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Oxidant Stress and Lipid Peroxidation in Acetaminophen Hepatotoxicity

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ABSTRACT | Acetaminophen (APAP) overdose is the most frequent cause of liver injury and acute liver failure in many Western countries. The mechanism of APAP-induced hepatocyte necrosis has been investigated extensively. The formation of a reactive metabolite and its binding to cellular proteins were initially thought to be responsible for cell death. A competing hypothesis was introduced that questioned the relevance of protein binding and instead suggested that cytochrome P450-derived oxidant stress and lipid peroxidation cause APAP-induced liver injury. However, work over the last 15 years has reconciled some of these apparent contradictory hypotheses. This review summarizes the present state of knowledge on the role of reactive oxygen species (ROS) in APAP hepatotoxicity. Detailed investigations into the sources and relevance of the oxidant stress have clearly shown the critical role of the electron transport chain of mitochondria as the main source of the oxidant stress. Other potential sources of ROS such as cytochrome P450 enzymes or NADPH oxidase in phagocytes are of limited relevance. The mitochondria-derived superoxide and peroxynitrite formation is initiated by the binding of the reactive metabolite to mitochondrial proteins and the amplification by mitogen-activated protein kinases. The consequences of this oxidant stress are the opening of the mitochondrial membrane permeability transition pore with cessation of ATP synthesis, nuclear DNA fragmentation, and ultimately cell necrosis. Lipid peroxidation is not a relevant mechanism of cell death but can be a marker of ROS formation. These mechanistic insights suggest that targeting mitochondrial oxidant stress is a promising therapeutic option for APAP hepatotoxicity.

KEYWORDS | Acetaminophen hepatotoxicity; Cytochrome P450; Lipid peroxidation; Mitochondria; Neutrophils; Reactive oxygen species

ABBREVIATIONS | AMAP, *N*-acetyl-*m*-aminophenol; APAP, acetaminophen; ASK1, apoptosis signal-regulating kinase 1; DAMP, damage-associated molecular pattern; GSH, reduced glutathione; GSSG, glutathione disulfide; HMGB1, high mobility group box 1; JNK, c-jun N-terminal kinase; LPO, lipid peroxidation; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; MLP3, mixed-lineage protein kinase 3; MPT, mitochondrial permeability transition; MPTP, mitochondrial permeability transition pore; NAC, *N*-acetylcysteine; NAPQI, *N*-acetyl-*p*-benzoquinone imine; ROS, reactive oxygen species; SOD, superoxide dismutase

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1. INTRODUCTION

Acetaminophen (APAP) is a widely used analgesic and antipyretic drug. It is considered safe at therapeutic doses but can cause liver injury and even acute liver failure and death upon overdosing [1, 2]. Overdose may occur either via taking a single large dose in a suicidal attempt or via unintentionally taking multiple large doses because APAP is present in numerous over-the-counter medications. As such, APAP is one of the most consumed drugs but is also one of the most important causes of drug-induced liver injury in the clinic. For this reason, the mechanisms of APAP hepatotoxicity have been investigated in animal models and in humans for more than 40 years [3, 4]. The first hypothesis to explain the toxicity was that a small part of APAP is being metabolized by cytochrome P450 enzymes to form a reactive metabolite, which can be detoxified by reduced glutathione (GSH), but when the capacity of hepatic GSH is exhausted, it also covalently binds to cellular proteins and causes cell death [5–7]. This early insight into the mechanism of cell death led to the development of *N*-acetylcysteine (NAC) as a clinical antidote [8]. However, the covalent binding hypothesis was also challenged by an oxidant stress and lipid peroxidation (LPO) hypothesis [9], creating a significant controversy at the time [10, 11]. This review discusses how this oxidant stress hypothesis has evolved and the current view of the co-existence of covalent binding and oxidant stress in the pathophysiology of APAP-induced cell death.

2. SOURCES OF OXIDANT STRESS: CYTOCHROME P450 ENZYMES

The first evidence of an oxidant stress after APAP overdose was reported in the late 1970s when it was shown that APAP-overdosed mice exhaled LPO products such as ethane and pentane [9]. Because it was newly recognized at the time that in a regenerating system in vitro, microsomal cytochrome P450 enzymes can produce superoxide and hydrogen peroxide [12], it was hypothesized that the metabolism of APAP triggered the oxidant stress [9]. This idea was supported by subsequent experiments showing reduced LPO and protection against APAP hepatotoxicity with cytochrome P450 inhibitors and the opposite effects with inducers [13]. Later studies indicated that the main cytochrome P450 enzyme involved in oxidative APAP metabolism is CYP2E1 [14], which also participates in ethanol metabolism where it has been postulated to be a major source of ROS in ethanol-induced liver injury [15]. Both leakage of ROS during ethanol metabolism and induction of CYP2E1 expression by ethanol with subsequent ROS formation through uncoupling have been shown [15]. Based on this insight from ethanol toxicity, it was assumed that the CYP2E1-dependent metabolism of APAP is responsible for the oxidant stress [16]. However, very early after a role of ROS was first shown, the relevance of a metabolism-dependent oxidant stress was questioned because no direct evidence for ROS formation was found. If superoxide and hydrogen peroxide are formed during the metabolism of APAP, some of them would be detoxified by copper-zinc superoxide dismutase (SOD1) and glutathione peroxidase, which would lead to glutathione disulfide (GSSG) formation in the cytosol. Such an oxidant stress would cause an increase in hepatic GSSG levels and release of GSSG into the bile and plasma as was shown for exposure to known oxidants such as *tert*-butyl hydroperoxide [17, 18] or the redox-cycling agent diquat [19]. However, APAP metabolism did neither cause elevated GSSG levels in the liver nor enhanced GSSG release into the bile or plasma indicating that there is no relevant oxidant

stress during the metabolism phase both in rats [17] and in mice [20]. In addition, when the formation of ROS was measured by 2′,7′-dichlorofluorescein (DCF) fluorescence in isolated mouse hepatocytes, an oxidant stress was observed only after GSH depletion at 3–4 h after APAP exposure [21]. This again suggests that there is no oxidant stress during the main metabolism phase of APAP in cultured hepatocytes [21]. Furthermore, when rats were exposed to a high overdose of APAP there was extensive depletion of hepatic GSH levels and formation of protein adducts indicating oxidative metabolism of APAP through the cytochrome P450 system [22]. However, despite this extensive metabolism, no evidence of an oxidant stress or liver injury was detected in these rats in vivo [22]. Taken together, attempts to detect direct evidence for ROS formation during the metabolism phase of APAP toxicity consistently failed to find any support in rats or mice and in cultured hepatocytes. The early observations that LPO and toxicity correlated with cytochrome P450 activities [13] does not contradict these findings because the formation of the reactive metabolite NAPQI and covalent binding to proteins are the initiating event in the toxicity. Thus, cytochrome P450 enzymes including CYP2E1 are unlikely a relevant source of the oxidant stress observed during APAP toxicity.

3. SOURCES OF OXIDANT STRESS: MITOCHONDRIA

During the controversial discussion whether an oxidant stress actually occurs in the course of APAP hepatotoxicity, it was discovered that hepatic GSSG levels increased substantially after the metabolism of APAP was over and GSH levels started to recover [23]. Interestingly, there was no GSSG release into the bile suggesting that GSSG must be formed in a compartment where it cannot be released into the cytosol [23]. Mitochondria are cell organelles that can only exchange GSH but not GSSG between the matrix and the cytosol [24]. Therefore, when mitochondria were isolated and GSSG was measured in these organelles at various times after APAP, it was estimated that almost all of the GSSG detected in the whole liver is actually located within mitochondria [23, 25]. This mitochondrial oxidant stress was confirmed in cultured hepatocytes using MitoSox Red [26]. In addition, after it was reported that the potent oxidant peroxynitrite was formed from superoxide and nitric oxide during APAP hepatotoxicity [27], it was shown that this peroxynitrite was also generated predominantly inside mitochondria [28]. Consistent with this observation, a loss of mitochondrial DNA and of SOD2, both located in the mitochondrial matrix, was found during APAP toxicity [28, 29]. Thus, there is extensive, direct evidence that the formation of superoxide, hydrogen peroxide, and peroxynitrite during APAP-induced liver injury occurs mainly within mitochondria.

The critical question whether this mitochondrial oxidative and nitrosative stress is actually relevant for the pathophysiology was subsequently addressed in a number of experiments. Support for the role of peroxynitrite was provided by the delayed treatment with sulfhydryl reagents GSH or NAC, which promoted the recovery of hepatic and mitochondrial GSH levels [30–32]. This mitochondrial GSH directly scavenged peroxynitrite [30] and detoxified ROS [30, 32] and protected against APAP-induced liver injury [30–32]. In addition, the SOD mimetic Mito-TEMPO effectively protected against APAP-induced liver injury by dismutating superoxide and preventing peroxynitrite formation [33]. The fact that the mitochondria-targeted Mito-TEMPO was an order of magnitude more potent than TEMPO, which does not accumulate selectively in mitochondria, further supports the critical role of mitochondrial oxidant stress [33]. The phenolic compound resveratrol, which is a known antioxidant [34], effectively protected against APAP hepatotoxicity by scavenging peroxynitrite and preventing mitochondrial dysfunction [35]. In addition to the pharmacological intervention to scavenge ROS, mice with a partial deficiency of mitochondrial SOD2 proved to be much more susceptible to APAP toxicity [36]. The enhanced susceptibility to APAP-induced liver injury in these mice was correlated with enhanced levels of GSSG, protein carbonyls, and nitrotyrosine adducts in the mitochondria [36]. More recently, several studies provided evidence that inhibiting ROS formation in mitochondria may be equally effective as scavenging them. Complex I of the mitochondrial electron transport chain is known to be an important source of ROS formation [37] and biguanides such as metformin are known to arrest complex I in the deactivated state thereby preventing ROS leakage [38]. Consistent with these observations, metformin profoundly protected against APAP hepatotoxicity

by inhibiting complex I thereby substantially attenuating the mitochondrial oxidant stress [39]. MCJ (methylation-controlled J protein), an endogenous regulator of complex I, reduces its activity [40]. Deficiency of MCJ in $CD8^+$ T cells increased complex I activity and mitochondrial respiration but not mitochondrial ROS formation by promoting the formation of electron transport chain supercomplexes [41]. Interestingly, mice with MCJ-deficiency were highly protected against APAP hepatotoxicity, which correlated with improved mitochondrial function and reduced mitochondrial oxidant stress [42]. In summary, the direct data for mitochondrial oxidant stress and peroxynitrite formation and the consistent beneficial effects when ROS are either scavenged or their formation is prevented, provide convincing evidence for the critical role of mitochondrial ROS derived from the electron transport chain in the pathophysiology of APAP-induced liver injury.

One aspect that has not been investigated in great detail is the question why the electron transport chain is leaking more electrons after APAP exposure. Early studies have shown that APAP can lead to inhibition of mitochondrial respiration [43]. Importantly, the mitochondrial dysfunction correlates with protein adduct formation in mitochondria [44]. A hepatotoxic dose of APAP caused extensive protein adducts formation in the whole liver and in mitochondria [44]. However, *N*-acetyl-*m*-aminophenol (AMAP), a non-toxic analog of APAP in mice, triggered extensive protein adducts formation only in the whole liver but not in mitochondria [44]. Interestingly, AMAP caused cytotoxicity in human hepatocytes, which correlated with mitochondrial protein adducts formation [45]. Although the specific adducted proteins in mitochondria that induce the ROS leakage from the electron transport chain have yet to be identified, the current data support the concept that the critical initiating event is the formation of the reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) and its reaction with mitochondrial proteins.

Although the initial protein adduct formation peaks by about 1–2 h after APAP treatment in the mouse [46, 47], the full blown mitochondrial oxidant stress and parenchymal cell necrosis occur several hours later [48]. This suggests that the initial oxidant stress is insufficient to trigger the mitochondrial permeability transition pore (MPTP) opening and cell death. More recently, it was recognized that the mitogen-activated protein kinase (MAPK) c-Jun N-terminal kinase (JNK) is activated (phosphorylated) and P-JNK translocates to the mitochondria [49]. P-JNK binds to Sab (SH3 domain-binding protein that preferentially associates with Btk) in the outer mitochondrial membrane and triggers through several additional proteins a further impairment of the electron transport chain and enhanced ROS formation [50]. Thus, the JNK activation pathway in vivo clearly represents an amplification cycle for oxidant stress and peroxynitrite formation in the mitochondria [51]. Although it was hypothesized that JNK is activated by oxidant stress, JNK is not a redox-sensitive kinase. Apoptosis signal-regulating kinase 1 (ASK1), which is sequestered by thioredoxin and can be activated by thioredoxin oxidation [52, 53] and mixed-lineage protein kinase 3 (MLK3) [54] are redox-sensitive MAP kinases, which activate JNK through the MAP2 kinase MKK4 [55]. Inhibition or deletion of each of these kinases attenuates APAP-induced liver injury suggesting that this amplification loop for the oxidant stress is critical for cell death in vivo [52–55].

4. SOURCES OF OXIDANT STRESS: NADPH OXIDASE

The extensive necrosis during APAP-induced liver injury leads to the release of damage-associated molecular patterns (DAMPs), which trigger a sterile inflammatory response with the formation of cytokines and chemokines and the activation and recruitment of phagocytes in the liver including Kupffer cells, neutrophils, and blood monocytes [56–58]. All these inflammatory cells contain NADPH oxidase (NOX2), which can release superoxide and hydrogen peroxide into the phagosome or to the extracellular space [59]. In addition, the neutrophils contain myeloperoxidase, which can form the potent oxidant and chlorinating agent hypochlorous acid from hydrogen peroxide and chloride [59]. In previous studies, it was clearly demonstrated that Kupffer cell-induced oxidant stress (mainly hydrogen peroxide) contributes to the early phase of hepatic ischemia-reperfusion injury [60]. Neutrophils are responsible for a second injury phase mainly by generating hypochlorite [61]. Although the ROS are generated outside of hepatocytes, due to the close proximity of the inflammatory cells to the target, these oxidants enter the cells and generate an intracellular oxidant stress in hepatocytes [62]. The presence of neutrophil-derived oxidants was

demonstrated by increased intracellular GSSG levels and chlorotyrosine protein adducts in hepatocytes during the neutrophil-induced injury phase in different models [61, 63–65]; this intracellular oxidant stress could be eliminated by inhibiting the neutrophil cytotoxicity [65, 66]. Moderate extracellularly derived oxidant stress results in mitochondrial dysfunction, mitochondrial oxidant stress, MPTP opening, and necrotic cell death [67, 68]. Thus, in order to cause tissue injury, the phagocytes need to be recruited into the tissue and generate these oxidants in sufficient quantities and long enough durations close to the target cells in order to be able to disturb the intracellular homeostasis of the cells and generate mitochondrial dysfunction leading to the MPTP opening and cell death [62].

The first report of Kupffer cell activation and its potential impact on the injury after APAP overdose was reported in rats [69]. However, these findings were never confirmed and are unlikely to be relevant as rats show a high resistance to APAP and are a poor model for the human pathophysiology [22]. Nevertheless, a second report using a mouse model suggested that inactivation of Kupffer cells with gadolinium chloride ($GdCl_3$) treatment eliminated nitrotyrosine staining and liver injury [70]. These findings, however, were questioned for a number of reasons. First, the most active Kupffer cells are located in the periportal region [71], which makes it unlikely that ROS and peroxynitrite generated by these cells can selectively cause cell death in the centrilobular area. Second, deficiency of NADPH oxidase activity (in $gp91^{phox}$ knockout mice), the principal enzyme responsible for ROS formation in Kupffer cells, did not prevent the oxidant stress or injury [31]. Third, elimination of Kupffer cells with clodronate liposomes did not protect against but actually even increased the injury [72]. Together, these data do not support the hypothesis that Kupffer cell-derived oxidant stress can be responsible for APAP-induced liver injury.

Neutrophils are recruited into the liver after the early injury phase [73]. Necrotic cells release DAMPs including high mobility group box 1 (HMGB1) protein, and mitochondrial DNA and nuclear DNA fragments [74, 75], which activate toll like receptors, such as TLR9 [57, 58], leading to activation of neutrophils by the formation of cytokines and chemokine [57, 58]. The controversial question is whether these neutrophils actually cause additional injury. In support of neutrophil-mediated injury, it was shown that neutropenia attenuates APAP hepatotoxicity [76, 77]. However, it was argued that early pretreatment with a neutropenia-inducing antibody actually causes a preconditioning effect that is responsible for the protection rather than the absence of neutrophils [78]. In addition, there is no neutrophil activation and priming for ROS formation during the early injury phase in mice or humans [79, 80]. There is no direct evidence for a neutrophil-induced oxidant stress (chlorotyrosine staining) [81] and interventions that reduce neutrophil transmigration (anti-CD18 antibodies, CD18- or ICAM-1-deficiency) [73, 79, 81] have no effect on the oxidant stress and injury during APAP hepatotoxicity. Although not every result published in favor of a neutrophil-mediated injury phase can be readily explained as being caused by an off-target effect, it is difficult to overlook the fact $gp91^{phox}$ knockout mice, which have neutrophils that are incapable of generating oxidant stress and causing cytotoxicity, show the same oxidant stress and peroxynitrite formation and liver injury after APAP overdose as wild type animals [31, 80]. Thus, the preponderance of experimental evidence does not support a neutrophil-induced injury during APAP hepatotoxicity but indicates that the main purpose of neutrophil recruitment into the injured liver is to assist in clean-up of necrotic tissue in preparation of regeneration [56, 57].

Blood-derived monocytes are also recruited into the liver of animals with APAP-induced liver injury. They accumulate mainly at later time points, i.e., after the peak of injury, and preferentially localize in the areas of necrosis [82, 83]. Although these cells contain NADPH oxidase and can generate ROS under certain conditions [84], in the case of APAP they develop an anti-inflammatory macrophage phenotype (M2) [82, 85, 86]. The recruitment occurs mainly through monocyte chemoattractant protein-1 (MCP-1, CCL2), which can be generated not only by macrophages but also by injured hepatocytes [82]. The receptor for MCP-1 on monocytes/macrophages is CCR2. Mice with CCR2 deficiency are not protected against APAP toxicity but show reduced regeneration and repair of the necrotic lesions [82, 83, 87, 88]. Similarly, animals deficient of MCP-1 experience the same injury as wild type animals but again have a delayed repair [82]. These results indicate that blood-derived monocytes/macrophages are important for the recovery from the injury and do not likely

participate in the injury process. Furthermore, the fact that gp91^{phox} knock out mice, which have a non-functional NADPH oxidase, show a similar recovery as wild type animals suggesting that the removal of necrotic cell debris by these phagocytes is independent of ROS formation [80].

In summary, all phagocytes have the capacity to generate ROS and all have been shown to be involved in various inflammatory tissue injury models including hepatic ischemia-reperfusion injury, obstructive cholestasis, and endotoxin shock [89]. However, extensive investigations into the role of these inflammatory cells in APAP hepatotoxicity did not provide any convincing evidence that inflammatory cell-derived oxidant stress is a relevant factor in the pathophysiology.

5. CONSEQUENCES OF OXIDANT STRESS: LIPID PEROXIDATION

Lipid peroxidation (LPO) is frequently, even today, invoked as a mechanism of cell death during APAP hepatotoxicity [90]. LPO is a free radical reaction process that is initiated by hydroxyl radical formation from hydrogen peroxide (Fe^{2+}-dependent Fenton reaction) and the generation of lipid radicals leading to the destruction of polyunsaturated fatty acids in lipid membranes [91]. LPO can cause rapid catastrophic breakdown of the membrane potential and ion gradients leading to cell necrosis. The lipid soluble vitamin α-tocopherol (vitamin E) is a highly effective radical scavenger that can interrupt the radical chain reaction and block LPO [91]. Early studies on LPO in the APAP mouse model used ethane and pentane exhalation and hepatic malondialdehyde as indicators of LPO [9, 13]. Massive LPO led to severe injury in these animals within 4 h [9, 13] and vitamin E pretreatment effectively inhibited LPO and injury induced by APAP [92]. Although these studies clearly support the hypothesis that LPO as the cause and not a consequence of the injury, one aspect that is frequently overlooked when discussing these experiments is that Wendel and coworkers used animals fed a vitamin E-deficient diet with a high content of polyunsaturated fatty acids [9, 13, 92]. However, a more recent follow-up study using animals on a regular diet found only minimal evidence for LPO after APAP and treatment with *d*-alpha-tocopheryl acetate, which increased hepatic tocopherol levels by almost 7-fold, had no effect on liver injury [93]. These data suggest that animals on a regular diet have enough lipid-soluble antioxidants to prevent extensive LPO and thus LPO is quantitatively not a relevant injury mechanism in APAP-induced liver injury [93]. However, the much higher susceptibility of animals on a diet low in vitamin E but high in polyunsaturated fatty acids indicates a potential danger under special circumstances of the oxidant stress generated after APAP overdose. On the other hand, these data also suggest that the mechanism of cell death even in vivo can be changed based on the experimental conditions. This means that special attention needs to be paid to the translational relevance of any experimental design.

6. OXIDANT STRESS AND MITOCHONDRIAL PERMEABILITY TRANSITION PORE OPENING

The mitochondrial permeability transition (MPT) is a phenomenon whereby the mitochondrial inner membrane becomes permeable to molecules of less than 1500 da [94], resulting in release of mitochondrial proteins into the cytosol and subsequent mitochondrial dysfunction. It has been well established that APAP overdose causes activation of the MPT, which results in the dissipation of the proton gradient across the inner mitochondrial membrane and the consequent loss of mitochondrial membrane potential, a collapse of ATP generation, leading to hepatocyte necrosis [95, 96]. The regulation of the MTP has been shown to be influenced by matrix calcium and free radicals, and is also dependent on electron flux through the electron transport chain, especially from complex I [97]. This is relevant in light of the earlier mentioned, beneficial effect of metformin against APAP hepatotoxicity [39], which inhibited complex I activity. The molecular composition of the mitochondrial permeability transition pore (MPTP) has been under intense investigation for decades and the current consensus implicates the proteins Bax and Bak as its components on the outer mitochondrial membrane [98, 99], which function along with subunits of the ATP synthase on the inner mitochondrial membrane [94].The concept that ATP synthase may form a component of the MPTP originated from the identification of the interaction of its lateral stalk (the Oligomycin Sensitivity Conferring Protein, OSCP)

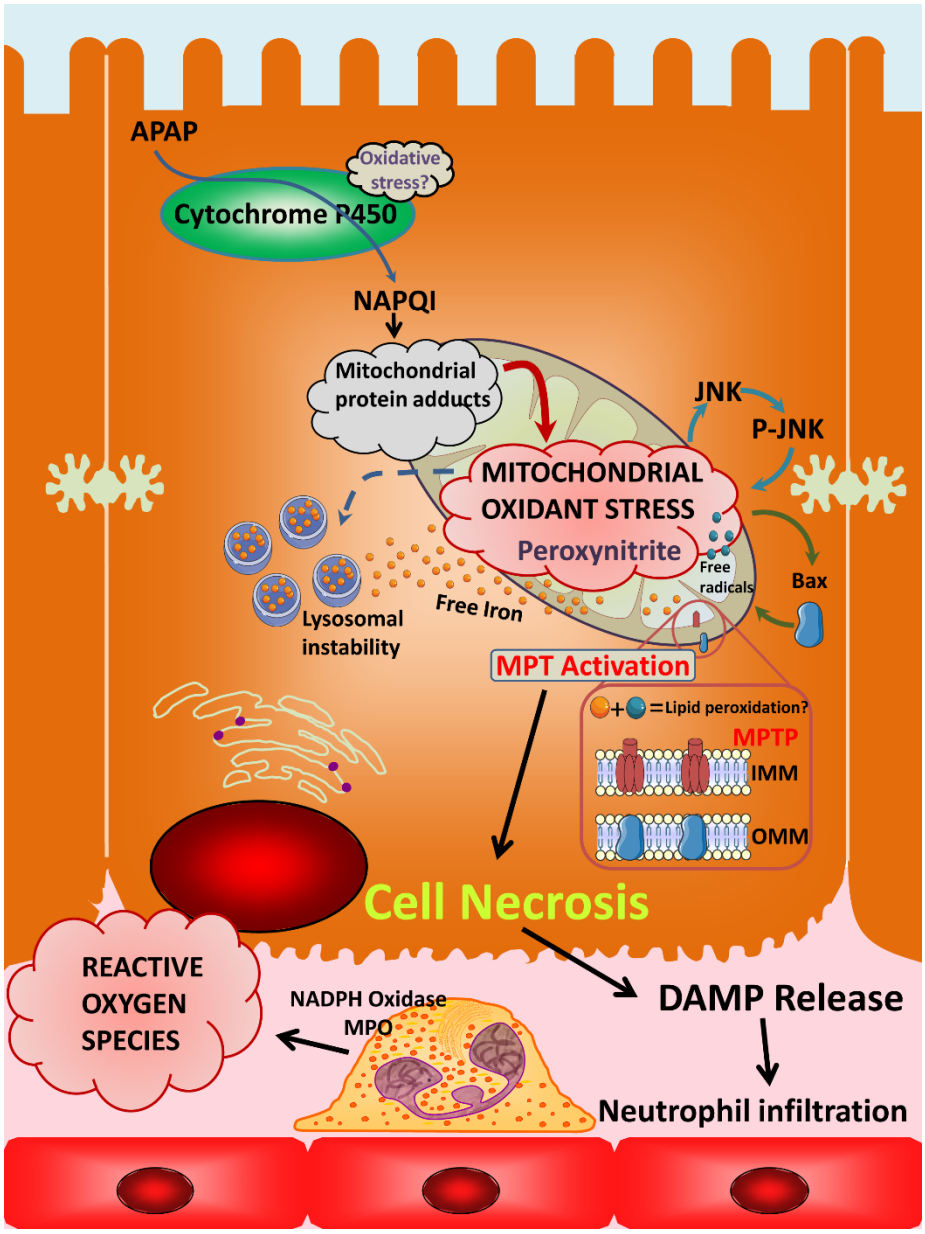

FIGURE 1. Reactive oxygen species and APAP hepatotoxicity. APAP is metabolized through the cytochrome P450 system to generate the reactive metabolite NAPQI, in a step that is unlikely to produce significant oxidant stress. NAPQI forms mitochondrial protein adducts, which induces mitochondrial oxidant stress and peroxynitrite formation and activates the MAPK JNK, which translocates to mitochondria and amplifies ROS production within mitochondria. This also causes lysosomal instability and release of free iron, which is taken up by mitochondria. Along with translocation of Bax to the mitochondrial outer membrane (OMM), this initiates the mitochondrial permeability transition (MPT) mediated by Bax on the OMM and components of the ATP synthase on the inner mitochondrial membrane (IMM). MPT activation subsequently causes cellular necrosis and release of DAMPs, which then result in neutrophil infiltration. Neutrophils contain NADPH oxidase and myeloperoxidase, which could then generate ROS outside the hepatocytes.

with cyclophilin D, the best characterized component of the MPTP [100]. Inhibition of cyclophilin D was shown to provide partial protection against APAP-induced hepatocyte necrosis in vitro [95]. However, this effect depends on the doses of APAP in vivo, with protection against liver injury at a moderate dose of 200 mg/kg [101], but no effect at a higher dose of 600 mg/kg [102]. This dose dependence of APAP's effects on the MPT was also seen using intravital microscopy, where a dose of 150 mg/kg APAP resulted in transient loss of membrane potential without cell necrosis [103]. In addition to calcium and ROS, cellular iron has also been shown to induce the MPT [104]. The ferric chelator deferoxamine (Desferal) was shown to delay APAP hepatotoxicity in mice [105] and an increase in cytosolic chelatable ferrous iron, presumably from disrupted lysosomes [106], which is taken up into polarized mitochondria to trigger the MPT and hepatocyte necrosis, was observed after APAP treatment of hepatocytes in vitro [107]. Further characterization of the lysosome to mitochondrial iron transfer revealed that lysosomally targeted iron chelation or inhibition of the mitochondrial calcium uniporter provided significant protection against mitochondrial depolarization and APAP hepatotoxicity [108] suggesting that cellular iron acts in concert with calcium and ROS to influence the MPT.

7. SUMMARY AND CONCLUSIONS

ROS and peroxynitrite are critical mediators of APAP-induced cell death (**Figure 1**). The electron transport chain in mitochondria is the principal source of the oxidant stress. Binding of NAPQI to mitochondrial proteins initiates the oxidant stress, which is further amplified through JNK activation. The dependence of the oxidant stress on reactive metabolite formation and protein binding reconciles previously competing hypotheses. Other sources of ROS such as cytochrome P450 enzymes and NADPH oxidase appear to be of limited relevance in the pathophysiology (**Figure 1**). The major consequence of the mitochondrial oxidant stress is the opening of the MPTP, which leads to cessation of ATP synthesis, nuclear DNA fragmentation, and ultimately necrotic cell death. LPO is quantitatively insufficient to cause cell death but can be considered as evidence for oxidant stress. Based on this mechanistic insight, potential therapeutic targets are interventions to prevent the mitochondria oxidant stress, to scavenge superoxide or peroxynitrite inside mitochondria, to prevent the MPTP formation, and to remove damaged mitochondria by autophagy and replace them by biogenesis.

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