms of mitochondrial protein release are activated in the face of sustained oxidant insult. A likely mechanism is that matrix swelling causes rupture of the outer membrane and release of the intermembrane proteins⁶³. Another cytosolic protein translocating to the mitochondria after APAP is the glycogen synthase kinase-3 β (GSK-3 β), which is a major regulator of glycogen synthase but has been shown to regulate other processes, including cell death⁶⁵. GSK-3 β also plays a role in APAP-induced liver injury, since silencing GSK-3 β was shown to attenuate JNK activation and inhibit APAP hepatotoxicity⁶⁵. Ultimately, both JNK and Bax translocation contributes to release of mitochondrial proteins into the cytosol and then their translocation to the nucleus as detailed below, to ultimately result in hepatocyte necrotic cell death (Fig. 1).

RELEASE OF MITOCHONDRIAL PROTEINS AND SUBSEQUENT DNA FRAGMENTATION

Translocation of phosphorylated JNK and Bax to the mitochondria amplifies mitochondrial oxidant stress and peroxynitrite formation and induces opening of the mitochondrial permeability transition pore (MPTP) (Fig. 1). Induction of the MPTP compromises mitochondrial ATP production, depolarizes the mitochondrial membrane, and consequently shuts down mitochondrial function^{66–68}. The MPTP has been suggested to consist of Bax, along with the protein Bak on the mitochondrial outer membrane^{69,70}, with the c-subunit ring of the F₁F₀ ATP synthase⁷¹ being one of the regulatory components within the inner membrane. However, the role of the c-subunit ring has been recently called into question⁷², so further studies may

be needed to confirm the MPTP structure. Another well-characterized regulatory component of the MTP is cyclophilin D⁷³, though its relevance with respect to pore opening induced by APAP seems to depend on the APAP dose. Pharmacological inhibition of cyclophilin D using cyclosporine A provided only temporary protection *in vitro*⁶⁶, but cyclophilin D-deficient mice were protected against liver injury when treated with a moderate APAP overdose of 200 mg/kg⁶⁸. However, no protection was evident when higher doses (600 mg/kg) were used⁷⁴, again suggesting that in the face of sustained mitochondrial insult due to either higher doses or long-term exposure, alternate regulatory molecules are probably recruited to induce downstream features of the cell signaling cascade to ultimately result in hepatocyte necrosis. Another trigger for the induction of the MPTP in the context of APAP-induced liver injury is lysosomal iron, whose translocation to the mitochondria has been shown to occur in mouse hepatocytes treated with APAP, where it then induced opening of the MPTP⁷⁵. This iron release could be due to lysosomal instability, which has been documented after APAP treatment⁷⁶, and lysosomal iron translocation seems to occur through the calcium uniporter, since treatment with either an iron chelator or an inhibitor of the uniporter prevented mitochondrial free radical generation and membrane depolarization⁷⁷. While the induction of the MPTP has been considered to be a catastrophic feature of APAP-induced cell signaling, recent research suggests that this too could be adjusted depending on the dose of APAP, where treatment of animals with a low 150-mg/kg overdose of APAP resulted in transient JNK activation and reversible induction of the MPTP²⁰, from which cells may be able to recover. This suggests that the cellular response to APAP, be it JNK activation, Bax translocation, or induction of the MPTP, can be calibrated to the dose of APAP the cells are exposed to.

Induction of the MPTP by the various stimuli detailed above ultimately results in release of a number of critical mitochondrial proteins into the cytosol, some of which, such as apoptosis-inducing factor (AIF) and endonuclease G, have nuclear localization signals⁷⁸, which result in their translocation to the nucleus⁷⁹. Others, such as cytochrome c, are essential for mitochondrial electron transport, and their loss from the mitochondria further contributes to disruption of ATP production and mitochondrial dysfunction. Endonuclease G cleavage of DNA in the nucleus results in DNA fragmentation indistinguishable from that seen during apoptosis³², and translocation of AIF to the nucleus results in chromatin condensation and DNA fragmentation⁸⁰. Its importance in APAP-induced DNA fragmentation is illustrated by the reduced liver injury seen in partial AIF-deficient mice⁸¹. Recent data also indicate that the extent of mitochondrial protein

release and DNA fragmentation could also dictate severity of liver injury in response to APAP between substrains of the common C57BL/6 mouse species used as an animal model of APAP-induced liver injury⁸².

Thus, mitochondrial protein adduct formation, oxidative stress, and, ultimately, induction of the MPTP are central to APAP-induced liver injury, and it is now evident that the organelle also plays a significant role in the recovery and regeneration process after injury. Recent evidence indicates that activation of mitochondrial biogenesis subsequent to liver injury plays a critical role in recovery after injury, and induction of mitochondrial biogenesis protects against APAP hepatotoxicity⁸³. Another feature of the recovery process is the removal of damaged mitochondria by a form of autophagy termed mitophagy^{84,85}, and inhibition of mitophagy by lysosomal cholesterol accumulation was shown to sensitize mice to APAP hepatotoxicity⁸⁶. From the spatial perspective, timely removal of damaged mitochondria by mitophagy may prevent induction of the cell death cascade, especially in hepatocytes at the border of the necrotic area⁸⁵. Activation of autophagy was shown to protect against APAP-induced hepatotoxicity, and inhibition of autophagy aggravated liver injury⁸⁴. Autophagy was also shown to help in the removal of APAP protein adducts, which may be most critical in protecting cells during long-term APAP treatment⁸⁷. Thus, while mitochondria are critical elements involved in the signaling cascade toward APAP-induced cell death, the organelle also plays an important role in the recovery and regeneration of liver function, especially in hepatocytes away from the central vein at the borders of the area of necrosis⁸³.

HEPATOCYTE CELL DEATH MECHANISMS AFTER APAP TOXICITY

The mode of cell death after APAP-induced liver injury was controversial initially, since a number of mechanistic steps in the process, such as mitochondrial Bax translocation, release of cytochrome c^{64,79,88}, and nuclear DNA fragmentation³², were similar to that seen during apoptosis (Fig. 1). However, a fundamental feature of apoptosis, namely, caspase activation, was absent^{64,89,90}. In addition, APAP-induced cell death has all the characteristics of necrosis both *in vitro* and *in vivo*^{66,91-93}. The lack of protection against liver injury by caspase inhibitors in APAP hepatotoxicity^{89-91,94} provides further evidence of the lack of apoptosis in this context. Furthermore, on closer examination, characteristics of DNA fragmentation are also distinct between apoptosis and APAP-induced necrosis⁹⁵⁻⁹⁷. Part of the reason for the early disregard for necrotic cell death was probably due to the fact that in contrast to apoptosis, necrosis was considered to be an unregulated form of cell death, which occurred once

cellular integrity was compromised due to a multitude of insults. This viewpoint has shifted due to emerging evidence that necrotic cell death can also be regulated, based on the discovery of a number of molecular mediators involved in the process, termed necroptosis⁹⁸. A central feature here is the formation of a multiprotein complex termed the “necrosome,” which includes, among others, the receptor-interacting protein kinases 1 and 3 (RIP1 and RIP3)⁹⁸ and activation of the pseudokinase mixed-lineage kinase domain-like protein (MLKL)⁹⁹. RIP1 and RIP3 form heterodimeric scaffolds in the complex, and while RIP1–RIP1 interactions are dispensable for necroptosis, RIP1–RIP3 or RIP3–RIP3 interactions are required for induction of necroptosis¹⁰⁰. Activated RIP3 phosphorylates MLKL¹⁰¹, and the phosphorylated MLKL translocates to the cell membrane where MLKL interferes with membrane integrity, causing necrotic cell death⁹⁹. RIP3 levels were found to be elevated after APAP overdose in mice^{102,103}, and RIP3 deficiency provided early protection against liver injury, though this was not sustained¹⁰². While RIP1 has also been implicated in APAP-induced liver injury^{56,102,104}, it has been suggested to act independently of the necrosome complex¹⁰⁵. In addition, the fact that MLKL¹⁰⁵ and tumor necrosis factor- α (TNF- α)¹⁰⁶ do not seem to be involved in APAP-induced cell death suggests that APAP-induced cell death should be termed “programmed necrosis” rather than necroptosis¹⁰⁷.

STERILE INFLAMMATION AND APAP HEPATOTOXICITY

The extensive cell necrosis after an APAP overdose leads to release of damage-associated molecular patterns (DAMPs) including mitochondrial DNA, nuclear DNA fragments, high-mobility group box 1 (HMGB1) protein, and many others^{108–110} (Fig. 2). DAMPs bind to pattern recognition receptors such as toll-like receptors (TLRs) on inflammatory cells and transcriptionally activate cytokine formation in inflammatory cells^{111,112}. Some of these inflammatory mediators are constitutively active, for example, TNF- α and interleukin-1 α (IL-1 α), whereas others are generated as a pro-form that requires proteolytic cleavage by caspase 1, for example, IL-1 β and IL-18¹¹¹. DAMPs like ATP can stimulate the purinergic receptor P2X7 on macrophages and activate the Nalp3 inflammasome, which triggers the activation of caspase 1¹¹². The proinflammatory cytokines and chemokines formed can activate neutrophils and monocytes and recruit these cells into the liver where they then may aggravate the existing cell necrosis¹¹². While there is general agreement in the literature that APAP-induced cell necrosis causes DAMP release, proinflammatory mediator formation, and recruitment of inflammatory cells into the liver, it is highly controversial whether this sterile inflammatory

response actually aggravates the injury or is beneficial by removing cell debris and promoting regeneration^{111–113} (Fig. 2).

There are two main areas of controversy. First, it is controversial whether neutrophils, the first responder to the initial cell necrosis, contribute to the injury. The principal support for this hypothesis comes from studies that showed neutropenia being protective against APAP hepatotoxicity¹¹⁴. However, this approach was criticized as prolonged neutropenia causes a preconditioning effect that is protective independent of the actual neutrophils¹¹⁵. In addition, interventions that inactivated neutrophils independent of neutropenia such as CD18 antibodies¹¹⁶, inhibitors of NADPH oxidase¹¹⁷, and use of mice deficient in CD18⁹⁴, intercellular adhesion molecule-1¹¹⁷, or NADPH oxidase¹¹⁸ all were not protective against APAP-induced liver injury. Furthermore, there was neither neutrophil activation nor a neutrophil-mediated oxidant stress observed in the liver during the injury phase^{94,117}. Most importantly, similar results regarding neutrophil activation were obtained in human patients¹¹⁹. Together, the findings strongly argue against a direct involvement of neutrophils in the injury phase.

A second area of controversy is the role of certain proinflammatory mediators, in particular IL-1 β . It was reported that DNA fragments promote pro-IL-1 β formation during APAP hepatotoxicity¹²⁰. In addition, the Nalp3 inflammasome was activated leading to caspase 1 activation and processing of pro-IL-1 β to the active cytokine¹²⁰. The pathophysiological relevance of these mechanisms and IL-1 β was established by the reduced APAP-induced liver injury in TLR9-, Nalp3-, and caspase 1-deficient mice¹²⁰. However, while the effect of TLR9 was confirmed by others¹²¹, the protection in mice deficient in Nalp3 or caspase 1 was not reproducible¹²². In addition, caspase inhibitors prevented IL-1 β formation but did not protect, and adding high doses of exogenous IL-1 β enhanced neutrophil recruitment but did not affect the APAP-induced liver injury¹²³. More recently, another study showed that neither mice deficient in IL-1 β nor treatment with an anti-IL-1 β antibody protected against APAP hepatotoxicity¹²⁴. Interestingly, these authors did not also find a protection in caspase 1- or Nalp3-deficient mice¹²⁴. The very limited IL-1 β formation as reported in mice^{123,124} was also confirmed in APAP overdose patients¹¹¹, suggesting that inflammasome activation and IL-1 β formation are of limited importance for APAP-induced liver injury in mice and humans. However, Zhang and coworkers¹²⁴ suggested a role for IL-1 α in the pathophysiology. IL-1 α is generated by Kupffer cells through TLR4 stimulation but not by TLR9- or TLR3-dependent mechanisms¹²⁴. In their hands, an anti-IL-1 α antibody protected and mice deficient in IL-1 α or the IL-1 receptor experienced less injury after an APAP

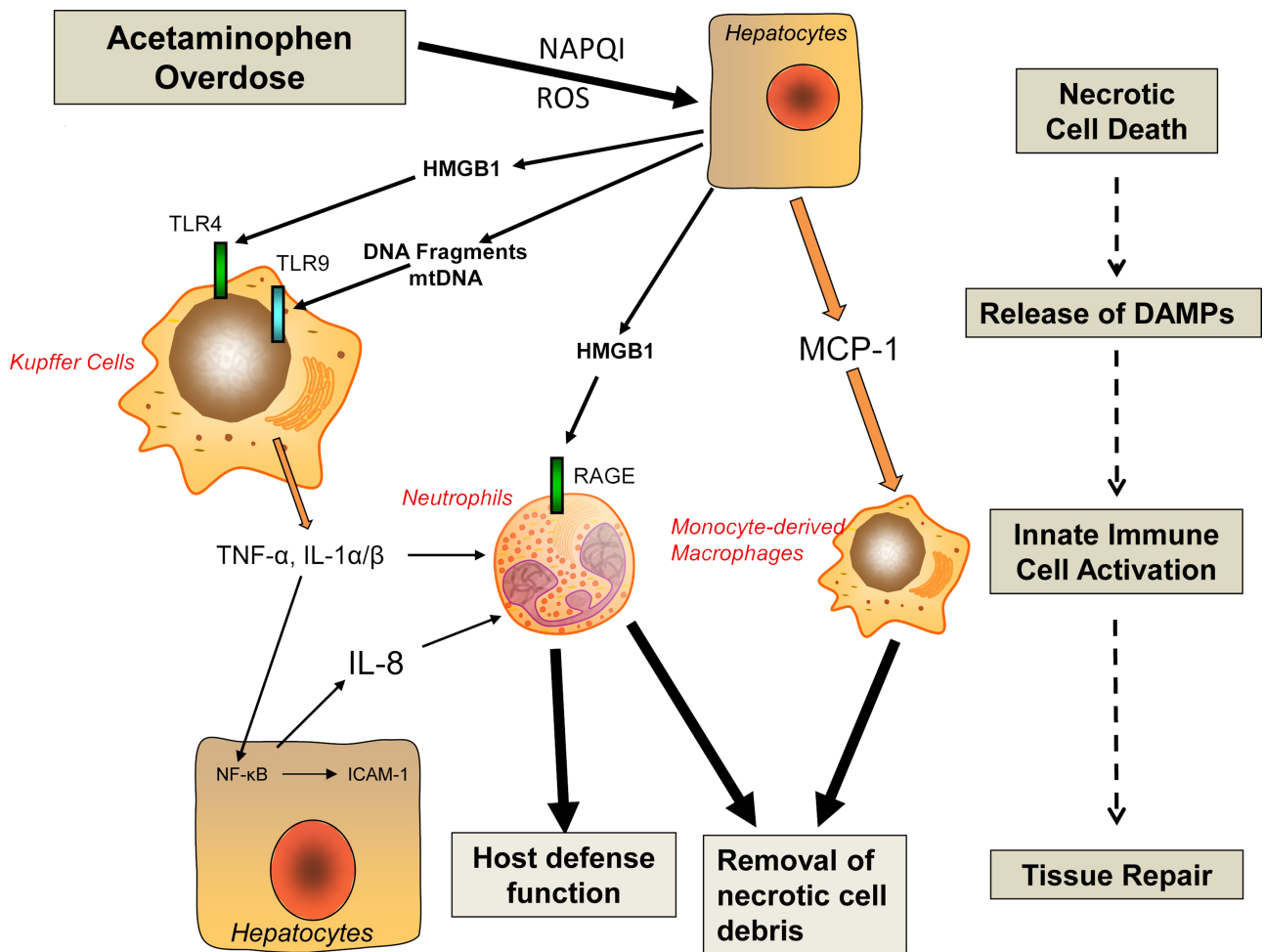


Figure 2. Sterile inflammation and liver regeneration. A sterile inflammatory response is initiated by release of damage-associated molecular patterns (DAMPs) from necrotic cells. DAMPs activate pattern recognition receptors such as toll-like receptors (TLRs), which induces the formation of cytokines and chemokines and the recruitment of inflammatory cells (see text for details). In APAP-induced liver injury, the preponderance of experimental and clinical evidence suggests that this sterile inflammatory response does not aggravate the original injury but causes the removal of necrotic cells and promotes regeneration (see text for details). HMGB1, high-mobility group box 1 protein; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; mtDNA, mitochondrial DNA; RAGE, receptor for advanced glycation end products; ROS, reactive oxygen species.

overdose¹²⁴. By again performing long-term neutropenia experiments, the authors concluded that IL-1 α -activated neutrophils contribute to the injury process¹²⁴. Although these conclusions appeared to be justified based on the reported experiments, it again raised the issue of questionable neutropenia experiments^{111,115}, and the findings contradicted previous data showing that IL-1 receptor-deficient mice were not protected¹²³, that total elimination of Kupffer cells actually enhanced APAP-induced liver injury^{125,126}, and that TLR9^{120,121} and TLR3¹²⁷ are important for the pathophysiology. Thus, there is not only a controversy between investigators who conclude that the sterile inflammatory response after APAP overdose is mainly involved in regeneration and those who believe that it aggravates the initial injury, but there are also

extensive contradictions between reported results and suggested mechanisms among investigators who believe in an inflammatory injury component in APAP-induced hepatotoxicity. Although it is not always obvious why so many contradictory results are being reported, it might be useful to focus on the clinically relevant aspects. In APAP overdose patients, proinflammatory cytokine formation is limited¹²⁸ and neutrophil activation does not occur during the injury phase but more during regeneration¹¹⁹. Consistent with these neutrophil findings, monocyte-derived macrophages that are recruited during APAP hepatotoxicity display a proregenerative phenotype in mice and in humans, suggesting that the inflammatory response is mainly geared toward recovery from the tissue injury¹²⁹⁻¹³¹.

FUTURE PERSPECTIVES

Over the last several decades, significant progress has been made in the understanding of the intracellular signaling mechanisms leading to APAP-induced cell death in hepatocytes in experimental animals and humans. Although more can be learned about various aspects of these mechanisms, it is important to keep in mind the potential effects of intervention strategies on drug metabolism, which can lead to misinterpretations. It is also critical to connect any newly discovered mediators and pathways to the established mechanisms. Furthermore, instead of assuming that a sterile inflammatory has to cause an innate immune cell-mediated injury, intracellular signaling mechanisms need to be more considered as targets for inflammatory mediators. Not only is APAP overdose a clinically relevant model to study hepatocyte cell death and liver injury, but it is also increasingly used to test potential therapeutic intervention strategies. The relevance of the studies will depend on the solid understanding of the toxicity mechanisms.

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