Central Mechanisms of Acetaminophen Hepatotoxicity: Mitochondrial Dysfunction by Protein Adducts and Oxidant Stress

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ABBREVIATIONS: APAP, acetaminophen, N-acetyl-p-aminophenol; ASK1, apoptosis signal-regulating kinase 1; Cyp2E1, cytochrome P450 2E1; gclc, γ-glutamylcysteine ligase catalytic subunit; GSH, glutathione; GSSG, glutathione disulfide; JNK, c-jun N-terminal kinase; K18, cytokeratin 18; Keap1, kelch-like ECH associating protein 1; LPO, lipid peroxidation; MAP3K, mitogen activated protein 3 kinases; MLK3, mixed-lineage kinase 3; MPTP, mitochondrial permeability transition pore; mtDNA, mitochondrial DNA; NAC, N-acetylcysteine; NAPQI, N-acetyl-p-benzoquinone imine; NO, nitric oxide radical; NOS, nitric oxide synthase; Nrf2, nuclear erythroid 2 p45-related factor 2; SOD, superoxide dismutase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling assay;
Abstract

Acetaminophen (APAP) is an analgesic and antipyretic drug used worldwide, which is safe at therapeutic doses. However, an overdose can induce liver injury and even liver failure. Mechanistic studies in mice beginning with the seminal papers published by B.B. Brodie’s group in the 1970s have resulted in important insight into the pathophysiology. Although the metabolic activation of APAP with generation of a reactive metabolite, glutathione depletion and protein adduct formation are critical initiating events, more recently the mitochondria came into focus as important target and decision point of cell death. This review provides a comprehensive overview of the induction of mitochondrial superoxide and peroxynitrite formation and its propagation through a mitogen activated protein kinase cascade, the mitochondrial permeability transition pore opening caused by iron-catalyzed protein nitration and the mitochondria-dependent nuclear DNA fragmentation. In addition, the role of adaptive mechanisms that can modulate the pathophysiology including autophagy, mitophagy, Nrf2 activation and mitochondrial biogenesis, are discussed. Importantly, it is outlined how the mechanisms elucidated in mice translate to human hepatocytes and APAP overdose patients, and how this mechanistic insight explains the mechanism of action of the clinically approved antidote N-acetylcysteine and led to the recent discovery of a novel compound, fomepizole, which is currently under clinical development.
Significance Statement

Acetaminophen (APAP)-induced liver injury is the most frequent cause of acute liver failure in western countries. Extensive mechanistic research over the last several decades revealed a central role of mitochondria in the pathophysiology of APAP hepatotoxicity. This review article provides a comprehensive discussion of a) mitochondrial protein adducts and oxidative/nitrosative stress, b) mitochondria-regulated nuclear DNA fragmentation, c) adaptive mechanisms to APAP-induced cellular stress, d) translation of cell death mechanisms to overdose patients, and e) mechanism-based antidotes against APAP-induced liver injury.
Introduction

Acetaminophen (N-acetyl-p-aminophenol, APAP, paracetamol) is a widely used analgesic and antipyretic present in numerous medications. Although APAP is considered safe at therapeutic levels, an overdose is dose-dependently hepatotoxic in animals and humans (Jaeschke, 2015). To identify therapeutic targets and develop effective antidotes, it is critical to understand the molecular mechanisms of liver injury and adaptive responses to this cellular stress. Mitochondria emerged as the central target of APAP-induced cell death mechanisms and this review gives an overview of major findings resulting from studying the role of mitochondria in APAP hepatotoxicity in mice, its translation to the human pathophysiology and its impact on antidote development.

Brief Historical Perspective

The first report of liver injury in a patient caused by an APAP overdose was published in 1966 (Davidson and Eastham, 1966). A few years later, investigators in B.B. Brodie’s laboratory at NIH developed a mouse model of APAP toxicity that reproduced the severe liver injury observed in patients (Jollow et al., 1973; Mitchell et al., 1973a,b; Potter et al., 1973). Importantly, these seminal publications established important mechanistic aspects of the toxicity that are still valid today 50 years later. Key findings include that APAP toxicity depends on drug metabolism by cytochrome P450 enzymes and formation of a reactive metabolite (Mitchell et al., 1973a; Potter et al, 1973), hepatic glutathione (GSH) depletion due to the scavenging of the reactive metabolite (Mitchell et al., 1973b) and, after >70% of the liver GSH is consumed, extensive covalent binding of the reactive metabolite to cellular proteins (Jollow et al., 1973; Potter et al., 1973). The relevance of the covalent protein binding of the reactive metabolite for APAP-induced cell
death in both mice and humans was shown by the protective effect of N-acetylcysteine (NAC) (Piperno and Berrsenbruegge, 1976; Corcoran et al., 1985a,b; Rumack et al., 1981). NAC is still the only clinically approved antidote against APAP overdose.

Despite the early enthusiasm with regards to the covalent binding hypothesis as an explanation for APAP-induced cell death, refinement of this mechanism required a search for specific cellular targets of the reactive metabolite, now hypothesized to be N-acetyl-p-benzoquinone imine (NAPQI) (Dahlin et al., 1984). Although a number of covalently adducted proteins were identified (Cohen et al., 1997; Qui et al., 1998), no specific protein could be identified that was sufficiently inactivated to unilaterally explain the cell death. The search for alternative mechanisms revealed that APAP overdose can cause impairment of mitochondrial function (Meyers et al., 1988; Ramsay et al., 1989), a mitochondrial oxidant stress (Jaeschke, 1990; Tirmenstein and Nelson, 1990) and evidence of DNA fragmentation (Ray et al., 1990). These early findings formed the basis for the current advanced understanding of intracellular signaling mechanisms around mitochondria as key events in APAP-induced cell death.

**Key Recent Advances**

**Oxidant Stress and Protein Adducts**

The protein binding hypothesis as first established by Mitchell and coworkers (Mitchell et al., 1973a,b; Jollow et al., 1973) was challenged by Wendel et al. (1979) who suggested that lipid peroxidation (LPO) and not protein adducts are responsible for APAP-induced cell death. LPO was thought to be initiated by reactive oxygen generated during cytochrome P450-dependent
metabolism of APAP (Wendel and Feuerstein, 1981). This triggered a long controversy about the role of oxidant stress/LPO versus protein adducts. However, in contrast to the earlier experiments that showed extensive LPO in animals fed a diet deficient in vitamin E and enriched in polyunsaturated fatty acids, which makes animals highly susceptible to LPO (Wendel et al., 1979; Wendel and Feuerstein, 1981), in animals on a standard diet LPO is very minimal and enhancing tissue vitamin E levels by 700% did not affect the injury (Knight et al., 2003). In addition, more direct assessment of oxidant stress during the metabolism phase provided no evidence for reactive oxygen formation in vivo or in isolated hepatocytes (Bajt et al., 2004; Smith and Jaeschke, 1989). Instead, the increase in mitochondrial GSSG levels after the metabolism phase indicated an oxidant stress in the mitochondrial matrix (Jaeschke, 1990). The fact that elimination of the mitochondrial oxidant stress without affecting the metabolic activation of APAP can protect (Jaeschke, 1990) and that mitochondrial protein adduct formation correlated with APAP-induced liver injury (Tirmenstein and Nelson, 1989; Xie et al., 2015b) suggested that both events are important for cell death and the oxidant stress is a consequence of the reactive metabolite NAPQI binding to proteins, especially mitochondrial proteins (Jaeschke et al., 2003).

**Reactive Oxygen and Reactive Nitrogen Species**

Although today the importance of oxidant stress for APAP-induced liver injury is no longer controversially discussed, the question which reactive oxygen species are responsible for cell death is critically important. It is well established that mitochondria can generate superoxide due to electron leaks from the mitochondrial electron transport chain (Loschen et al., 1974). The absence of relevant LPO after APAP overdose and the presence of Mn-SOD, glutathione
peroxidase 1, thioredoxin 2 and peroxiredoxin 3 in the mitochondria indicates that antioxidant defense mechanisms can deal with any excessive superoxide being generated (Jaeschke and Ramachandran, 2018). Thus, under normal circumstances, reactive oxygen species are effectively detoxified and are unlikely to be directly responsible for mitochondrial dysfunction and triggering of the mitochondrial permeability transition pore (MPTP) opening. However, superoxide radicals can also readily react with nitric oxide radicals (NO) to form the potent oxidant and nitrating species peroxynitrite (Radi, 2018). Early studies with APAP overdose in rats showed enhanced expression of inducible nitric oxide synthase (iNOS) and increased formation of NO (Gardner et al., 1998). In addition, centrilobular staining of nitrotyrosine protein adducts were observed after APAP overdose in mice (Hinson et al., 1998). Given the generation of superoxide in the mitochondrial matrix, it was then not surprising to find evidence for peroxynitrite formation almost exclusively in mitochondria (Cover et al., 2005). However, nitrotyrosine staining was also observed in the absence of iNOS induction (Knight et al., 2001; Saito et al., 2010a). In addition, neither deficiency of the iNOS gene nor specific iNOS inhibitors showed protective effects against APAP hepatotoxicity in mice (Burke et al., 2010; Hinson et al., 2002; Michael et al., 2001; Saito et al., 2010a). However, independent of the source of NO, the most critical question was whether peroxynitrite was the actual oxidant responsible for mitochondrial dysfunction and the MPTP opening. As this could not be addressed by inhibiting iNOS, we treated animals intravenously with GSH, which was degraded in the kidney, and the amino acids were then used to re-synthesize GSH in the liver (Knight et al., 2002). When GSH was given shortly after APAP, i.e., during the metabolism phase of APAP, the re-synthesized GSH effectively scavenged NAPQI and completely prevented liver injury (Knight et al., 2002). However, when animals were treated with GSH after the metabolism phase, the re-synthesized
GSH was imported into mitochondria, scavenged peroxynitrite and protected against APAP hepatotoxicity (Knight et al., 2002). Similar effects were also reported with NAC posttreatment (James et al., 2003). In addition, when GSH or NAC were given in higher doses than needed to re-synthesize GSH, the excess amino acids were used to generate Krebs cycle intermediates and improve mitochondrial bioenergetics (Saito et al., 2010b). Together, these experiments strongly support the conclusion that peroxynitrite is indeed the critical oxidant generated in mitochondria responsible for APAP toxicity.

The critical role of peroxynitrite in APAP-induced cell death was further confirmed more recently. First, animals partially deficient in the mitochondrial MnSOD showed a dramatic aggravation of APAP hepatotoxicity compared to wild type animals (Fujimoto et al., 2009; Ramachandran et al., 2011). Second, the mitochondrial targeted SOD mimetic Mito-Tempo eliminated nitrotyrosine staining and effectively protected against APAP overdose (Du et al., 2017a, 2019). Similar observations were recently made with Mitoquinone another SOD mimic that accumulates inside mitochondria (He et al., 2023). Importantly, these mitochondria-targeted interventions were highly effective in eliminating mitochondrial dysfunction and cell death without affecting protein adduct formation and other events upstream of mitochondria (Du et al., 2017a). SOD accelerates the dismutation to superoxide to form hydrogen peroxide, which is a substrate for the Fenton reaction and initiation of LPO. On the other hand, the rapid removal of superoxide prevents the reaction with NO to form peroxynitrite. Thus, the fact that mitochondrial SOD activity limits or prevents injury, can only be explained with peroxynitrite being the critical oxidant for APAP hepatotoxicity (Du et al., 2016a; Ramachandran and Jaeschke, 2021).
Some reports investigating the role of iron seem to contradict the findings that peroxynitrite is the most important oxidant. Ferrous iron (Fe$^{2+}$) is well-known to be the catalyst that can reduce hydrogen peroxide to generate hydroxy radicals (Fenton reaction), the initiation reaction for LPO. Several studies were published showing protection with iron chelation implying the importance of LPO as injury mechanism in APAP toxicity (Sakaida et al., 1995; Yamada et al., 2020) even though LPO is quantitatively insufficient to cause cell death in this model (Jaeschke et al., 2003, 2021). However, recently more detailed mechanistic studies shed light onto this controversy. APAP overdose causes lysosomal instability (Woolbright et al., 2012), which triggers the release of ferrous iron from this cell organelle (Kon et al., 2010). Selective chelation of lysosomal iron reduced the MPTP opening and cell death in vitro (Kon et al., 2010). The uptake of ferrous iron released from lysosomes into mitochondria is facilitated by the mitochondrial electrogenic Ca$^{2+}$, Fe$^{2+}$ uniporter (Hu et al., 2016; Hu and Lemasters, 2020). Interestingly, it is known that protein nitration by peroxynitrite requires transition metal catalysis (Campolo et al., 2014). Consistent with this observation, we could show that iron chelation with deferoxamine and inhibition of mitochondrial iron uptake by minocycline eliminated nitrotyrosine staining and protected against APAP-induced liver injury but had no effect on LPO (Adelusi et al., 2022). This further supports the critical role of peroxynitrite as essential oxidant in the pathophysiology of APAP under normal conditions. However, in case of an APAP overdose with iron overload, both LPO and protein nitration contribute to the injury process (Adelusi et al., 2022). Furthermore, if the critical antioxidant vitamin E is depleted and additionally the membrane content of polyunsaturated fatty acids is increased, massive LPO is the dominant mechanism of cell death (Wendel and Feuerstein, 1981; Wendel et al, 1979, 1982). These observations indicate that LPO can be a relevant contributor to the injury process after an
APAP overdose only under conditions of iron overload or enhancing the susceptibility to oxidant stress. While it is very difficult to achieve vitamin E deficiency in humans, iron overload is possible when iron supplements are taken together with the APAP overdose (Nye and Singh, 2022).

The role of peroxynitrite in APAP hepatotoxicity is now well established but the source of NO is less clear. Although iNOS can be induced after APAP (Gardner et al., 1998), APAP toxicity can occur in the absence of iNOS expression (Knight et al., 2001) and contribution to the pathophysiology (Burke et al., 2010; Michael et al., 2001; Saito et al., 2010a). It was reported that endothelial NOS-deficient mice were protected (Salhanik et al., 2006) but we could not reproduce these findings (Ramachandran and Jaeschke, unpublished observation). However, Hinson’s group showed protection against APAP hepatotoxicity both in vivo and in vitro with various neuronal NOS (nNOS) inhibitors (Banerjee et al., 2015; 2017; Burke et al., 2010) and in vivo in nNOS-deficient mice (Agarwal et al., 2012). These data suggest that nNOS could be the main source of NO formation after an APAP overdose. However, whether a mitochondrial NOS activity exists and if this is nNOS as was suggested (Kanai et al., 2001) is controversial (Lacza et al., 2003). The main argument against a distinct mitochondrial NOS enzyme is the fact that mtDNA does not encode such an enzyme and the nuclear DNA encoded genes of eNOS, iNOS or nNOS have no mitochondrial localization sequence (Lacza et al., 2009). However, NO is a freely diffusible gas that may not need to be generated in mitochondria but could be produced in the cytosol or even in adjacent cells and still can react with superoxide in mitochondria to form peroxynitrite in the mitochondrial matrix.
Initiation and Propagation of the Mitochondrial Oxidant Stress

Mitochondria as the source of APAP-induced oxidant stress were discovered by the observation that the increase in hepatic GSSG levels was caused almost exclusively by GSSG accumulation inside of mitochondria and the absence of an increased biliary efflux of GSSG (Jaeschke, 1990). This suggested an oxidant stress in the mitochondrial matrix and not in the cytosol. However, this concept was challenged when the role of JNK in APAP hepatotoxicity was recognized (Gunawan et al., 2006). Although JNK cannot be directly activated by reactive oxygen, it relies on upstream cytosolic mitogen activated protein 3 kinases (MAP3K), such as apoptosis signal-regulating kinase 1 (ASK1) and mixed-lineage kinase 3 (MLK3), which can be activated by a mild oxidant stress (Nakagawa et al., 2008; Sharma et al., 2012). As recently recognized, protein adducts formation on mitochondria causes a bidirectional leakage of electrons and formation of superoxide by complex III after an APAP overdose (Nguyen et al., 2021). This limited reactive oxygen formation in the intermembrane space is responsible for ASK1 activation in the cytosol. In turn, ASK1 activates the MAP2K MKK4, which then phosphorylates JNK (Zhang et al., 2017). Phosphorylated JNK translocates to the mitochondria where it binds to an anchor protein Sab on the outer mitochondrial membrane (Win et al., 2011) and triggers inactivation of Src on the inner mitochondrial membrane (Win et al., 2016), which further impairs the electron transport chain and enhances superoxide formation towards the matrix leading to amplified mitochondrial peroxynitrite formation (Hanawa et al., 2008; Nguyen et al., 2021; Saito 2010a). Both complex III and complex I contribute to the amplified oxidant stress in the mitochondrial matrix (Du et al., 2016b; Nguyen et al., 2021). Thus, a sustained mitochondrial oxidative and nitrosative stress that can induce necrotic cell death requires a sustained amplification of reactive oxygen formation through the JNK activation loop in vivo. An additional detrimental effect of
prolonged JNK activation can lead to degradation of γ-glutamylcysteine ligase catalytic subunit (gclc), which delays hepatic GSH recovery (Win et al., 2023). The induction rate of gclc and hence recovery rate of hepatic GSH level determine the scavenging capacity of peroxynitrite in the mitochondria and thus the injury progression (Du et al., 2014; Ryan et al., 2012).

**Regulation of Nuclear DNA Fragmentation by Mitochondria**

Nuclear DNA fragmentation is a characteristic of APAP-induced necrosis and is considered the point of no-return to cell death (Kirkland et al., 2021). Evidence of DNA damage after APAP overdose was first shown by DNA ladder *in vivo* (Ray et al., 1990) and in primary hepatocytes (Shen et al., 1991). This was followed by a positive terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Lawson et al., 1999; Gujral et al., 2002) and the detection of nuclear DNA fragments in the cytosol and circulating blood with an anti-histone ELISA (Lawson et al., 1999; Gujral et al., 2002; Cover et al., 2005). In addition, staining for Ser139 phosphorylation of histone H2AX (pH2AX) detects DNA double strand breaks during APAP hepatotoxicity (Borude et al., 2018). A caveat of these assays is that they are not specific for a certain cell death modes but simply provide evidence for DNA damage independent of the mode of cell death. In this respect, the most controversial assay is the TUNEL assay, which is frequently considered evidence for apoptosis. However, it is known for many years that it shows DNA damage independent of the mode of cell death (Grasl-Kraupp et al., 1995) and is used as indicator for APAP-induced nuclear fragmentation during necrosis (Lawson et al., 1999; Gujral et al., 2002). Although apoptotic and necrotic cells can be TUNEL-positive, there are fundamental differences. During apoptosis, the TUNEL assay selectively stains the nucleus or apoptotic bodies because the small DNA fragments that are released (Jahr et al., 2001) are not
recognized as substrates by the terminal deoxynucleotidyl transferase of the TUNEL assay (Jaeschke et al., 2011, 2018). In contrast, during necrotic cell death, larger DNA fragments are released from the nucleus (Jahr et al., 2001), which results in staining of the nuclear remains and the DNA fragments in the cytosol causing a fundamentally different staining pattern of necrosis compared to apoptosis (Gujral et al., 2002; Jaeschke et al., 2011; 2018). The mechanistic reason for this difference is that the DNA fragmentation during apoptosis is caused by a caspase-activated DNase resulting in small nucleosome units (Nagata, 2005). In striking contrast, nuclear DNA fragmentation during APAP-induced necrosis is triggered by endonuclease G and apoptosis-inducing factor (AIF) released from the mitochondrial intermembrane space into the cytosol and, due to the nuclear localization sequence, enter the nucleus (Bajt et al., 2006, 2011). Thus, nuclear DNA damage after APAP overdose is critically linked to mitochondrial dysfunction and not to caspase activation (Cover et al., 2005). Interestingly, endonuclease G and AIF are released initially through Bax and Bid pores formed in the outer mitochondrial membrane through mitochondrial translocation (Bajt et al., 2008; Maxa et al., 2019). The early release of the mitochondrial endonucleases accelerates cell death after APAP (Bajt et al., 2008). However, because Bax does not affect the mitochondrial oxidant stress, Bax deficiency only delays cell death but does not prevent it (Bajt et al., 2008). When the mitochondrial oxidant stress and peroxynitrite triggers the MPTP opening (Kon et al., 2004), matrix swelling and rupture of the outer membrane will then release intermembrane proteins and cause DNA fragmentation independent of Bax (Bajt et al., 2008). A similar result with early protection and reduced DNA fragmentation was obtained by silencing Bid (Maxa et al., 2019); however, the long-term beneficial effect of Bid knock-down was not evaluated (Maxa et al., 2019). Thus, nuclear DNA fragmentation during APAP hepatotoxicity, which can be considered the point of
no return of necrotic cell death, is caused by release and nuclear translocation of mitochondrial intermembrane proteins endonuclease G and AIF initially through Bax/Bid pores in the outer membrane or later through matrix swelling and rupture of the outer membrane.

**Adaptive Mechanisms Modulating APAP-induced Liver Injury**

*Autophagy.* While formation of APAP protein adducts is the critical initiator of mitochondrial dysfunction after an APAP overdose, their formation *per se* does not condemn cells to embark on a pathway of cell death. Numerous adaptive responses attempt to mitigate the consequence of protein adduct formation and allow cellular survival (Figure 1). The earliest of these mechanisms would be the activation of autophagy to consolidate adducted proteins and degrade them by lysosomal fusion. Autophagy is a cellular phenomenon which is exquisitely responsive to cellular stress and initiated by formation of double-membrane phagophores which surround adducted proteins and form an autophagosome. Fusion with the lysosome then forms an autolysosome which allows degradation of the adducted proteins and recycling of the amino acids (Qian et al., 2021; Ni et al., 2016). The critical role of autophagy in adaptation after exposure to APAP overdose is illustrated by the exacerbation of APAP-induced liver injury by pharmacological inhibition of autophagy (Ni et al., 2012). This was further confirmed by the protection against APAP-induced liver toxicity seen on induction of autophagy (Lin et al., 2014; Ni et al., 2012). Though autophagy is very sensitive to cellular stress and is activated in an attempt to curtail harmful effects of APAP-protein adduct formation, it can be overwhelmed when large doses of APAP induce significant NAPQI generation and subsequent protein adduct formation on mitochondrial proteins. As described, protein adduct formation on mitochondria are critical for APAP-induced hepatocyte cell death and additional adaptive mechanisms function to
mitigate detrimental effects of mitochondrial protein adduct formation and its effects on the mitochondrial respiratory chain and membrane potential. Initial adaptation is through reversible alterations in mitochondrial morphology, which occur in response to the mild drop in mitochondrial membrane potential to facilitate maintenance of the proton motor gradient and mitochondrial function. Mitochondria undergo transition from elongated forms to “donut” shaped spherical mitochondria to maintain respiratory function despite the decrease in membrane potential (Umbaugh et al., 2021a; Ramachandran and Jaeschke, 2023). This change in mitochondrial morphology also allows the organelle to resist removal by autophagy (Zhou et al., 2020), as long as mitochondrial function can be maintained. However, if mitochondrial function deteriorates in response to persistent protein adduct formation which is not curtailed by change in morphology, the next level of adaptation is initiated, which attempts to selectively remove damaged and dysfunctional mitochondria allowing functioning organelles to supply cellular energy requirements for survival. This is mediated by a variation of autophagy called mitophagy, which is mediated by elevated localization of the protein PARKIN on mitochondria, which, along with its partner PINK1 induces the envelopment of defective mitochondria within autophagic vacuoles for subsequent degradation through fusion with lysosomes (Chao et al., 2018; Wang et al., 2019).

*Nrf2 activation.* Additional adaptive mechanisms respond to the release of oxygen free radicals from mitochondria to induce canonical anti-oxidant responses mediated by activation of the nuclear erythroid 2 p45-related factor 2 (Nrf2) (Aleksunes and Manautou, 2007). This transcription factor is a central player in the response to an APAP overdose since it mediated recovery of hepatic glutathione stores (Aleksunes and Manautou, 2007), which is the primary
scavenger of reactive metabolite NAPQI formed through CYP2E1-mediated metabolism of APAP (Lee et al., 1996). Under baseline conditions, Nrf2 is retained in the cytosol by its binding to the kelch-like ECH associating protein 1 (Keap1). However, modification of cysteine residues on Keap1 during oxidant stress results in the dissociation of Nrf2 and its translocation to the nucleus (Levonen et al., 2004). There, it binds to the anti-oxidant response element (ARE) on several genes (Klaassen and Reisman, 2010), including those involved in glutathione synthesis to enhance glutathione production and enable scavenging of NAPQI. The essential role of Nrf2 in responding to enhanced NAPQI formation and subsequent oxidative stress is illustrated by the exacerbated APAP-induced liver injury seen in Nrf2 deficient mice (Chan et al., 2001; Enomoto et al., 2001) and resistance to injury evident in mice with defective Keap1, which have enhanced nuclear Nrf2 accumulation (Okawa et al., 2006).

Mitochondrial biogenesis. Most of the adaptive responses thus discussed are in response to initial formation of protein adducts or mitochondrial dysfunction and oxidative stress, all of which are components of the cascade of liver injury induced by APAP. Since these signaling events are predominantly occurring in hepatocytes surrounding the central vein with increased expression of CYP2E1, these centrilobular hepatocytes undergo necrosis, which radiates outwards towards the portal area. The next adaptive mechanism occurs in a spatially distinct manner, where the robust capacity of hepatocytes for regeneration after injury is harnessed by surviving cells surrounding areas of necrosis. These cells are thus important for liver recovery after APAP-induced injury and a central feature critical for their success is induction of mitochondrial biogenesis (Jaeschke et al., 2019; Ramachandran and Jaeschke, 2019). Selective induction of mitochondrial biogenesis in surviving hepatocytes bordering areas of necrosis was shown to be essential for liver recovery.
after an APAP overdose (Du et al., 2017b). This was further reiterated by the enhanced recovery seen in animals with pharmacological activation of mitochondrial biogenesis (Du et al., 2017b). Thus, several adaptive mechanisms at various levels of the signaling cascade induced by APAP overdose attempt to mitigate the harmful effects of the reactive metabolite NAPQI and prevent hepatocyte cell death. Additional mechanisms such as mitochondrial biogenesis also facilitate recovery of surviving hepatocytes to repopulate areas of necrosis and regain hepatic homeostasis.

**Sterile Inflammation.** It is well established that the severe APAP-induced hepatocellular necrosis causes the release of damage-associated molecular patterns, which includes mitochondrial DNA and nuclear DNA fragments, triggering a sterile inflammatory response with activation of resident macrophages (Kupffer cells) and the recruitment of innate immune cells such as neutrophils and monocytes (Woolbright and Jaeschke, 2017). Although the role of these immune cells in the injury process and recovery is controversially discussed for years (Jaeschke and Ramachandran, 2020), an in-depth review of the newest developments in this area is provided by another review in this series (Luyendyk and Copple, 2023).

**Translation of Liver Injury Mechanisms in Mice to Patients**

APAP hepatotoxicity in the mouse is generally considered the best model for the human pathophysiology because the degree of liver injury is very similar although the time course in humans is more delayed compared to mice (Jaeschke, 2015; Larson, 2007). In contrast, although APAP is metabolized in rats similarly as in mice and humans, even a severe overdose does not cause mitochondrial dysfunction, DNA fragmentation and extensive cell necrosis (McGill et al., 2012b). Thus, most of the insight into the human pathophysiology is derived from comparing
mouse studies with results in human hepatocytes and from circulating mechanistic biomarkers in patients.

**Human hepatocytes.** Formation of protein adducts, which can be detected in circulation, is also a hallmark of APAP exposure in patients (Davern et al., 2006; Heard et al., 2016; Xie et al., 2015a), which suggests that any relevant *in vitro* model needs to be capable of forming the reactive metabolite NAPQI by Cyp2E1. In fact, freshly isolated primary human hepatocytes show a time- and dose-dependent cell death after exposure to 5-20 mM APAP (Xie et al., 2014). There was a time-dependent depletion of GSH levels followed by an increase in overall cellular protein adducts and adducts in mitochondria, which triggered a progressive mitochondrial dysfunction as indicated by the collapse of the mitochondrial membrane potential (Xie et al., 2014). Like mouse hepatocytes, there was JNK activation observed at 6 h and later, P-JNK translocation to mitochondria at 15 h (Xie et al., 2014). Cell death was observed beginning at 24 h but peaking mainly around 48 h. No caspase activation was detected during APAP-induced cell death and pancaspase inhibitors did not protect indicating that human hepatocytes die by necrosis not apoptosis (Xie et al., 2014). Thus, the sequence of events was very similar as in mouse hepatocytes, but the events were delayed reflecting the difference in the time course of cell death development between mice and humans. This also explains the fact that the therapeutic window of NAC is much wider in primary human hepatocytes compared to mouse hepatocytes (Xie et al., 2014; Bajt et al., 2004).

The human HepaRG cells behave very similar to primary human hepatocytes in terms of time- and dose-dependent metabolism, adducts formation and mitochondrial dysfunction (McGill et
al., 2011). In addition, Mito-SOX staining indicated a mitochondrial oxidant stress. HepaRG cells die by necrosis not apoptosis by 24-48 h. Thus, HepaRG cells are a valuable human model for APAP toxicity with the advantage that they are derived from a single donor and are easier available than primary human hepatocytes. In addition, HepaRG cells show expression of P450 enzyme levels and other drug metabolism enzymes that is close to human hepatocytes (Aninat et al., 2006; Nelson et al., 2017). However, there is no JNK activation in response to APAP (Xie et al., 2014). However, this may be an issue of cells cultured under room air, i.e., hyperoxic conditions, which increases reactive oxygen generation (Yan et al., 2010). Thus, HepaRG cells are not dependent on the amplification of the mitochondrial oxidant stress by JNK. Even primary human hepatocytes appear to rely less on JNK activation to generate mitochondrial oxidant stress than hepatocytes in vivo (Xie et al., 2014).

Many other cell lines are being used with variable results. However, metabolic capabilities of these cells need to be investigated before use. For example, HepG2 cells have low cytochrome P450 activities, which results in no GSH depletion, no protein adducts formation and no mitochondrial dysfunction after APAP exposure (Xie et al., 2014); the cell eventually dies by apoptosis not necrosis and NAC does not protect in this cell line (Manov et al., 2004). Thus, mechanistic data generated with HepG2 cells and many other uncharacterized cell lines are not applicable to primary hepatocytes and patients.

Mechanistic biomarkers in patients. Due to the bleeding risk in patients with severe liver injury, liver biopsies are generally contraindicated. Therefore, insight into mechanisms of APAP-induced liver injury in patients must be based mainly on circulating biomarkers. APAP protein
adducts are detectable in plasma after therapeutic or toxic doses suggesting the formation of a reactive metabolite NAPQI in patients (Davern et al., 2006; Heard et al., 2016; Xie et al., 2015a). Clinically, these adduct levels can be used to confirm that liver injury was due to an APAP overdose and was not caused by other drugs or chemicals (Davern et al., 2006). High levels of high mobility group box 1 protein, miR-122 and cytokeratin 18 (K18) in APAP overdose patients are indicators of cell necrosis especially when there is very limited or no increase of caspase-cleaved K18 or circulating active caspase-3 activities (Antoine et al., 2013; McGill et al., 2012a). In addition, mitochondrial DNA (McGill et al., 2012a), glutamate dehydrogenase (McGill et al., 2012a) and carbamoyl phosphate synthase 1 (Kwan et al., 2023; Weerasinghe et al., 2014) are all located in the mitochondrial matrix and the release of these compounds indicates mitochondrial damage. Whether this release is caused by rupture of the mitochondrial membranes or is achieved by stress-induced macropore formation requires further studies (Kim et al., 2023). Nevertheless, the correlation of higher levels of mitochondrial damage biomarkers with negative outcome suggests that mitochondrial damage is a key event in the pathophysiology in patients (McGill et al., 2014b). Further evidence for mitochondrial dysfunction comes from elevated acylcarnitine levels, which reflect impairment of the mitochondrial fatty acid uptake system (Bhattacharyya et al., 2014; McGill et al., 2014a). In addition, the presence of nuclear DNA fragments is indirect evidence for mitochondrial dysfunction (McGill et al., 2012a, 2014b) due to the release of endonucleases from the mitochondria (Bajt et al., 2006). Taken together, both circulating mechanistic biomarkers and studies in primary human hepatocytes or the metabolically competent hepatocyte cell line HepaRG confirm all mechanisms of cell death and liver injury observed in mice or mouse hepatocytes with one major difference that the timeline of events in humans is more delayed.
Mechanism-based Antidotes against Acetaminophen Hepatotoxicity

*N-acetylcysteine* (NAC). After the recognition of NAPQI formation and GSH depletion as critical early events in APAP toxicity (Jollow et al., 1973; Mitchell et al., 1973a,b), several reagents were tested that supported the recovery of hepatic GSH levels (Piperno and Berssenbruegge, 1976). NAC emerged as the most effective and best tolerated agent, that had the advantage of being already an approved drug (Rumack and Bateman, 2012). As a result, NAC was quickly tested in patients. However, it was approved for intravenous administration in the UK (Prescott et al., 1977) but only for oral treatment in the US (Rumack and Petersen, 1978). Nevertheless, both treatment regimen proved highly effective, and NAC was officially approved as antidote against APAP overdose in the US in 1985 (Rumack and Bateman, 2012) after successful large-scale trials (Rumack et al., 1981; Smilkstein et al., 1988). Mechanistic studies in mice demonstrated the capacity of NAC treatment to scavenge the reactive metabolite NAPQI thereby preventing protein adduct formation and toxicity (Corcoran et al., 1985a,b). Importantly, NAC does not directly react with NAPQI but requires GSH synthesis (Corcoran and Wong, 1986). Later it was recognized that peroxynitrite is formed as part of the pathophysiology (Hinson et al., 1998) and that peroxynitrite is the actual cytotoxic agent generated inside mitochondria (Knight et al., 2002; Cover et al., 2005). Since GSH is a potent direct scavenger of peroxynitrite (Knight et al., 2002), NAC triggers GSH synthesis in the cytosol and this GSH requires uptake into mitochondria to be effective as peroxynitrite scavenger (Saito et al., 2010b). Interestingly, if more NAC is administered than used for GSH synthesis, NAC is being degraded to Krebs cycle intermediates, which support the impaired mitochondrial bioenergetics (Saito et al., 2010b). Together, these mechanisms expand the therapeutic window for NAC. Nevertheless, there are
some limitations of NAC treatment. In humans, NAC is highly effective when administered within 10 h after an overdose. However, after that time, the efficacy gradually diminishes (Rumack et al., 1981; Smilkstein et al., 1988). Additional issues can be potential side effects of NAC treatment (Bebarta et al., 2010) and the fact that a standard dose of NAC may be insufficient in patients with severe APAP overdoses (Marks et al., 2017). Thus, there is a need for additional antidotes to cover some of the therapeutic gaps.

4-Methylpyrazole (Fomepizole). More recently, a patient with severe APAP overdose was successfully treated with NAC and fomepizole because of assumed alcohol poisoning (Zell-Kanter et al., 2013). Fomepizole is an alcohol dehydrogenase inhibitor, which is a clinically approved antidote against ethylene glycol and methanol poisoning (McMartin, 2010). Later it was hypothesized that fomepizole may have contributed to the positive outcome in this APAP overdose patient (Yip and Heard, 2016) because it may also be a Cyp2E1 inhibitor \textit{in vitro} (Hazai et al., 2002). Studies with Cyp2E1-deficient mice (Lee et al., 1996) and with cyp2e1-humanized transgenic mice (Cheung et al., 2005) directly demonstrated the central role of this P450 enzyme for the bioactivation of APAP in mice and humans. Subsequently, it was shown in a mouse model \textit{in vivo} that co-treatment of APAP with fomepizole effectively protected due to prevention of the oxidative metabolism of APAP and the almost complete elimination of protein adduct formation, which is consistent with inhibition of Cyp2E1 as the cause of protection (Akakpo et al., 2018). This beneficial effect could also be confirmed in primary human hepatocytes (Akakpo et al., 2018) and in human volunteers where more than 90% of the oxidative metabolites of APAP could be eliminated by clinically approved doses of fomepizole in a cross-over study (Kang et al., 2020). However, besides inhibition of Cyp2E1, it was also
discovered that delayed treatment with fomepizole after the metabolism phase still effectively protected because of inhibition of JNK activation and prevention of the mitochondrial oxidant stress (Akakpo et al., 2019). This mechanism of action significantly expanded the therapeutic window of fomepizole as shown by the high efficacy even when given 18 h after APAP in primary human hepatocytes (Akakpo et al., 2021). This effect was superior to NAC treatment (Akakpo et al., 2021). In addition, in contrast to NAC (Slitt et al., 2004), fomepizole was also effective in preventing APAP-induced acute kidney injury (Akakpo et al., 2020). Furthermore, as the history of fomepizole use in patients for almost 20 years documented, there are no relevant side effects (Rasamison et al., 2020). Because of these positive results, fomepizole is currently used off-label for severe overdose patients where there is a chance that NAC may not be sufficient (Filip and Mullins, 2023), although in the absence of a successful clinical trial this is still controversial (Dear, 2023). Together, the detailed understanding of the mechanism of action and the observations that fomepizole is at least as effective or even better than NAC in experimental animals and human hepatocytes (Akakpo et al., 2022), justifies the current ongoing clinical trial to assess its efficacy as adjunct therapy to NAC in patients.

**Current Challenges and Knowledge Gaps**

Although the mechanisms of cell death in APAP-induced liver injury and the central role of mitochondria are well understood, there are still areas that require further studies including more information on the mitochondrial proteins that are being adducted by NAPQI resulting in superoxide formation and proteins modified by nitrotyrosine that cause the MPTP opening. In addition, the molecular mechanisms of mitochondrial biogenesis and liver regeneration need further investigation (Bhushan and Apte, 2019). Also, little is known on the impact of the spatial
transcriptomics and proteomics of hepatocytes and non-parenchymal cells on these injury and regeneration events (Umbaugh et al., 2021b). Another gap in our understanding of the pathophysiology is the transition from injury to regeneration versus development of acute liver failure. Furthermore, both diagnostic and prognostic biomarker research is underdeveloped. Although progress was made in the diagnostic area with the development of a potential bed-side assay to measure adducts (Roberts et al., 2017), other approaches such as a microRNA panel to distinguish APAP-induced liver injury from ischemic hepatitis (Ward et al., 2014) and earlier prediction of liver injury after an overdose by measuring miR-122 and other sensitive biomarkers (Antoine et al., 2013; Vazquez et al., 2020) are feasible. However, the time delay in measuring some of these parameters may make them less usable in clinical practice. Similar problems exist for prognostic biomarkers that could predict acute liver failure and death early enough to be able to save the patient with a liver transplant. Some biomarkers such as mtDNA, glutamate dehydrogenase, lactate dehydrogenase and carbamoyl phosphate synthase 1 were shown to have predictive value for negative outcome in overdose patients (Kim et al., 2013; McGill et al., 2014b; Vazquez et al., 2022). However, their predictive specificity is limited and some of these biomarkers require too much time to measure to be clinically useful. Thus, despite the substantial progress in understanding mechanisms of APAP hepatotoxicity, there are significant gaps in our knowledge that require more research.

**Perspective on Future Directions**

Based on the discussion of the known mechanisms of APAP-induced liver injury and acute liver failure and the gaps of knowledge identified in the previous paragraph, future research needs to
be prioritized. The most important areas of future research are better understanding of the mechanisms of regeneration and the development of compounds that promote this process. Closely related to that is the question of the mechanisms of acute liver failure development and potential molecular targets to prevent this detrimental event. Also, biomarkers are needed that predict early and with high accuracy and specificity negative outcome in APAP-induced acute liver failure but also can be measured easily and in time to have an impact on the clinical decision process. Together, these research directions will have the most translational impact.

**Summary and Conclusions**

Decades of intensive research has uncovered extensive information regarding cell signaling mechanisms involved in hepatocyte cell death after an APAP overdose. The critical requirement for cytochrome P450 mediated reactive metabolite formation as well as the central role of mitochondrial dysfunction in the process provided foundational knowledge in understanding mechanisms of cell death. The more recent discovery of the role of peroxynitrite in mediating mitochondrial damage as well as the requirement of cellular iron for formation of nitrotyrosine protein adducts provide further nuanced insight into the APAP pathophysiology. The recognition of various adaptive responses which attempt to mitigate damaging effects of the reactive metabolite on mitochondria or enhance liver recovery after central hepatocyte necrosis through induction of mitochondrial biogenesis highlight the complexities of the hepatic response to an APAP overdose. All this information gathered over years of research allowed deeper mechanistic understanding for development of therapeutic strategies to prevent liver injury induced by excessive APAP, resulting in the introduction of NAC, as well as use of newer therapeutics such as fomepizole, which benefit from the extensive studies in the mouse model that replicate the
human pathophysiology. Ongoing research into APAP pathophysiology has the potential to uncover further nuances of the hepatocyte response to an overdose and help develop additional therapeutics which can complement NAC and fomepizole, especially drugs that can promote regeneration.
Data Availability Statement

This review article contains no dataset generated or analyzed.

Authorship Contributions

Participated in research design: Jaeschke, Ramachandran.

Wrote or contributed to the writing of the manuscript: Jaeschke, Ramachandran.
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Figure Legend

**Figure 1: Steps in APAP-induced necrosis and adaptive responses.** APAP induced hepatotoxicity affects hepatocytes surrounding the hepatic central vein and is initiated by cytochrome P450 catalyzed formation of a reactive metabolite NAPQI, which overwhelms hepatic glutathione stores and subsequently forms protein adducts within the cell. Adducts on mitochondria induce superoxide generation from the organelle targeted to the cytosol which activates the MAP kinase JNK, causing its translocation to mitochondria. This amplifies the mitochondrial oxidant stress, where superoxide and nitric oxide formation results in production of the highly damaging free radical peroxynitrite. Uptake of lysosome-derived iron into mitochondria facilitates nitrotyrosine protein adduct formation by peroxynitrite. This ultimately induces the mitochondrial permeability transition pore opening with release of mitochondrial proteins such as endonuclease G (EndoG) and apoptosis inducing factor (AIF), which translocate to the nucleus, causing DNA fragmentation and hepatocyte necrosis. Several adaptive responses attempt to mitigate the effects of NAPQI formation starting with induction of autophagy to scavenge NAPQI cellular protein adducts. When this is overwhelmed and mitochondrial adducts are formed, the decrease in membrane potential induces adaptive changes in mitochondrial morphology which attempt to prevent mitochondrial dysfunction. Even after amplification of mitochondrial oxidant stress, the Nrf2 mediated activation of the antioxidant response further attempts to preserve cellular function and prevent cell death. Additional responses such as induction of mitochondrial biogenesis are activated in surviving hepatocytes surrounding areas of necrosis to facilitate liver recovery and a return to homeostasis. (Created with Biorender.com)