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Acetaminophen Hepatotoxicity: Paradigm for Understanding Mechanisms of Drug-Induced Liver Injury

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Abstract

Acetaminophen (APAP) overdose is the clinically most relevant drug hepatotoxicity in western countries, and, because of translational relevance of animal models, APAP is mechanistically the most studied drug. This review covers intracellular signaling events starting with drug metabolism and the central role of mitochondrial dysfunction involving oxidant stress and peroxynitrite. Mitochondria-derived endonucleases trigger nuclear DNA fragmentation, the point of no return for cell death. In addition, adaptive mechanisms that limit cell death are discussed including autophagy, mitochondrial morphology changes, and biogenesis. Extensive evidence supports oncotic necrosis as the mode of cell death; however, a partial overlap with signaling events of apoptosis, ferroptosis, and pyroptosis is the basis for controversial discussions. Furthermore, an update on sterile inflammation in injury and repair with activation of Kupffer cells, monocyte-derived macrophages, and neutrophils is provided. Understanding these mechanisms of cell death led to discovery of *N*-acetylcysteine and recently fomepizole as effective antidotes against APAP toxicity.

INTRODUCTION AND CLINICAL RELEVANCE

Acetaminophen (APAP) (in the United States and Japan) or paracetamol (in Europe and the rest of the world) is a common, widely available analgesic and antipyretic drug that has been in use for decades (1) and is safe and effective at therapeutic doses (2). However, an overdose of APAP can lead to severe liver injury, which could progress to acute liver failure (ALF) and death (3). Many cases of APAP overdose are due to attempts at self-harm, such that APAP overdose is the number one cause of ALF in the United States and the United Kingdom (4), highlighting the significance of the clinical problem. This persistence of APAP overdose as a major cause of liver injury is also attributed to its presence in many combination medications. This results in individuals overdosing on APAP accidentally without realizing that the drug is a constituent of multiple medications they may be consuming. Such accidental overdoses usually result in more severe injury and are more frequently associated with fatal outcome (5). Epidemiological studies from the United States show that almost half of APAP overdoses are unintentional, largely due to opioid-acetaminophen combinations while attempting better pain relief (6). Since regulatory approaches seem to have limited benefit in blunting the numbers of APAP-induced liver injury cases, there is immense interest in developing therapeutic options to supplement the only US Food and Drug Administration (FDA)-approved antidote, *N*-acetylcysteine (NAC). This requires better mechanistic insight into APAP pathophysiology, and several recent findings highlight nuances in cellular responses to APAP of clinical relevance; these are discussed in subsequent sections.

DRUG METABOLISM AND METABOLIC ACTIVATION OF ACETAMINOPHEN (APAP)

Therapeutic doses of APAP are almost completely metabolized in the liver by glucuronidation and sulfation to nontoxic metabolites and excreted by the kidneys into urine. Glucuronidation is the major metabolic pathway, accounting for up to 70% of the administered therapeutic dose (7). Sulfation accounts for the rest (~30%) of APAP metabolism after a therapeutic dose, and this is mediated by sulfotransferase (SULT) enzymes, which use 3′-phosphoadenosine-5′-phosphosulfate as a donor of the sulfate group transferred to APAP (7). While glucuronidation and sulfation are the predominant modes of metabolism after a therapeutic dose of APAP, a minor component is also metabolized by the cytochrome P450 system to generate an electrophilic metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI), which rapidly reacts with cellular sulfhydryl groups (7). The minor amounts of this reactive metabolite generated under therapeutic doses is efficiently scavenged by hepatic stores of glutathione, which far exceed concentrations of NAPQI generated after therapeutic doses. Hence, provided that glucuronidation and sulfation are active and hepatic glutathione stores are adequately maintained, therapeutic doses of APAP do not produce liver injury since all NAPQI generated is efficiently scavenged. However, the presence of preexisting conditions such as acute viral hepatitis, treatment with antitubercular drugs, or chronic alcoholism could cause liver injury even with therapeutic doses of APAP (8), though there are nuances to be considered in these scenarios (8).

When APAP is taken as an overdose, metabolism through the cytochrome P450 system becomes significantly more important since the rapid generation of large amounts of NAPQI overwhelms hepatic glutathione stores. Subsequently NAPQI reacts with cysteine groups on proteins, forming protein adducts (9). Among the cytochrome P450 enzymes, Cyp2E1 has been identified as the major form involved in metabolism of APAP to NAPQI, though other family members such as 1A2 and 3A4 may play roles under specific circumstances (7). The central role of Cyp2E1 was further established when mice deficient in CYP1A2 were not protected (10), while those lacking Cyp2E1 showed almost complete protection against APAP-induced liver injury (11). While protein adducts can also form after therapeutic doses of APAP, the critical difference

between such benign elevations in adducts and those indicating hepatic injury is the magnitude of elevation, with levels of protein adducts >1 μM generally indicative of APAP-induced toxicity (12). However, a recent report indicated that subjects who had ingested therapeutic doses of APAP displayed APAP-cysteine concentrations as high as 2.8 μM along with alanine aminotransferase (ALT) elevation from ischemic hepatitis (13). Thus, comorbidities could probably influence release of adducts from hepatocytes and modulate absolute concentrations in circulation. In cases where patients present with ALF of unclear causes however, such APAP adduct measurements are useful for definitive diagnosis, especially in the pediatric population (14). Protein adduct formation is required for induction of hepatocyte necrosis and is thus a hallmark of APAP pathophysiology, and adducts on specific organelles rather than specific proteins seem to be critical for hepatocyte cell death. While mitochondrial protein adducts are recognized to cause mitochondrial dysfunction after APAP overdose (15), it is now evident that this response is much more nuanced than earlier appreciated, with the initial response to protein adduct formation being attempts at mitochondrial and cellular adaptation to the stress.

ADAPTIVE MECHANISMS MODULATING TOXICITY

Autophagy

Initial adaptation to the stress of protein adduct formation is an activation of autophagy, which is a cellular process tailored toward degrading modified cellular components after cellular stress to prevent the accumulation of modified molecules, which could endanger cellular survival. Specialized versions of autophagy such as mitophagy also target damaged organelles such as mitochondria, as discussed below. Induction of autophagy is very responsive to cellular stress, and it is initiated by formation of double-membrane phagophores, which gradually elongate to surround the cargo of damaged or modified proteins to form an autophagosome. This autophagosome then fuses with the lysosome to form an autolysosome, allowing the cargo molecules to be degraded by lysosomal enzymes (16).

APAP-induced formation of protein adducts on cellular proteins necessitates their removal for maintenance of cellular homeostasis, and APAP administration was found to increase the number of autophagosomes, especially in the pericentral area (17) where hepatocytes are more susceptible to formation of the reactive metabolite due to increased expression of Cyp2E1 (18). Further analysis also revealed a unique zonated pattern of changes, with the increase in autophagosome formation adjacent to areas of necrosis around the central vein (19) probably being an attempt to restrict progression of necrosis and facilitate liver recovery. APAP protein adducts were also found to localize to autophagosomes and lysosomes in APAP-treated primary hepatocytes (20), suggesting that activation of autophagy was involved in removal of APAP protein adducts. Pharmacological inhibition of autophagy further exacerbated APAP-induced hepatotoxicity, while increasing autophagy protected against APAP-induced liver injury (17, 20). This highlights the essential nature of autophagy for removal of APAP-protein adducts and handling cellular stress after an APAP overdose to protect against cellular injury.

Alterations in Mitochondrial Morphology, Mitophagy, and Mitochondrial Biogenesis

Although several cytosolic proteins undergo adduct formation after excess NAPQI formation, it is formation of adducts on mitochondrial proteins that are central to APAP pathophysiology. This likely occurs when production of NAPQI-mediated generation of protein adducts overwhelms the initial autophagic response to clear them, allowing dysfunctional mitochondria to accumulate. However, it is now becoming clear that several adaptive responses also occur at the mitochondrial

level to try and prevent catastrophic functional failure. A relatively early change detected after exposure to an APAP overdose is an alteration in mitochondrial morphology to a donut-like topology in response to a decrease in mitochondrial membrane potential without compromised respiratory chain function (21). This is a reversible process once APAP is removed, and it has been reported that the donut topology maintains mitochondrial membrane potential and helps mitochondria resist autophagy (8). Thus, the second level of adaptation to formation of APAP-protein adducts once autophagic removal has been overwhelmed seems to be at the level of the mitochondria, which attempt to alter morphology and maintain respiratory function to tide over the functional deficits caused by mitochondrial protein adduct formation. Once mitochondria are exposed to persistent NAPQI generation and formation of protein adducts, it ultimately results in severe compromise of mitochondrial function (8), as detailed in subsequent sections. These defective organelles are then targeted for replacement through PARKIN-mediated mitophagy (22), which has been observed after APAP overdose (17).

Thus, it is now evident that the initial response to excessive protein adduct formation after an APAP overdose is the robust induction of autophagic pathways to rapidly remove cellular protein adducts to mitigate the detrimental effects on cellular function. This could be occurring through secretory autophagic pathways (23), since exosomes released from hepatocytes after toxic exposure to APAP did not have appreciable APAP-protein adducts (24). This secretion would explain the presence of circulating protein adducts in humans after a therapeutic dose (25), which would indicate robust activation of the autophagic process to expel protein adducts from hepatocytes. This was experimentally proven in mice given multiple therapeutic doses of APAP, which typically produce no injury but are hepatotoxic when autophagy is inhibited (26). While the effects of defective autophagy in human drug-induced liver injury (DILI) are not well characterized, polymorphisms in autophagy-related genes have been shown to be prevalent in patients with other liver disorders such as nonalcoholic fatty liver disease (27). Thus, it is probable that such defects would significantly increase susceptibility to liver injury in the context of an APAP overdose in humans.

An additional adaptive mechanism relevant to APAP pathophysiology is mitochondrial biogenesis, which is a spatially distinct response in specific hepatocyte populations. APAP-induced hepatocyte necrosis is centrilobular, with cells surrounding the central vein being most susceptible due to increased expression of cytochrome P450 2E1 and lower glutathione stores (18). Hence, surviving hepatocytes surrounding areas of necrosis are critical for liver recovery since these cells are important for hepatocyte regeneration to repopulate areas of necrosis. Mitochondrial biogenesis is important for recovery after APAP-induced liver injury (28–30), and this was found to occur selectively in the surviving hepatocytes around areas of necrosis (31). The importance of mitochondrial biogenesis for recovery after APAP-induced liver injury is also illustrated by the enhanced liver recovery and regeneration after pharmacological activation of mitochondrial biogenesis (31).

Early Mitochondrial Superoxide Generation

The formation of mitochondrial APAP-protein adducts results in early superoxide formation from mitochondrial respiratory complex III, which is directed away from the mitochondrial matrix and inner membrane toward the intermembrane space and cytosol, preserving mitochondrial respiratory chain function (8, 32). Antioxidant systems such as manganese superoxide dismutase (MnSOD) would scavenge any free radicals leaking toward the mitochondrial matrix and hence preserve functional integrity of mitochondria. However, this directed superoxide generation into the cytosol induces a mild oxidant stress in that compartment, which triggers activation of redox

sensitive kinases such as apoptosis-signal-regulating kinase 1 (ASK1) (33). This activates additional kinases within the cytosol such as mitogen-activated protein kinase kinase 4 (MKK4) to ultimately cause activation and phosphorylation of c-Jun N-terminal kinase (JNK) (34). Once activated in the cytosol, phosphorylated JNK (p-JNK) translocates to the mitochondrial outer membrane and binds to the protein Sab (35).

The release of mitochondrial superoxide into the cytosol additionally activates glycogen synthase kinase-3β (GSK3β), which also translocates to the mitochondria (36), and silencing GSK3β was found to significantly reduce JNK activation and protect mice against APAP hepatotoxicity (36). This indicates that GSK3β activation was upstream of JNK, since suppressing phosphorylation of GSK3β and the upstream kinase MKK4 in mice protected against APAP hepatotoxicity with blunted JNK phosphorylation (37). Activation of JNK in the cytosol also results in phosphorylation of the cytosolic 14–3–3 proteins, which tether Bax in the cytosol (38), resulting in detachment of Bax and its translocation to the mitochondria (39), which occurs along a similar time course as JNK after an APAP overdose (40).

Antioxidant Responses and Glutathione Recovery

The mild oxidative stress induced by release of mitochondrial superoxide into the cytosol activates several cellular antioxidant responses including the recovery of cellular glutathione depleted by scavenging of NAPQI. The predominant mechanism involved in glutathione recovery is likely activation of the transcription factor nuclear erythroid 2 p45-related factor 2 (Nrf2), which is sequestered in the cytosol by its binding partner kelch-like ECH associating protein 1 (Keap1) under baseline conditions but dissociates from it during oxidative stress due to modification of cysteine residues (41). Nrf2 then translocates to the nucleus, where it binds the antioxidant response element (ARE), activating a series of genes involved in the cellular antioxidant response (42). An APAP overdose was shown to initiate nuclear translocation of Nrf2 in vivo, with maximum levels by 1 h, in a dose-dependent manner, even at doses below those causing overt liver damage (43). Interestingly, glutathione (GSH) depletion alone seemed insufficient for Nrf2 activation, suggesting the involvement of direct interactions such as modifications of Nrf2 or Keap1 (41, 43). Lending credence to the direct activation, it was also shown that NAPQI could directly activate the Nrf2 pathway in mouse liver cells, inducing an adaptive defense response (44). The importance of Nrf2-mediated pathways is highlighted by the high sensitivity of Nrf2 knockout (KO) mice to APAP hepatotoxicity, which was associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes (45). The lack of glutathione recovery due to a decrease in gene expression of glutathione resynthesis enzymes in Nrf2-deficient mice contributed to this increased susceptibility (45). The relationship of Nrf2 to Keap1 binding is also reiterated by the resistance to APAP hepatotoxicity in mice with hepatocyte-specific disruption of the *Keap1* gene, which had enhanced nuclear accumulation of Nrf2 (46). Upregulation of APAP scavenging mechanisms could also protect mice overexpressing hepatitis C virus structural proteins (47), and Nrf2-mediated induction of Mrp3 and Mrp4 could facilitate regulation of detoxification after APAP overdose to limit toxicity (48). Recent evidence also indicates that activated JNK interacts directly with GCLC, a critical enzyme involved in glutathione resynthesis, causing its proteolytic degradation to impair GSH recovery after APAP overdose and allow continued activation of JNK (49). This would then further amplify the mitochondrial oxidant stress. Thus, the initial cellular response to an APAP overdose seems to be the activation of a multitude of adaptive pathways, which attempt to circumvent critical mitochondrial dysfunction and maintain cellular function (**Figure 1**). However, these mechanisms are overwhelmed in the face of persistent NAPQI formation at higher APAP doses, which ultimately exacerbates mitochondrial injury.

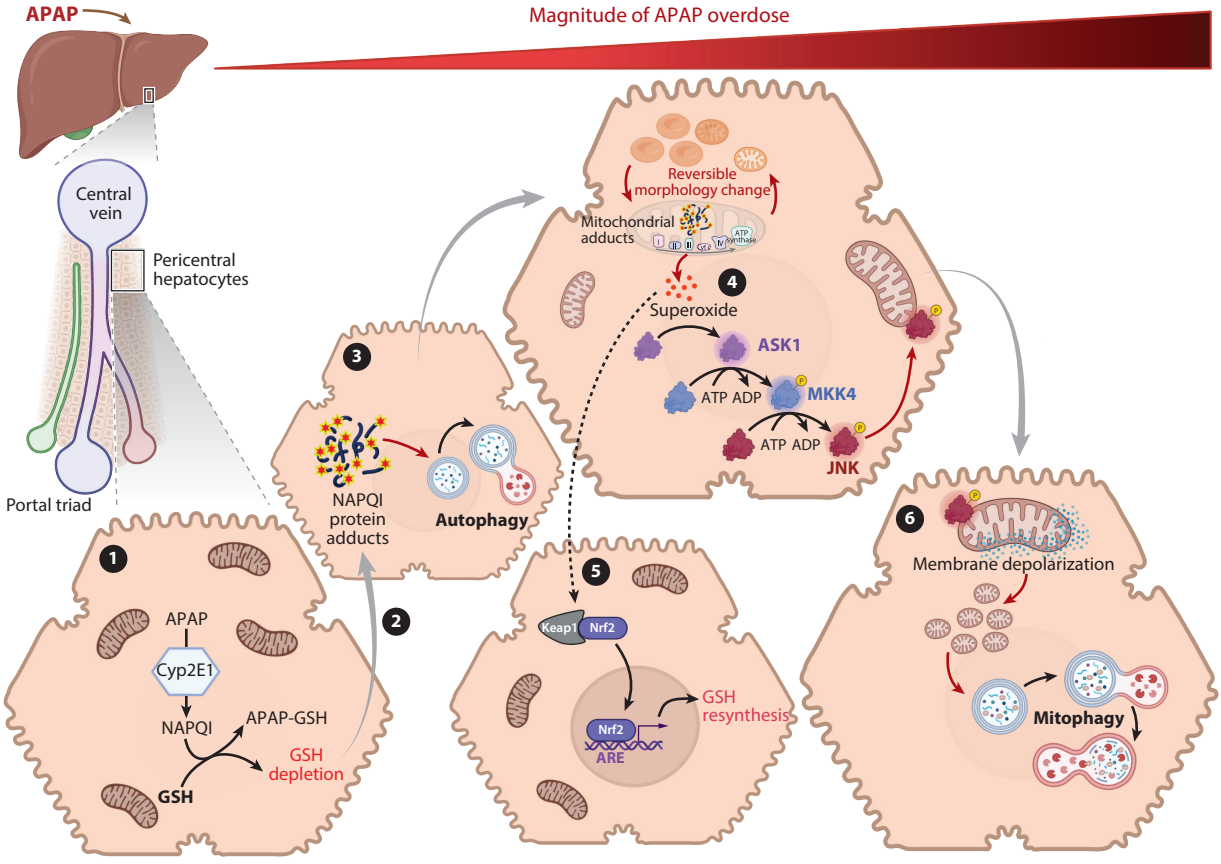

Figure 1

Adaptive responses after APAP overdose. APAP hepatotoxicity after an overdose mainly affects (①) centrilobular hepatocytes where Cyp2E1 generates the reactive metabolite NAPQI, which is scavenged by hepatic glutathione stores. This results in a depletion of glutathione, which (②) allows formation of NAPQI protein adducts. (③) This molecular modification activates autophagic pathways, which remove adducted proteins and attempt to limit cellular damage. (④) When this adaptive pathway is overwhelmed, NAPQI adducts on mitochondrial proteins induce directional release of superoxide into the cytosol from respiratory complex III, which activates a MAP kinase cascade culminating in the activation of JNK and its mitochondrial translocation. This is accompanied by a decrease in mitochondrial membrane potential, which induces a reversible change in mitochondrial morphology. (⑤) The mild cytosolic oxidant stress also likely activates antioxidant responses mediated by Nrf2, which detaches from its binding partner Keap1 to translocate to the nucleus and induce transcription of genes involved in glutathione resynthesis after binding to the ARE. (⑥) Mitochondrial JNK translocation also induces mitochondrial membrane depolarization and induction of mitophagy, which attempts to remove damaged organelles and maintains cellular function. Red arrows illustrate critical steps within the cell signaling cascade. The dashed arrow indicates that the relationship may not be direct and includes multiple steps that are not discussed. Abbreviations: APAP, acetaminophen; ARE, antioxidant response element; ASK1, apoptosis-signal-regulating kinase 1; cyt c, cytochrome complex; Cyp2E1, cytochrome P450 2E1; GSH, glutathione; JNK, c-Jun N-terminal kinase; Keap1, kelch-like ECH associating protein 1; MKK4, mitogen-activated protein kinase kinase 4; NAPQI, *N*-acetyl-*p*-benzoquinone imine; Nrf2, nuclear erythroid 2 p45-related factor 2. Figure adapted from images created with BioRender.com.

MITOCHONDRIAL AMPLIFICATION OF OXIDATIVE AND NITROSATIVE STRESS

Once phosphorylated and activated in the cytosol, p-JNK translocates to the outer mitochondrial membrane and binds to the scaffold protein Sab (50). In parallel, GSK3β moves to the

mitochondrial outer membrane and likely interacts with VDAC2, as has been demonstrated in cardiomyocytes exposed to oxidative stress (51), but Bax likely inserts directly into the mitochondrial outer membrane (52). Binding and phosphorylation of Sab by p-JNK on the outer mitochondrial membrane leads to an SHP1- and DOK4-dependent inactivation of p-Src on the inner membrane, which in turn inhibits electron transport and increases reactive oxygen species (ROS) release (50). Thus, in contrast to the earliest release of mitochondrial superoxide into the cytosol, which seems to be from respiratory complex III, the subsequent mitochondrial oxidant stress due to JNK translocation seems to be predominantly from respiratory complex I, since treatment with metformin showed significant inhibition of complex I activity, accompanied by an attenuation of mitochondrial oxidant stress and subsequent mitochondrial dysfunction, protecting against APAP hepatotoxicity (53).

Thus, the major consequence of the translocation of JNK and proteins such as GSK3β onto the mitochondria is an inhibition of the respiratory chain and enhanced production of superoxide from the organelle (54). This is distinct from the earlier spatially directed superoxide formation, which spared mitochondrial respiration. The critical nature of mitochondrial superoxide generation is illustrated by the effects seen in mice where mitochondrial superoxide handling is compromised. Mice heterozygous for SOD2 deficiency showed fourfold higher ALT activities and necrosis, along with enhancement of all parameters of mitochondrial oxidant stress and more prolonged JNK activation (55). Animals overexpressing SOD or plasma glutathione peroxidase demonstrated dramatic resistance to APAP toxicity (56), and adenoviral extracellular *SOD* gene transfer significantly attenuated release of liver enzymes and inhibited necrosis after APAP overdose (57). The targeted mitochondrial SOD mimetic Mito-TEMPO also protects against APAP overdose in mice (58) by attenuating the mitochondrial oxidant stress, peroxynitrite formation, and subsequent mitochondrial dysfunction (59). Adaptive modulation of mitochondrial bioenergetics by upregulation of mitochondrial uncoupling proteins such as UCP2 can also prevent mitochondrial dysfunction despite JNK translocation (60).

Despite excessive superoxide formation, mitochondrial antioxidant defenses typically would prevent catastrophic mitochondrial dysfunction. However, it is now evident that the reaction of superoxide radicals generated after mitochondrial JNK translocation with nitric oxide (NO) to form the highly reactive peroxynitrite are the cause for irreversible damage of mitochondrial function (61–63). It should be noted that the early release of superoxide into the cytosol is not accompanied by peroxynitrite formation (32), unlike that seen after mitochondrial JNK translocation, when mitochondrial respiration is inhibited. This formation of peroxynitrite is aided by nitration and inhibition of MnSOD activity (64), which presumably allows greater availability of superoxide for preferential reaction with NO, enhancing peroxynitrite formation and causing mitochondrial DNA damage and dysfunction (61). One possible candidate contributing NO for peroxynitrite formation is neuronal nitric oxide synthase (nNOS), since its pharmacological inhibition prevented early cell death after APAP exposure in freshly isolated mouse hepatocytes (65). However, this protection does not seem to be sustained long term since nNOS deficiency in vivo only delayed induction of liver injury after APAP overdose without influencing metabolism (66). Thus, it is possible that alternate sources of NO could be involved in contributing to peroxynitrite formation, but this is an area that needs additional investigation. In any case, the importance of peroxynitrite in mediating mitochondrial dysfunction and ultimately hepatocyte necrosis is illustrated by the protection when the radical is scavenged by mitochondrial glutathione (63, 67) or resveratrol (68) or prevented from forming by accelerated superoxide dismutation (58, 59). Additional mechanistic clarity on the damage due to peroxynitrite formation was revealed by recent studies implicating cellular iron in the formation of nitrotyrosine adducts (69), which is discussed in detail in the section on ferroptosis below.

MITOCHONDRIAL PERMEABILITY TRANSITION AND ITS CONSEQUENCES

Persistent peroxynitrite formation coupled with loss of mitochondrial membrane potential ultimately activates the mitochondrial permeability transition (70–72), which is mediated by a calcium-activated megachannel whose exact identity is still being worked out (73). Current candidates for the mitochondrial permeability transition pore (MPTP) include subunits of ATP synthase and the adenine nucleotide translocase (ANT) on the mitochondrial inner membrane (73). Despite the uncertainty on the exact molecular makeup of the MPTP, there is consensus that an important regulator of the pore is cyclophilin D, which has been shown to bind to both ATP synthase subunits as well as ANT and regulate pore opening (74). Though long-term opening of the MPTP had been implicated in mitochondrial dysfunction and cell injury, it is now evident that transient openings or flickering behavior of the pore are also of physiological significance (73). This is relevant to APAP-induced mitochondrial alterations since it has been shown that modulation of the MPTP has transient effects as demonstrated by the early protection against MPTP by inhibition of cyclophilin D, which, however, was overcome at later time points after APAP overdose (70). Effects on the MPTP were also dependent on dose of APAP since transient MPTP opening was evident after a low 150-mg/kg dose, in contrast to irreversible induction at 300 mg/kg (15). Mice deficient in cyclophilin D were also protected against liver injury at a dose of 200 mg/kg (72), but not after a severe APAP overdose of 600 mg/kg (75). JNK residency on the mitochondria is transient, typically peaking at 6 h and decreasing by 8 h and essentially disappearing by 24 h after a 300-mg/kg dose (76). Hence, accelerating JNK inactivation by dephosphorylation can also modulate downstream events such as induction of the MPTP. In support of this concept, it was demonstrated that mice deficient in mitogen-activated protein kinase phosphatase 1 (MKP-1), which inactivates JNK, show exacerbated liver injury and sustained JNK activation after APAP overdose (77). Activation of the MPTP is accompanied by mitochondrial fission after APAP overdose, mediated by induction of the canonical fission protein Drp1 (dynamin-related protein 1) and its translocation to mitochondria (78). Mitochondrial fission seems to be contingent on JNK activation and its mitochondrial translocation since deficiency of Sab, its mitochondrial binding partner, prevents mitochondrial translocation of Drp1 (79). Mitochondrial fission can also be influenced by enhanced mitochondrial iron (80) as seen after toxic APAP exposure. Recent evidence also indicates that mitochondrial fission can influence induction of the MPTP since inhibition of fission by modulation of Drp1 attenuates the MPTP (81).

Persistent induction of the MPTP after APAP overdose ultimately results in mitochondrial swelling and release of several mitochondrial intermembrane proteins into the cytosol. These include cytochrome c, Smac (second mitochondria-derived activator of caspase), endonuclease G, and apoptosis inducing factor (AIF). Interestingly, apoptosis is not a major mechanism of cell death due to APAP despite the release of these mitochondrial proteins, which also play a role in apoptosis, as discussed in subsequent sections. This is probably because nitrotyrosine modification of these mitochondrial proteins may impede their ability to function as proapoptotic molecules. In fact, tyrosine nitration of cytochrome c has been shown to block its ability to function in the apoptotic cascade and activate caspase 9 (82), which may then render the release of Smac to be inconsequential. Moreover, several nonapoptotic roles have been identified for Smac (83), suggesting that the proapoptotic roles of these proteins become relevant only when a multitude of signals induce them along that pathway, which is not the case after APAP overdose. The release of endonuclease G and AIF, on the other hand, have significant consequences in APAP pathophysiology due to the presence of nuclear localization signals (84), which promote their movement to the nucleus once released from the mitochondria (85). Endonuclease G then cleaves nuclear DNA, resulting in DNA fragmentation (61), while AIF facilitates DNA fragmentation and chromatin condensation

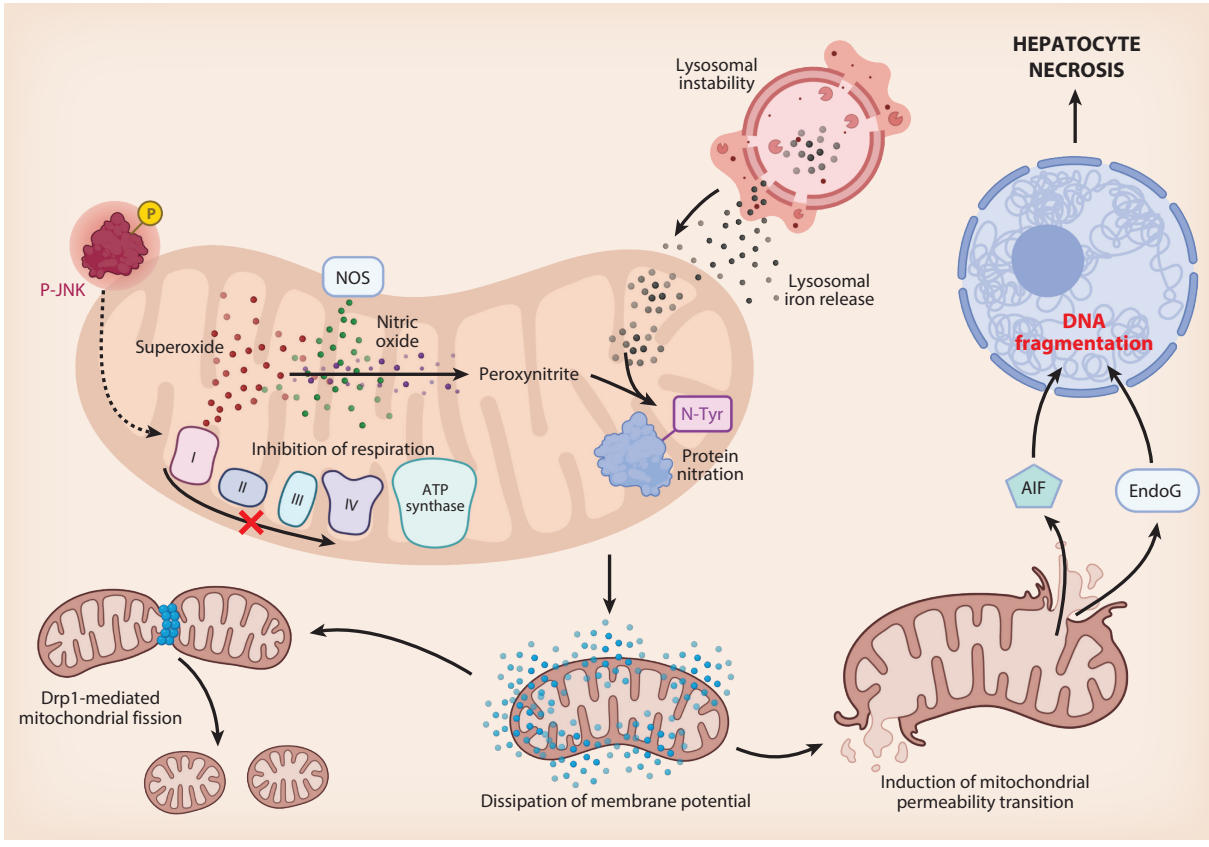

Figure 2

Mitochondrial JNK translocation amplifies mitochondrial dysfunction. Persistent JNK activation and translocation to the mitochondria inhibits mitochondrial electron transport and respiration, causing release of superoxide from respiratory complex I, which affects the mitochondrial matrix proteins unlike the earlier superoxide generation from complex III. Reaction of superoxide with nitric oxide generates highly reactive peroxynitrite, which modifies mitochondrial proteins by tyrosine nitration, a process requiring that lysosomal iron be taken up into the mitochondria. The formation of nitrotyrosine on mitochondrial proteins ultimately causes complete dissipation of mitochondrial membrane potential and induction of Drp1-mediated mitochondrial fission. Ultimately, activation of the mitochondrial permeability transition results in release of EndoG and AIF, which translocate to the nucleus, causing DNA fragmentation and ultimately hepatocyte necrosis. The dashed arrow indicates that there are multiple steps that are not depicted. Abbreviations: AIF, apoptosis inducing factor; Drp1, dynamin-related protein 1; EndoG, endonuclease G; JNK, c-Jun N-terminal kinase; NOS, nitric oxide synthase; N-Tyr, 3-nitro-L-tyrosine; p-JNK, phosphorylated JNK. Figure adapted from images created with BioRender.com.

(86). AIF release and nuclear translocation seems essential for APAP-induced cell death since mice with partial AIF deficiency were protected against APAP-induced DNA fragmentation and liver injury (87). Extensive DNA fragmentation is considered the point of no return for APAP-induced cell death (88) (**Figure 2**).

MODES OF CELL DEATH IN ACETAMINOPHEN HEPATOTOXICITY

Oncotic Necrosis or Programmed Necrosis

The mode of APAP-induced cell death was a controversial topic for the last several decades. Before the 1990s, it was necrosis or oncotic necrosis characterized by cell and organelle swelling,

karyorrhexis, karyolysis, cell content release, and inflammation. The early hypothesis was that cell death was triggered by a catastrophic event such as massive lipid peroxidation (LPO) or collapse of ion homeostasis with extensive Ca^{2+} accumulation. However, when more details of the intracellular signaling events became known, the term programmed necrosis was introduced (89, 90). This reflects more accurately the morphological characteristics and acknowledges that this is not caused by a random catastrophic accident but rather that the stress involves very distinct intracellular signaling pathways (91). In addition, cells can adapt to such a stress by upregulation of defense genes through, for example, Nrf2 activation or enhanced removal of damaged proteins and organelles by autophagy as discussed above. Thus, although not entirely occurring through predetermined signaling pathways, the stress caused by an APAP overdose distinctly disturbs the normal functioning of the cell, eventually leading to cell death.

Apoptosis

Only a few studies initially hypothesized that APAP-induced liver injury involved apoptosis (92). However, the validity of these studies was questioned because there was no morphological evidence of cell shrinkage and formation of apoptotic bodies (93). In addition, no relevant caspase activation (increased enzyme activity; procaspase cleavage) was detectable (93, 94). Most importantly, highly effective pancaspase inhibitors did not protect against APAP toxicity (93, 94). It was also demonstrated that the DNA fragmentation during APAP hepatotoxicity, as indicated by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay, DNA ladder, and antihistone ELISA (enzyme-linked immunoassay) (61), was caused by mitochondria-derived endonucleases that translocated to the nucleus (61, 85, 87) and not caspase-activated deoxyribonuclease (61). Although other intermembrane proteins such as cytochrome c and Smac/DIABLO (direct inhibitor of apoptosis-binding protein with low pI) are also released during this process (40), there is no evidence of caspase activation (93). Importantly, this applies not only to mice but also to humans (95) and human hepatocytes (96).

Ferroptosis

One form of programmed necrosis that came recently into focus is ferroptosis. It is characterized by iron-dependent LPO, which is facilitated by depletion of GSH and inhibition of glutathione peroxidase 4 (GPx4) activity due to cosubstrate loss (97). The latter enzyme is responsible for reduction of lipid hydroperoxides using reducing equivalents from GSH, thereby interrupting the free radical chain of LPO. Although the term ferroptosis is relatively new, the underlying mechanism of LPO and iron was investigated more than 40 years ago. At the time, Wendel & Feuerstein (98) hypothesized that massive LPO was the main mechanism of cell death after APAP overdose with reactive oxygen derived from cytochrome P450–dependent metabolism as the initiating stress. In these experiments, LPO parameters (ethane, pentane) and liver injury were amplified by vitamin E deficiency and a diet high in unsaturated fatty acids (98). However, subsequent studies in animals kept on a regular diet did not confirm the extent of LPO after a dose of 300–400 mg/kg of APAP, which still triggered extensive centrilobular necrosis but no mortality between 6 and 24 h after APAP administration (99, 100); not unexpectedly, pretreatment that increased the hepatic vitamin E levels by 700% did not protect against APAP-induced hepatocellular necrosis (100). Instead of LPO, it was shown that the intracellular oxidant stress led to peroxynitrite formation in the mitochondria, which triggered the MPTP opening and cellular necrosis (61).

The role of iron in the pathophysiology of APAP hepatotoxicity was controversial. Recently, it was recognized that APAP overdose causes lysosomal instability and that lysosomal iron contributes to the MPTP opening and cell death (101). The iron uptake into the mitochondria is facilitated by the mitochondrial electrogenic Ca^{2+}, Fe^{2+} uniporter (102, 103). However, the role of

iron in catalyzing the Fenton reaction as an initiating event for LPO appeared to contradict other observations such as very limited LPO and a prominent role of peroxynitrite in the mechanisms of cell death. This controversy was solved when it was recognized that peroxynitrite-mediated protein nitration requires a reduced metal ion such as Fe^{2+} (104). The iron chelator deferoxamine and the inhibitor of the Ca^{2+}, Fe^{2+} uniporter, minocycline, prevented nitrotyrosine protein adduct formation and protected against APAP-induced liver injury without effect on LPO (69). This suggested that after an APAP overdose, lysosomal iron translocates to the mitochondria and catalyzes the formation of nitrotyrosine protein adducts, which trigger the MPTP opening and cell necrosis independent of LPO. However, in case of an iron overload with APAP, the iron can facilitate nitrotyrosine formation and LPO, with both mechanisms contributing to cell death (69). Thus, under normal circumstances, APAP-induced cell death does not involve ferroptosis. However, in the case of iron overload or severe impairment of antioxidant defense mechanisms, LPO may become a contributing factor.

Necroptosis

The important characteristics of necroptosis include the activation of receptor interacting protein kinase 1 (RIPK1) and RIPK3 leading to phosphorylation of mixed lineage kinase domain-like (MLKL), which then translocates to the cell membrane and forms pores leading to cell lysis (105). Although RIPK1 and RIPK3 activation were shown during APAP toxicity and deletion/inhibition of these kinases proved to be at least in part protective (78, 79), MLKL KO mice were not protected (79). Thus, these data do not support the conclusion that necroptosis is a critical mode of cell death in APAP toxicity.

Pyroptosis

More recently, pyroptosis was suggested as the main mode of cell death during APAP hepatotoxicity (106). The key characteristics of pyroptosis include the activation of the Nlp3 (NOD-, LRR-, and pyrin domain-containing protein 3) inflammasome leading to formation of the active caspase-1, which cleaves the proforms of interleukin 1β (IL-1β) and IL-18 to the active cytokine and also cleaves gasdermin D (GSDMD) (107). The N-terminal cleavage product of gasdermin D ($GSDMD^{NT}$) translocates to the cell membrane and forms a pore, which is used to release IL-1β and IL-18 from the cell. However, these GSDMD pores can progress to cause cell lysis with release of cell contents (108). Although some studies provide evidence for these events to be present to various degrees in the liver after APAP overdose (106), critical experiments did not support the conclusion that this cell death is pyroptosis. First, pancaspase inhibitors, which also block caspase 1, did not protect against cell death (109). Second, IL-1β formation is minimal, that is, well below levels that can affect the injury in mice (109) or humans (110). Third, GSDMD and gasdermin E KO mice are not protected against cell death (111, 112). Fourth, the expression of proteins needed to cause pyroptosis are predominantly located in liver macrophages not in hepatocytes, which are the main target of APAP toxicity (113).

In summary, there is strong morphological evidence for necrosis and there are distinct signaling events leading to oncotic necrosis or programmed necrosis after APAP overdose in mice, humans, and their respective hepatocytes. Although there is partial overlap in signaling pathways to other modes of cell death including apoptosis, necroptosis, ferroptosis, and even pyroptosis, APAP-induced cell death does not fulfill all the key characteristics of any of these different modes of cell death. Most of the conclusions in the literature regarding these modes of cell death are based on correlations with unspecific parameters and not solid causality assessment. Thus, there is no credible evidence for any other cell death mode than oncotic necrosis or alternatively termed programmed necrosis after an APAP overdose (89, 90).

STERILE INFLAMMATION AFTER APAP-INDUCED NECROSIS

Cytokine and Chemokine Formation

It is well established that the extensive necrosis caused by an APAP overdose triggers a sterile inflammatory response with activation of the resident macrophages (Kupffer cells) and the recruitment of neutrophils and monocyte-derived macrophages as the main infiltrating cells. Necrotic cells release damage-associated molecular patterns (DAMPs), which include high mobility group box 1 protein (HMGB1), mitochondrial and nuclear DNA, ATP, and many more in both animals and humans (95, 114, 115). DAMPs can bind to pattern recognition receptors such as Toll-like receptors (TLRs) (115, 116). Specifically, there is evidence for a role of TLR9 (receptor for DNA fragments) (117) and TLR4 (receptor for HMGB1) (118) in the pathophysiology although other receptors such as receptor for advanced glycation end-products (RAGE) can also recognize HMGB1 (119). Binding of DAMPs to TLRs mainly on macrophages results in the transcriptional activation of cytokine and chemokine gene expression. Although most of these inflammatory mediators are directly released into the circulation after formation, there are a few cytokines that are generated as an inactive proform, for example, IL-1β and IL-18. These cytokines require proteolytic cleavage of the proform by caspase-1 to generate the active cytokine (115). Generation of the active caspase-1 involves the stimulation of purinergic receptor P2X7 by binding of ATP, which then triggers the assembly of the Nlp3 inflammasome complex consisting of Nlp3, CAD, and procaspase-1 (120). The active caspase-1 also cleaves GSDMD to generate N-terminal fragments, which translocate to the cell membrane and form a pore through which active IL-1β and IL-18 can be released from the cell (108). Interestingly, there is a lot of emphasis on primary cytokines such as tumor necrosis factor alpha (TNF-α), IL-1α, and IL-1β in the literature (117, 121); however, the plasma levels of these cytokines in both mice (109, 121, 122) and humans (110) are only modestly elevated after APAP overdose, which raises the question of whether this limited increase can have a relevant impact on the pathophysiology (109). In contrast, there is substantial activation of complement and the chemotactic chemokines for neutrophils [CXCL2/MIP-2 (C-X-C motif chemokine ligand 2/macrophage inflammatory protein 2)] and monocytes [CCL2/MCP-1 (chemokine ligand 2/monocyte chemotactic protein 1)] in mice (123–125) and in humans (110). This would indicate that these latter inflammatory mediators are more relevant for the recruitment of neutrophils and monocyte-derived macrophages and their impact on injury and repair.

Role of Neutrophils in APAP Hepatotoxicity

As a cell type of the innate immune system that can respond quickly to any threat anywhere in the body, neutrophils are highly mobile and have the capacity to phagocytose, kill, and digest any infectious agent. Neutrophils also respond to necrotic cell death, and, although the main purpose of a sterile inflammatory response is to clean up the cell debris and assist in the repair process, neutrophils can aggravate the existing injury as was shown in cases of hepatic ischemia-reperfusion injury, endotoxemia, and obstructive cholestasis (126). This potential for a dual function of neutrophils led to a long-term controversy regarding the role of these inflammatory cells in APAP hepatotoxicity. As reviewed in detail elsewhere (115, 127, 128), the preponderance of experimental evidence using direct interventions against neutrophil functions indicates that neutrophils do not contribute to APAP-induced liver injury after moderate overdoses. However, some conclusions claiming a role of neutrophils after a moderate overdose are based on interventions with off-target effects or are just consequences of reduced injury (115, 127, 128).

Most studies using the APAP model in the mouse use doses between 200 and 400 mg/kg in fasted animals (or slightly higher doses in fed animals). Under these conditions, the animals develop severe liver injury between 6 and 12 h after APAP administration, and regeneration starts

at 24 h, leading to complete repair of the damaged liver by 72–96 h (129, 130). This is the most relevant model for humans who develop liver injury but regenerate and survive (131). Although the human condition of ALF cannot be completely reproduced in this mouse model, one important feature of ALF, that is, lack of regeneration, can be achieved with a higher dose of APAP in mice (130). Interestingly, this dose of 600 mg/kg of APAP in C57BL/6J mice induces much higher CXCL2 levels compared with the standard 300-mg/kg dose, resulting in earlier and more severe neutrophil recruitment (124). In addition, a CXCL2 antibody administered 2 h after APAP attenuated both neutrophil recruitment and injury at 24 h, suggesting that the enhanced neutrophil response after the high dose of APAP aggravated liver injury (124). These newer findings indicate that the neutrophil response and its impact on the injury after APAP poisoning may be dose dependent. Importantly, this applies to both male and female mice, as the initial injury and the progression is similar in both sexes for the higher dose (124).

The presence of neutrophils in the regenerating liver after a moderate overdose but the absence of a negative effect on the injury (132) led to the hypothesis that neutrophils as phagocytes may have a beneficial effect on the recovery. Recently, more direct evidence for this conclusion was provided. Yang et al. (133) showed that neutrophils recruited into the liver after a dose of 400 mg/kg of APAP did not affect the injury but promoted the conversion of the phenotype of monocyte-derived macrophages from the proinflammatory to the proregenerative phenotype, which are predominantly responsible for the removal of necrotic cell debris and enhancement of repair. This indirect proregenerative effect of neutrophils is caused by NADPH oxidase 2 (NOX-2)-derived ROS (133). However, a more direct effect of neutrophils on regeneration has also been proposed. Chauhan et al. (134) showed that blocking platelet recruitment and activation increased the number of neutrophils but not the number of other leukocytes in the liver and attenuated liver injury at 24 h after a dose of 350 mg/kg of APAP. Importantly, these additional neutrophils showed enhanced phagocytic capacity, suggesting a proregenerative effect by accelerated removal of cell debris and promoting of regeneration by neutrophils (134). These experimental data are consistent with clinical findings where activation including phagocytosis of circulating neutrophils was observed in APAP overdose patients selectively during the regeneration phase (132). However, the role of neutrophils in nonsurviving ALF patients is unknown.

Role of Kupffer Cells

Earlier, there was some controversy about the potential role of the resident macrophages of the liver (Kupffer cells) in APAP toxicity, mainly related to the type of intervention used to block Kupffer cell function (127). However, the elimination of Kupffer cells with clodronate liposomes demonstrated that these cells do not directly cause toxicity but are beneficial (135). This may be related to limiting proinflammatory cytokine formation and inducible NOS induction by Kupffer cell–derived IL-10 (136). However, an important role for Kupffer cells is related to CCL2 (MCP-1) generation that recruits monocytes into the necrotic areas of the liver (125, 137) where neutrophil-derived ROS (133) and other mediators (138) promote the phenotypic conversion of these monocytes to proregenerative macrophages critical for removal of cell debris. In addition, Kupffer cells can directly induce via IL-10 CXCR2 expression and a proregenerative gene expression profile in hepatocytes surrounding the necrotic areas to stimulate proliferation of these hepatocytes for replacement of the dead cells (139). Thus, the resident macrophages of the liver play a critical role in the initiation and progression of the sterile inflammatory response after an APAP overdose. Although generally geared toward a repair function, a higher overdose can lead to an exaggerated chemokine response with excessive recruitment of neutrophils and aggravation of the initial DILI (124).

Monocyte-Derived Macrophages in APAP Hepatotoxicity

Another important cell type accumulating in the liver during a sterile inflammatory response is monocytes, which transition to a macrophage phenotype once they arrive in the areas of necrosis (137, 140). Monocytes are recruited through monocyte chemotactic protein 1 (CCL2/MCP-1) generated by stressed hepatocytes and activated macrophages (Kupffer cells) acting on the C-C chemokine receptor type 2 (CCR2) receptor on monocytes (125, 137, 140). Although long thought to be recruited late, that is, 24–48 h after APAP overdose (125, 137, 140), more recent reports suggested that the first monocytes start to arrive at 6 h with progressive accumulation by 12 and 24 h, which corelates with CCL2 formation (124, 141). Interestingly, the newly accumulating monocytes or monocyte-derived macrophages ($Ly6C^{hi}$; Cx_3CR1^{low}) express a proinflammatory phenotype, which changes to a prorestorative phenotype ($Ly6C^{low}$; Cx_3CR1^{high}) with downregulation of proinflammatory genes and induction of prorestorative wound healing genes (140, 142) after arrival in the injured liver. These monocyte-derived macrophages are critical for tissue repair through phagocytic removal of necrotic cell debris, induction of apoptosis and removal of proinflammatory neutrophils, and promotion of hepatocyte proliferation and angiogenesis (137, 140, 141, 143). However, some reports indicate that the selective prevention of monocyte-derived macrophage recruitment alone delays tissue repair and recovery (137, 140, 141), while others have shown no effect and the need for additional Kupffer cell elimination to delay regeneration (125, 143). The latter observation would be consistent with the fact that Kupffer cells can proliferate and contribute to the phagocytic removal of necrotic cell debris (140, 144); in addition, Kupffer cells promote the expression of proregenerative genes in CXCR2-expressing hepatocytes surrounding the areas of necrosis (139). These are the hepatocytes that show the most prominent evidence for cell cycle activation and are the main cells that replace the necrotic centrilobular hepatocytes (129, 130). Although all studies using CCR2-deficient mice showed no effect on APAP-induced liver injury at 24 h, there is evidence for a transient increase in tissue damage at 12 h (141). Since the mechanisms of this increased injury are unknown and the injury catches up to wild-type animals by 24 h, the relevance for the overall pathophysiology remains unclear.

Most of the mentioned studies used moderate overdoses of APAP with significant injury but also full recovery. However, after a severe overdose, monocyte recruitment is substantially delayed during the first 24 h but is then accelerated by 48 h (124). Although this delayed monocyte recruitment may contribute to the impairment of regeneration under these conditions, it cannot be the main reason (129). In fact, in nonsurviving APAP ALF patients, there is prolonged generation of CCL2 (110) suggesting a continuous, albeit futile, effort to recruit more monocytes for tissue repair. In contrast, development of senescence in viable hepatocytes has been implicated as the cause of limited regeneration and recovery, which is observed in mice and humans after APAP overdose (145). Transforming growth factor beta 1 (TGF-β1), generated by $Ly6C^{hi}$ monocyte–derived macrophages, may be one of the mediators for hepatocyte senescence (145). This would indicate that a limited transitioning of the infiltrating monocyte-derived macrophages to the proregenerative phenotype may promote senescence. More studies are necessary to investigate the detailed mechanisms of the sterile inflammatory response that mediates a coordinated repair of the necrotic tissue or, when this process fails, causes ALF after a severe APAP hepatotoxicity.

REGENERATION AFTER ACETAMINOPHEN TOXICITY

Besides the removal of necrotic cell debris by inflammatory cells, regeneration of lost hepatocytes and nonparenchymal cells is vital for the recovery of the liver and restoration of its function. For detailed discussion on mechanisms of liver regeneration after APAP toxicity, the reader is referred to a recent review (129). Several key characteristics are worth summarizing. First, regeneration is

an adaptive response to injury with a close correlation between the severity of liver damage and the extent of compensatory regeneration (129). However, there is an inflection point of this correlation where, with further increase in injury, regeneration declines. At this point, the tissue damage is no longer repaired, leading to ALF. Second, hepatocyte proliferation is regulated by primary mitogens such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), and TGF-α and assisted by auxiliary mitogens such as TNF-α and IL-6 (129). EGF and TGF-α signal through the EGF receptor (EGFR), and HGF signals through c-MET. Inhibition or receptor deletion of these growth factors and cytokines, which are all activated early after an APAP overdose, generally impair regeneration. However, there are also differences. For example, an EGFR inhibitor attenuated regeneration but also inhibited APAP-induced liver injury (146), which indicates complex regulation of the regeneration process that requires further investigations. The Wnt/β-catenin pathway has also been implicated in APAP regeneration in mice and human patients (130, 147), likely through binding of β-catenin to the cyclin D1 promoter in the nucleus and thereby causing cell cycle activation (130). On the other hand, the inhibition of regeneration after high overdoses of APAP may be caused by activation of cell cycle inhibitors p21 and p53 through formation of TGF-β by macrophages leading to cellular senescence (145). However, Kupffer cells surrounding the area of necrosis can produce IL-10, and sinusoidal endothelial cells generate HGF through induction of vascular endothelial cell growth factor, which promotes proliferation of both hepatocytes and sinusoidal endothelial cells (139, 148). Furthermore, activated hepatic stellate cells support hepatic regeneration by formation of HGF (149). Thus, there is a complex interaction of mediators generated by a variety of resident liver cells and infiltrating leukocytes that orchestrates the promotion or inhibition of liver regeneration (150). This is an area that requires further investigation, as it is critical for the overall outcome after APAP hepatotoxicity and provides potential therapeutic targets.

THERAPEUTIC INTERVENTIONS AGAINST APAP TOXICITY

A key observation in APAP hepatotoxicity is the fact that the basic mechanisms of cell death in mouse livers effectively translate to the human pathophysiology (131). Thus, it is highly likely that a mechanism-based intervention strategy successful in mice will also be effective in APAP overdose patients (**Figure 3**).

N-Acetylcysteine

After the pioneering studies in B.B. Brodie's laboratory at the US National Institutes of Health showing the importance of cytochrome P450-dependent formation of a reactive metabolite, GSH depletion, and protein binding (9, 151), it was shown that cysteamine, a GSH-like nucleophile, prevented protein adduct formation and effectively protected against an APAP overdose in mice (152). This led to more testing of GSH precursors and the discovery of NAC as an effective antidote in preclinical models (153). Because NAC was already a clinically approved drug, it could be quickly used in APAP overdose patients. The results of clinical trials using a 72-h oral treatment protocol in the United States (154) and a 21-h intravenous administration regimen in the United Kingdom (155) showed impressive beneficial effects when administered within 10 h after the overdose. These data led to the official approval of NAC as antidote against APAP overdose by regulatory agencies worldwide; NAC remains up to now the only clinically approved antidote for this indication. Although NAC is highly effective when administered early (<10 h), it gradually loses efficacy when patients present at later time points (156); in addition, NAC can have some side effects such as anaphylactic reactions (157, 158). Nevertheless, NAC remains the standard of care for APAP toxicity, although today there are many variations of the original dosing protocols (159). Follow-up mechanistic studies demonstrated that the early treatment of NAC promotes

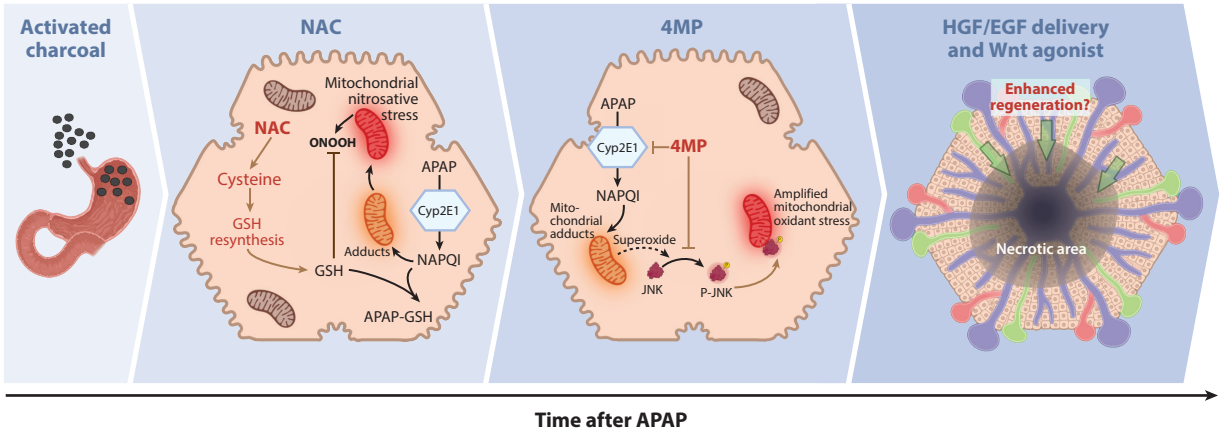

Figure 3

Mechanistic timeline for therapeutic options after an APAP overdose. Since oral overdose of APAP is most common in humans, the earliest intervention typically administered in the ER on presentation is activated charcoal to prevent absorption of the drug. However, this would be relevant only if the patient presents immediately after the overdose before the drug has been completely absorbed. For patients presenting up to 10 h after an overdose, NAC would be effective since it provides cysteine for glutathione resynthesis enabling scavenging of the reactive metabolite NAPQI generated from APAP. For severe overdoses, 4MP provides additional benefit since it is mechanistically distinct from NAC, inhibiting NAPQI formation by inhibition of Cyp2E1. 4MP would likely be beneficial for patients presenting beyond the 10-h window, since it protects through additional mechanisms such as inhibition of JNK activation, which would prevent amplification of mitochondrial oxidant stress. Recent data suggest that HGF/EGF delivery and Wnt agonists can also provide benefit in patients with delayed presentation, where necrosis has destroyed hepatocytes surrounding the central vein. These interventions could enhance the regenerative response to facilitate repopulation of areas of necrosis and enhance recovery. Black arrows depict steps in signaling to toxicity; brown arrows show steps involved after the intervention; dashed arrow indicates that there are multiple steps that are not depicted. Abbreviations: 4MP, 4-methylpyrazole; APAP, acetaminophen; Cyp2E1, cytochrome P450 2E1; EGF, epidermal growth factor; ER, emergency room; GSH, glutathione; HGF, hepatocyte growth factor; JNK, c-Jun N-terminal kinase; NAC, *N*-acetylcysteine; NAPQI, *N*-acetyl-*p*-benzoquinone imine; ONOOH, peroxynitrous acid; p-JNK, phosphorylated JNK. Figure adapted from images created with BioRender.com.

the scavenging of the reactive metabolite NAPQI (160) but also scavenges peroxynitrite inside of mitochondria, and degradation products of excessive NAC can support the mitochondrial bioenergetics (63). Importantly, NAC does not react directly with NAPQI or peroxynitrite but promotes hepatic GSH synthesis (161).

4-Methylpyrazole (Fomepizole)

Given the limited therapeutic window of NAC, there was always the hope that with better understanding of the pathophysiology of APAP-induced liver injury, additional therapeutic intervention strategies might be discovered. A few years ago, fomepizole (4-methylpyrazole), an approved drug against methanol and ethylene glycol poisoning, came into focus as a potential antidote (158) because of its effect as a specific Cyp2E1 inhibitor in vitro (162). Thus, we investigated whether fomepizole can inhibit APAP toxicity in mice in vivo. The first study involved cotreatment of fomepizole with APAP. Using a single bolus dose of 50 mg/kg of fomepizole in mice (human equivalent dose: 4 mg/kg), which is below the clinically approved dose of 15 mg/kg, it was shown that fomepizole eliminated the formation of all oxidative metabolites and prevented protein adduct formation, leading to complete protection against APAP-induced liver injury (163). These results suggested that fomepizole effectively inhibited Cyp2E1 in vivo. This protective effect was also confirmed in primary human hepatocytes (163). However, inhibition of cytochrome P450 activity alone would limit the therapeutic window for this drug. Therefore, other potential protective

mechanisms were investigated. When mice were treated with fomepizole after the metabolism phase for a dose of 300 mg/kg of APAP was over, fomepizole still protected against liver injury almost completely without inhibiting protein adduct formation (164). The mechanism of protection of the delayed treatment included inhibition of JNK and prevention of mitochondrial p-JNK translocation, which effectively attenuated the mitochondrial oxidant stress and prevented cell death (164). Again, this delayed protection could be confirmed in primary human hepatocytes (165). Interestingly, fomepizole was still effective when treatment was delayed by 18 h in human hepatocytes, which appeared to be beyond the therapeutic window of NAC (165). In addition, fomepizole cotreatment was also very effective in protecting against kidney injury, which is an additional problem especially after high overdoses of APAP in mice and humans (166). However, NAC does not appear to be protective against kidney injury, at least in mice (167). Thus, the preclinical studies suggest that fomepizole is extremely effective against APAP toxicity due to its dual mechanism of protection, that is, inhibition of Cyp2E1 and JNK. The inhibitory effect of Cyp2E1 is the main reason for the protection against kidney injury, which means that in mice and human hepatocytes fomepizole is at least as effective as NAC in preventing APAP-induced liver injury, with possibly a wider therapeutic window and the additional benefit of inhibiting kidney injury.

The advantage of repurposing a clinically approved drug is that mandatory safety studies are completed and there is clinical experience with the use of the drug. In the case of fomepizole, the drug had been on the market for more than 20 years and proved to be well tolerated with only very minor side effects (168). Thus, as a first step, a crossover volunteer study was performed where each person was exposed to a mild overdose of APAP (70 mg/kg) with and without concomitant treatment of the standard doses of fomepizole (169). Oral doses of APAP resulted in detection of the major APAP-glucuronide and -sulfate metabolites in plasma of each volunteer but also APAP-cysteine and APAP-NAC metabolites, which are derived from the detoxification of the reactive metabolite NAPQI by GSH. The cotreatment of APAP with fomepizole did not significantly affect the glucuronide and sulfate metabolites but reduced the oxidative metabolites by >90%, suggesting that fomepizole effectively inhibited Cyp2E1 in humans (169). As a result of the detailed understanding of the mechanisms and the safety of the drug, it is already used off-label for high-risk APAP overdose patients (170); a phase III clinical trial is currently in progress, which may provide the basis for the approval of the first new antidote against APAP hepatotoxicity in more than 40 years.

Additional Emerging Therapeutics

Despite many studies over the last decades identifying numerous potential therapeutic targets, very few compounds emerged that may have a realistic chance to make it to the clinic. Any intervention that targets the injury phase has to compete with the existing antidote NAC and the emerging drug fomepizole (158). However, the new frontier in drug development will be interventions that promote regeneration. In this respect, it was recently shown that a nucleoside-modified, lipid-nanoparticle-encapsulated mRNA of HGF and EGF administered 24 h after APAP overdose substantially enhanced regeneration and recovery (171). In addition, a Wnt agonist injected 32 h after 600 mg/kg of APAP overcame the impaired regeneration observed and promoted recovery through induction of cyclin D1 expression (172). However, there is one caveat to consider with this approach. Because the Wnt/β-catenin pathway regulates Cyp2E1 expression (147), administration of the Wnt agonist at 12 h after APAP enhanced liver injury, potentially through Cyp2E1 induction (172). The adenosine A2B receptor agonist BAY 60-6583 administered 6 h after APAP attenuated the late injury and promoted regeneration by enhanced recruitment of reparative macrophages (173). Although the mechanisms are less clear, a novel pegylated thrombopoietin mimetic peptide (PEG-TPO), which is under clinical development, was shown to reduce

the late injury and advance regeneration in the murine APAP toxicity model (174). Together, these emerging approaches indicate enhanced efforts to target regeneration after APAP hepatotoxicity.

SUMMARY AND FUTURE PERSPECTIVES

As outlined in this review, substantial progress has been made in the understanding of the molecular mechanisms of APAP-induced hepatocyte cell death and liver injury in experimental animals and cultured cells. The intracellular signaling events and inflammatory mechanisms elucidated with APAP may also apply to many other chemical-induced liver toxicities. Importantly, most of this mechanistic insight obtained with APAP could be translated to the human pathophysiology of APAP overdose patients, which has allowed for the identification of novel therapeutic targets and advancement of new drugs that limit APAP toxicity. However, there are still some gaps that need to be addressed. These include studies of signaling events in regeneration, assessment of the still mainly unknown mechanisms of the development of ALF, and identification of clinically applicable prognostic biomarkers. The future research focus should shift from the injury mechanisms toward the recovery phase and the development of drugs that promote regeneration.

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