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Role of Mitochondrial Iron Uptake in Acetaminophen Hepatotoxicity †

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Abstract

Overdose of acetaminophen (APAP) produces fulminant hepatic necrosis. The underlying mechanism of APAP hepatotoxicity involves mitochondrial dysfunction, including mitochondrial oxidant stress and the onset of mitochondrial permeability transition (MPT). Reactive oxygen species (ROS) play an important role in APAP-induced hepatotoxicity, and iron is a critical catalyst for ROS formation. This review summarizes the role of mitochondrial ROS formation in APAP hepatotoxicity and further focuses on the role of iron. Normally, hepatocytes take up Fe^{3+}-transferrin bound to transferrin receptors via endocytosis. Concentrated into lysosomes, the controlled release of iron is required for the mitochondrial biosynthesis of heme and non-heme iron-sulfur clusters. After APAP overdose, the toxic metabolite, NAPQI, damages lysosomes, causing excess iron release and the mitochondrial uptake of Fe^{2+} by the mitochondrial calcium uniporter (MCU). NAPQI also inhibits mitochondrial respiration to promote ROS formation, including H_2O_2, with which Fe^{2+} reacts to form highly reactive •OH through the Fenton reaction. •OH, in turn, causes lipid peroxidation, the formation of toxic aldehydes, induction of the MPT, and ultimately, cell death. Fe^{2+} also facilitates protein nitration. Targeting pathways of mitochondrial iron movement and consequent iron-dependent mitochondrial ROS formation is a promising strategy to intervene against APAP hepatotoxicity in a clinical setting.

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acetaminophen; iron; mitochondria; NAPQI; •OH; oxidative stress

1. Introduction

1.1. Epidemiology of Acetaminophen Hepatotoxicity

Acetaminophen (also known as Tylenol®, paracetamol, and N-acetyl-para-aminophenol and commonly abbreviated for the latter as APAP) is one of the most used antipyretic and analgesic medications and is often combined with cough-and-cold remedies and narcotic pain relievers. APAP is generally very safe in therapeutic doses. However, an overdose of APAP causes severe liver injury, leading to elevations of serum transaminases (ALT and AST), hepatic necrosis, and even acute liver failure requiring liver transplantation [1]. APAP hepatotoxicity is the leading cause of acute liver failure in the United States, and up to 50% of cases are unintentional [2]. The currently recommended maximal therapeutic dose is 4 g/day. However, it is estimated that 6% of adults in the USA are taking over 4 g/day due to APAP combination medications [3].

1.2. Metabolism of APAP

At therapeutic doses in humans, 85–90% of APAP becomes conjugated with sulfate and glucuronide and is excreted in urine. Only a small portion of APAP is metabolically activated by cytochrome P450 enzymes (mainly CYP2E1) to the toxic and reactive metabolite, *N*-acetyl-*p*-benzoquinoneimine (NAPQI). Under normal conditions, NAPQI is efficiently detoxified by conjugation with glutathione (GSH) [4]. After an overdose of APAP, the sulfate and glucuronide pathways become saturated, and CYP450 produces relatively more NAPQI. Subsequently, GSH becomes depleted by conjugation with NAPQI, and additional NAPQI can no longer be detoxified, which then leads to liver damage [5,6].

1.3. Risk Factors of APAP Hepatotoxicity

APAP toxicity shows a threshold dose dependence such that therapeutic doses are completely non-toxic, but the threshold dose causing liver damage varies between individuals. Not all individuals with APAP overdose progress to acute liver failure. Moreover, even at a therapeutic dose, APAP hepatotoxicity can occur under certain conditions. Accordingly, the safe upper limit of APAP for therapeutic indications remains controversial [7–9]. Genetic variation within the CYP450 system can cause differing sensitivity to APAP hepatotoxicity, as well as to other risk factors [10,11].

Malnutrition, fasting, and chronic liver disease may increase the risk of APAP hepatotoxicity by decreasing hepatic levels of GSH. A 6 h fast depletes hepatic GSH levels in mice by 44% [12]. Patients with already low GSH stores as a result of fasting or malnutrition can develop severe hepatotoxicity at recommended doses of APAP [13]. Infants and adults who are alcoholic or who take certain CYP450-inducing drugs may also be more prone to liver injury from APAP [14–16]. Commonly used upregulating CYP450 drugs include rifampin, isoniazid, and phenobarbital. Chronic alcohol use also causes CYP450 enzyme induction

with the increased toxic metabolism of APAP to NAPQI and enhanced hepatotoxicity, even at therapeutic doses. Fibrates, nonsteroidal anti-inflammatory drugs (NSAIDs), and alcohol are associated with a higher incidence of death in patients with APAP-associated liver injury [17]. Nonalcoholic fatty liver disease (NAFLD), recently renamed metabolic dysfunction-associated steatotic liver disease (MASLD) [18], is also associated with increased CYP2E1 activity and is accompanied by an increased risk of APAP-induced hepatotoxicity [19].

1.4. Treatment for APAP Hepatotoxicity

Early diagnosis means early intervention, which is crucial to prevent APAP-induced acute liver failure (ALF). *N*-acetylcysteine (NAC) is the preferred antidote for APAP toxicity. NAC prevents hepatotoxicity by replenishing GSH stores, binding with NAPQI, and enhancing sulfate conjugation [20]. NAC may further limit APAP toxicity through antioxidant and anti-inflammatory effects. For maximal protection against liver injury, NAC should be given within 8 h after an APAP overdose in patients whose plasma APAP levels are above the “possible hepatic toxicity” line of the Rumack–Matthew nomogram [21,22]. NAC can be given intravenously or by mouth with similar efficacy for improving outcomes in APAP overdoses [23]. However, the indications and dosage for NAC are debated. Other treatments include activated charcoal and liver transplantation. Activated charcoal can be used within 4 h after taking APAP to limit the gastrointestinal absorption of APAP [24]. However, this treatment is ineffective in most cases because of the rapid absorption of APAP. Liver transplantation is the ultimate treatment for patients with ALF [25].

2. Role of Mitochondria in Pathogenesis of APAP Hepatotoxicity

The toxic metabolite NAPQI, rather than APAP itself, causes hepatotoxicity [26]. The main mechanism causing liver injury is thought to be covalent NAPQI protein adduct formation, which leads to mitochondrial dysfunction, oxidative stress due to GSH depletion by conjugation with NAPQI, and cell death [27].

2.1. Mitochondrial Permeability Transition in APAP Hepatotoxicity

Mitochondria are a primary target of NAPQI. The expression of some CYP2E1 in the mitochondrial inner membrane rather than the endoplasmic reticulum may account, at least in part, for mitochondrial NAPQI protein adduct formation [28–30]. Mitochondrial protein adduct formation with NAPQI causes oxidative stress, which leads to various mitochondrial dysfunctions, including respiratory inhibition, decreased hepatic ATP, decreased mitochondrial membrane potential ($\Delta\Psi$), and the onset of the mitochondrial permeability transition (MPT) [31,32]. Interestingly, low-dose APAP, which does not cause necrosis in vivo, can still produce MPT-dependent mitochondrial depolarization, which is reversible [33,34].

The MPT is an abrupt increase in the permeability of the mitochondrial inner membrane to molecules of less than about 1500 Daltons in molecular weight [35,36]. Ca^{2+} activates MPT onset, whereas cyclosporin A (CsA) and non-immunosuppressive analogs like NIM811 inhibit permeability transition (PT) pore opening [37,38]. In one model, PT pores are formed by the voltage-dependent anion channel (VDAC) in the outer membrane, the adenine

nucleotide translocator (ANT) in the inner membrane, and cyclophilin D (CypD) in the matrix. However, the genetic deletion of ANT1/ANT2 and VDAC does not prevent the onset of the MPT [39–41], although more recent studies in triple ANT1, 2, and 4 and CypD-deficient mice and cell lines indicate that the MPT requires ANT and CsA-binding CypD [42,43]. Other studies suggest that dimers or oligomers of the mitochondrial F_1Fo-ATP synthase or the c-rings of the F_O subunit of the synthase form PT pores [44–46], but other studies show that Ca^{2+}-induced PT pore-opening persists after genetic interventions that prevent assembly ATP synthase monomers, dimers, or oligomers [47–49]. Another recent study concludes that the ATP synthase is a negative rather than a positive regulator of PT pores [50]. In addition, regulated and unregulated conductance modes for PT pores have been described: one activated by Ca^{2+} and inhibited by CsA and the other not requiring Ca^{2+} for activation and not inhibited by CsA [51]. Consistent with regulated and unregulated pore opening, a different model of pore formation and gating proposes that PT pores are created by misfolded integral membrane proteins damaged by oxidants and other stresses. These misfolded proteins aggregate at exposed hydrophilic surfaces within the membrane bilayer to form aqueous channels. Chaperone-like proteins, including CypD, a peptidyl-prolyl cis-trans isomerase or foldase, initially block conductance through these misfolded protein clusters. However, increased Ca^{2+} acting on CypD opens these regulated PT pores, which is an effect blocked by CsA. When protein clusters exceed chaperones available to block conductance, unregulated pore opening occurs [51,52]. Thus, in this proposal, PT pores comprise multiple different molecular species, which is a conclusion increasingly made in experimental studies [42,53–55]. Nonetheless, the precise molecular composition of the PT pore or pores remains controversial.

CsA specifically blocks the MPT by binding to CypD [56]. NIM811 (N-methyl-4-isoleucine cyclosporin) is a non-immunosuppressive derivative of CsA that inhibits the MPT equivalently to CsA in isolated mitochondria [38,57]. NIM811 is protective to cultured hepatocytes and livers in vivo after a variety of injurious stresses, including ischemia/reperfusion injury, transplantation, massive hepatectomy, and cholestatic injury [58–61]. CsA and NIM811 also inhibit the MPT and attenuate APAP hepatotoxicity both in vivo and in vitro [31,33,62,63]. As discussed above, PT pores have two open conductance modes—a Ca^{2+}-activated and CsA-sensitive regulated mode associated with early PT pore opening and an unregulated mode occurring later, which does not require Ca^{2+} and is not inhibited by CsA [51]. In cultured mouse hepatocytes, CsA and NIM811 delay but do not prevent APAP-induced mitochondrial depolarization, indicating that APAP initially induces a regulated MPT that is later superseded by an unregulated MPT [31]. Ultimately, the release of proapoptotic mitochondrial proteins, together with the cessation of ATP production, leads to cell death [31,64,65].

2.2. Apoptosis and Necrosis in APAP Hepatotoxicity

Whether apoptosis or necrosis is the major mode of cell death in APAP hepatotoxicity has been a controversial topic. The MPT plays an important role in the development of both necrotic and apoptotic cell death [66]. Specifically, the uncoupling of oxidative phosphorylation after the MPT causes ATP depletion, which leads to necrotic cell killing, whereas the mitochondrial outer membrane rupture after MPT-induced mitochondrial

swelling causes cytochrome *c* release and apoptosis. In vitro, APAP mainly induces necrosis in cultured mouse hepatocytes. However, apoptosis increases when necrotic cell death is blocked [67]. Animal studies suggest that APAP-induced hepatic damage is predominantly oncotic necrosis rather than apoptosis [68]. Although modest caspase activation resulting from the release of mitochondrial proteins may occur after APAP, it is insufficient to actually cause significant apoptotic cell death [69]. Nonetheless, a human study reported increased serum apoptotic markers in patients with APAP-induced acute liver failure and suggested the predictive role of apoptotic markers in the progression of acute liver failure after APAP overdose [70].

2.3. c-Jun N-Terminal Protein Kinase Activation in APAP Hepatotoxicity

In mice and cultured mouse hepatocytes after APAP exposure, c-Jun N-terminal protein kinase (JNK), a mitogen-activated protein kinase (MAPK), becomes phosphorylated, signifying activation [71]. Phospho-JNK (p-JNK) then translocates to mitochondria by binding and phosphorylating the outer membrane protein SAB, an abbreviation for the SH3 domain-binding protein that preferentially associates with Bruton's tyrosine kinase [72,73]. The subsequent release of protein tyrosine phosphatase nonreceptor type 6 (PTPN6) from SAB in the intermembrane space leads to the dephosphorylation of mitochondrial tyrosine-protein kinase c-SRC [74]. Decreased phospho-c-SRC leads to the inhibition of the respiratory chain, which enhances the generation of reactive oxygen species (ROS) [73,75]. The amplified oxidant stress then causes sustained JNK activation and promotes an APAP-induced MPT [32,76]. Platanosides, a botanical drug combination, decrease liver injury from APAP overdose in mice, possibly by preventing sustained JNK activation [77]. After low-dose APAP is given to mice, reversible hepatic mitochondrial dysfunction occurs associated with transient JNK activation [33].

3. Role of Oxidative Stress in APAP Hepatotoxicity

Oxidative stress is a principal mediator of toxicity and has been suggested as an important mechanism in APAP-induced hepatotoxicity. ROS formation increases after APAP exposure and agents that augment antioxidant defenses and scavenge ROS protect against APAP toxicity in vitro and in vivo [78]. The formation of ROS like $O_2^{\bullet -}$ occurs selectively in mitochondria after the initial metabolism of APAP and originates at least in part from Complex III of the respiratory chain [79–82].

The Fenton or iron-catalyzed Haber–Weiss reaction is critical following oxidative stress during APAP toxicity [83]. Initially, superoxide ($O_2^{\bullet -}$) may be formed by activated NADPH oxidase, loosely coupled CYP2E1, and the NAPQI-dependent disruption of the mitochondrial respiratory chain. Dismutation catalyzed by superoxide dismutase (SOD) converts $O_2^{\bullet -}$ to H_2O_2. After an APAP overdose, H_2O_2 cannot be completely detoxified by glutathione peroxidase since its cofactor, GSH, becomes depleted by NAPQI. $O_2^{\bullet -}$ also reduces ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). Fe^{2+}, thus, formed reacts rapidly with H_2O_2 to form the highly reactive hydroxyl radical (•OH) [27,81,83]. •OH, in turn, damages protein and DNA, as well as causing lipid peroxidation and the breakdown of membranes. However,

the most critical effect of this oxidative stress is the induction of the MPT, which produces bioenergetic failure and, ultimately, cell death [31,63].

4. Iron Metabolism

Iron is essential in the catalysis of many, if not most, enzymatic reactions that involve electron transfer and play a critical role in cellular survival. However, free iron is toxic due to its ability to generate free radicals via the Fenton reaction and to catalyze lipid peroxidation chain reactions [83,84]. Thus, the control of this necessary but potentially toxic metal is important for human health and disease. Iron homeostasis is tightly controlled by the regulation of its cellular import, storage, and intracellular movement [85,86].

4.1. Cellular Iron Metabolism

In animal cells, non-heme iron is transported into cells through two main pathways: transferrin (Tf)-bound iron uptake and non-Tf-bound iron (NTBI) uptake. NTBI uptake occurs when the body absorbs dietary iron from the intestinal lumen, or when Tf becomes saturated with iron because of iron overload. Although the exact NTBI uptake pathway is unclear, it is proposed that reductases, such as duodenal cytochrome *b* (Dcytb), reduce Fe^{3+} to Fe^{2+}, which is then imported into cells via divalent metal transporter 1 (DMT1) or ZRT/IRT-like proteins (ZIPs) [87–89].

Under physiological conditions, almost all serum iron is bound to Tf. The uptake of Tf-bound iron through Tf receptor-1 (TfR1) is the major pathway for the delivery of iron into cells [85,86]. Tf-dependent iron delivery begins with the binding of diferric Tf to TfR1 on the cell surface, followed by the endocytosis of the Tf-TfR1 complex. As pH decreases during endosome maturation and fusion with lysosomes, Fe^{3+} dissociates from Tf, and both Tf and TfR1 recycle to the cell surface for another round of iron uptake. A ferrireductase (Steap3) then reduces dissociated Fe^{3+} to Fe^{2+} within the endosomal/lysosomal compartment. Fe^{2+} subsequently exits the endosomal/lysosomal compartment into the cytosol via DMT1 or ZIP14 [90,91]. The release of Fe^{2+} from endosomal/lysosomal membranes appears to involve an Fe^{2+}/H^{+} exchange mechanism [92]. Iron released to the cytosol is in a soluble, chelatable state, which constitutes the labile iron pool (LIP). From this pool, iron can be stored in ferritin, utilized for metabolism (*e.g.*, imported into mitochondria for the synthesis of heme and Fe-S clusters), used to generate ROS, or exported from the cell by ferroportin 1 (FPN1) [85,86]. Notably, lysosomes are additionally involved in intracellular iron recycling because of the degradation of many macromolecules containing iron inside the lysosomal lumen [93].

4.2. Mitochondrial Iron Metabolism

Mitochondria utilize iron for the synthesis of heme and Fe-S clusters [94–97]. Iron moves into mitochondria using the following hypothesized mechanisms: (i) Iron-loaded endosomes/lysosomes interact directly with mitochondria by a “kiss-and-run” mechanism, leading to mitochondrial iron uptake [98]. (ii) Iron from ferritin transfers into mitochondria after ferritin complex degradation [99–101]. These mechanisms remain incompletely understood and need further study.

Two transporters, the mitochondrial calcium uniporter (MCU) and the two isoforms of mitoferrin (Mfrn1/2), play essential roles in transporting iron across the inner membrane. MCU catalyzes the electrogenic mitochondrial uptake of both Ca^{2+} and Fe^{2+} driven by the negative inside mitochondrial $\Delta\Psi$, which is blocked by the specific MCU inhibitor, Ru360 [81,102–104]. Mfrn1 and its paralog Mfrn2 also mediate mitochondrial iron uptake in erythroid and non-erythroid cells, respectively [105,106]. Because mitochondrial iron uptake is needed for heme synthesis, the deletion of Mfrn1 in hematopoietic tissues leads to anemia [106]. Some evidence indicates that Mfrn2 physically interacts with MCU, possibly as a component and/or regulator of the MCU complex [107].

Once imported into mitochondria, iron is utilized for the synthesis of heme and Fe-S clusters, which are incorporated into respiratory and other enzymes inside the mitochondria or exported to the cytosol to become prosthetic groups for cytosolic enzymes. Mitochondrial iron is also stored in mitochondrial ferritin (FTMT) [108].

4.3. Role of Iron in Common Models of Acute Liver Injury

However, when mitochondrial iron uptake results in iron overload and simultaneously H_2O_2 is generated by mitochondrial respiration that cannot be detoxified by antioxidant systems, Fe^{2+} and H_2O_2 react to form •OH, leading to lipid peroxidation, mitochondrial dysfunction, DNA damage, and a form of necrotic cell death now called ferroptosis [83,104,109,110]. Iron chelators like desferal and starch-desferal decrease mitochondrial ROS formation, MPT opening, and cell killing in cultured rat hepatocyte models of hypoxia/ischemia [104]. Desferal also protects against lethal injury to cultured hepatocytes from *tert*-butyl hydroperoxide, as does the lipid radical scavenger, N,N-diphenyl-p-phenylenediamine (DPPD) [111,112]. Another iron chelator, deferasirox, protects against concanavalin A-induced hepatic injury and fibrosis in rats [113]. Cytoprotection by iron chelators against hypoxia/ischemia, oxidative stress, and APAP hepatotoxicity infers a critical role for iron in the pathogenesis of injury, most likely by catalyzing •OH formation and subsequent lipid peroxidation [104,111,114–117].

5. Iron and Acetaminophen Hepatotoxicity

5.1. Evidence for Mitochondrial Iron Uptake in Acetaminophen Hepatotoxicity

After APAP overdose, the mitochondrial generation of ROS is a critical factor triggering the MPT, and iron promotes this oxidative stress [81]. Iron chelators and antioxidants that scavenge ROS protect against APAP toxicity in vitro and in vivo [114,118–121]. Treatment with the iron chelator, desferal (also called deferoxamine or desferrioxamine), increases the time required for APAP to induce ROS and mitochondrial dysfunction in cultured mouse hepatocytes [122]. After iron chelation with desferal, the addition of iron to the culture medium restores the sensitivity of hepatocytes to APAP toxicity in vitro [114,120]. Moreover, the treatment of mouse hepatocytes with the iron donor 3,5,5-trimethyl-hexanoyl ferrocene (TMHF) causes APAP-induced ROS formation and mitochondrial dysfunction to occur at earlier time points than APAP treatment alone, which is partially prevented by desferal [122].

Several fluorescent probes can visualize intracellular iron movement between organelles. The exogenously added calcein-acetoxymethylester (AM) is de-esterified in the cytosol to release calcein-free acid, whose fluorescence is quenched by chelatable Fe^{2+} [92,104,123]. Mitoferrofluor (MFF) is another iron indicator that accumulates electrophoretically into mitochondria in response to $\Delta\Psi$ and then binds covalently to mitochondrial proteins. Like green-fluorescing calcein, red-fluorescing MFF is quenched by chelatable Fe^{2+} [124]. Calcein and MFF can be used together or in combination with fluorescent indicators of mitochondrial $\Delta\Psi$, such as red-fluorescing tetramethylrhodamine methylester (TMRM) and green-fluorescing rhodamine 123 (Rh123) [81,124]. To visualize lysosomes, cells can be pre-loaded with red-fluorescing rhodamine-dextran, which is taken up via endocytosis and delivered to the lysosomes [116].

In cultured mouse hepatocytes, APAP causes lysosomes to rupture and release rhodamine-dextran into the cytosol within 4 h (Figure 1, top row). The mechanism underlying APAP-induced lysosomal rupture is not known. The APAP metabolite, NAPQI, may react covalently with lysosomal membrane components to cause the rupture. In parallel, cytosolic calcein fluorescence becomes quenched, though this is not the case for the fluorescence of calcein-free acid added to the extracellular medium, indicating an increase in cytosolic Fe^{2+} due to its release from lysosomes (Figure 1, bottom row) [81]. Starch-desferal suppresses the increase in cytosolic and mitochondrial Fe^{2+} after APAP [81]. Since membrane-impermeant starch-desferal is taken up via endocytosis into the lysosomal/endosomal compartment like rhodamine-dextran, the prevention of APAP-induced increases in cytosolic and mitochondrial Fe^{2+} by starch-desferal confirms that endosomes/lysosomes are the source of mobilizable chelatable iron entering the cytosol and mitochondria during APAP hepatotoxicity. Other sources of iron may promote the Fenton reaction in mitochondria. For example, ROS promote heme oxygenase 1 (HO-1) translocation to mitochondria in cardiomyocytes, leading to iron release from heme [125]. Further study is needed to determine whether HO-1 is involved in APAP hepatotoxicity.

5.2. Role of the Mitochondrial Calcium Uniporter in Mitochondrial Iron Uptake during Acetaminophen Hepatotoxicity

Movement into the mitochondria of Fe^{2+} released from ruptured lysosomes is mediated by MCU, an electrogenic Ca^{2+} transporter that also conducts Fe^{2+}, since the MCU inhibitors, Ru360 and minocycline, block MFF quenching but not calcein quenching after APAP [81]. Further support for this role of MCU is provided by studies using mice with a hepatocyte-specific MCU (hsMCU) deficiency. In wildtype hepatocytes, mitochondrial MFF fluorescence is bright but subsequently progressively decreases after APAP exposure, beginning within 4 h and becoming virtually complete after 12 h (Figure 2A, bottom row). In parallel, mitochondrial depolarization (the loss of Rh123 fluorescence), signifying the onset of the MPT, begins to occur within 8 h and is complete within 12 h (Figure 2A, top row). By contrast, in hsMCU KO hepatocytes that are deficient in MCU, mitochondrial MFF quenching and mitochondrial depolarization are suppressed after APAP (Figure 2B). Nonetheless, cytosolic calcein fluorescence is just as strongly quenched after APAP in MCU-deficient hepatocytes as in wildtype hepatocytes showing that lysosomes still release Fe^{2+} (Figures 1 and 3). Both in vitro and in vivo, lysosomal iron chelation with starch-

desferal and the inhibition of MCU-mediated mitochondrial iron uptake protect against APAP-induced hepatotoxicity [81,116,117,126]. Notably, both the global- and hepatocyte-specific deficiency of MCU decreases APAP hepatotoxicity in vivo as assessed by ALT release and necrosis by histology without altering hepatic APAP metabolism [126]. In addition, the co-treatment of APAP with $FeSO_4$ dramatically increases APAP-induced hepatotoxicity, which is prevented by desferal [27].

5.3. Possible Roles of Kupffer Cells and JNK in Iron-Dependency of Acetaminophen Hepatotoxicity

Kupffer cells are liver-resident macrophages that are involved in the phagocytosis of senescent red blood cells and the recycling of iron [127]. Kupffer cells are also a potential source of oxidant stress promoting cell death [128]. Human and mouse studies indicate that Kupffer cells and infiltrating monocyte-derived macrophages have both injury-promoting and injury-repair functions after APAP overdose [129–133]. Although MCU deficiency in hepatocytes decreases liver necrosis and ALT release after APAP in mice, MCU deficiency in Kupffer cells does not alter APAP hepatotoxicity [126].

JNK activation in the cytosol and translocation of p-JNK to mitochondria are important early events promoting the MPT and cell death in APAP hepatotoxicity [32]. Recent in vivo studies in mice show that neither desferal nor Fe^{2+} treatment affects JNK activation and its translocation to mitochondria after APAP overdose [27]. These findings suggest that the effect of iron is not at the early stages of the response to APAP but specifically at later events within mitochondria.

5.4. “Two Hit” Hypothesis

Overall, these results support a “two hit” hypothesis for the role of oxidative stress and iron in APAP hepatotoxicity (Figure 4) [81] (see also [104]). In the first hit, CYP2E1 metabolizes APAP to NAPQI, which induces mitochondrial protein adduct formation, the disruption of mitochondrial respiration, and consequent generation of ($O_2^{\bullet-}$ and H_2O_2. These ROS also activate JNK, which translocates to mitochondria to further inhibit respiration with the feed-forward effect of enhancing mitochondrial ROS generation even more. In the second hit, toxic NAPQI causes lysosomal breakdown and the release of chelatable Fe^{2+} into the cytosol. Fe^{2+} is then taken up into mitochondria via MCU. In the presence of $O_2^{\bullet-}$ and H_2O_2, such mitochondrial Fe^{2+} loading induces •OH formation via the Fenton reaction, which in turn causes MPT onset, mitochondria depolarization, bioenergetic failure, and cell death. Iron imported into mitochondria also facilitates protein nitration by peroxynitrite ($ONOO^-$), which is formed from the reaction of $O_2^{\bullet-}$ with nitric oxide (NO) [27].

5.5. Ferroptosis during Acetaminophen Hepatotoxicity

Iron has long been known to promote lipid peroxidation and cell death in various models of cell injury (see [112,115,120,134,135]). During APAP toxicity to cultured hepatocytes, DPPD, a scavenger of lipid radicals, prevents both lipid peroxidation and cell death [111,136]. Similarly, ferrostatin-1, a scavenger of alkoxyl radicals that propagate lipid peroxidation chain reactions, protects against APAP-induced hepatotoxicity in mice [137]. Non-apoptotic iron-dependent cell death involving lipid peroxidation and mitochondrial

iron-loading has more recently been named ferroptosis [110,138]. A novel ferroptosis inhibitor, mifepristone, prevents APAP-induced hepatotoxicity in vitro and in mice in vivo [139], and growth arrest-specific 1 (GAS1) overexpression promotes ferroptosis and aggravates APAP-induced hepatocellular injury both in vitro and in vivo [140].

5.6. Role of Peroxynitrite and Protein Nitration in Acetaminophen Hepatotoxicity

Protein nitration is an important pathophysiological event in APAP hepatotoxicity [141,142]. During APAP overdose, respiratory chain dysfunction leads to the generation of $O_2^{\bullet -}$, which reacts with NO to form reactive and toxic $ONOO^-$ in the mitochondrial matrix [27,143]. The mitochondrial uptake of iron released from lysosomes then promotes $ONOO^-$-dependent nitration of protein tyrosine residues to form nitrotyrosine protein adducts [27,144]. This stress further induces the MPT in APAP toxicity (Figure 5). Consistent with this mechanism in vivo after APAP overdose, desferal and the MCU blocker, minocycline, attenuate immunostaining for nitrotyrosine protein adducts and the release of the mitochondrial intermembrane protein, cytochrome *c*, which is a consequence of mitochondrial swelling after MPT onset [27]. The co-treatment of APAP with $FeSO_2$ in mice further increases nitrotyrosine staining and the release of cytochrome *c*, as well as causing lipid peroxidation, which desferal inhibits [27]. Moreover, the mitochondria-specific SOD mimetic, mito-TEMPO, protects against APAP-induced liver injury and nitrotyrosine protein adduct formation in mice [145].

5.7. Aldehydes as Drivers of Acetaminophen Hepatotoxicity

•OH from Fenton chemistry reacts with unsaturated lipids to initiate a lipid peroxidation chain reaction with the formation of lipid radicals (L•), lipid peroxides (LOOH), and peroxyl radicals (LOO•). Iron is an important catalyst to then promote a subsequent alkoxyl radical (LO•) and more LOO• formation. Notably, the spontaneous non-enzymatic beta-scission of LO• generates a variety of aldehydes, including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which are often used as biomarkers for lipid peroxidation. However, MDA, 4-HNE, and other aldehydes formed downstream of lipid peroxidation are toxic, reactive, and mutagenic, with MDA reported to be the most mutagenic and 4-HNE the most toxic [146–148].

Lipid peroxidation in APAP hepatotoxicity was initially indicated by the appearance of exhalated hydrocarbons in mice in vivo and by MDA formation in liver homogenates in vitro that inducers and inhibitors of P450 enzymes, respectively, up and down modulate [149,150]. However, these studies were performed with mice fed a vitamin E-deficient diet high in polyunsaturated fatty acids that made the animals sensitive to lipid peroxidation induced by APAP [150,151]. A follow-up study with mice fed a regular diet showed minimal evidence for lipid peroxidation after APAP [152]. Furthermore, mice fed a diet high in vitamin E diet do not show decreased APAP hepatotoxicity, suggesting that endogenous defense mechanisms are normally sufficient to prevent excessive lipid peroxidation after APAP [152]. Additionally, the co-treatment of Fe^{2+} with APAP increases lipid peroxidation in vivo in mice, which desferal almost completely prevents [27,153]. Nonetheless, other reports show that APAP stimulates lipid peroxidation in isolated mouse and rat hepatocytes in vitro [154,155], and mass spectroscopy reveals lipid peroxides derived from n-6 fatty

acids, mainly from arachidonic acid, after APAP overdose [137]. Moreover, 4-HNE adduct formation increases after APAP in mice fed normal chow [156].

N-(1,3-benzodioxol-5-ylmethyl)-2,6-dichlorobenzamide (Alda-1) is an activator of mitochondrial aldehyde dehydrogenase-2 (ALDH2) and is responsible for detoxifying aldehyde oxidation to fatty acids [157]. After APAP in vivo, Alda-1 decreases 4-HNE adduct formation, APAP-induced liver injury, and mitochondrial dysfunction, indicating that lipid peroxidation-derived aldehydes are important mediators of APAP hepatotoxicity. Lipid peroxidation may occur relatively selectively in mitochondria that are the source of •OH from Fenton chemistry and whose membranes are enriched in arachidonic acid.

6. Summary and Conclusions

Iron-catalyzed free radical generation in mitochondria plays an important role in APAP toxicity (Figure 5). Initially, the toxic APAP metabolite, NAPQI, binds to mitochondrial proteins to inhibit mitochondrial respiration. Inhibited respiration leads to increased levels of ubisemiquinone and flavin semiquinone, which transfer their unpaired electrons to oxygen to form $O_2•^-$. Respiratory inhibition is further amplified through JNK activation, leading to greater $O_2•^-$ generation. $O_2•^-$ reacts with nitric oxide to produce peroxynitrite or is converted to H_2O_2 by SOD. Since NAPQI depletes GSH after APAP overdose, GSH is no longer available to detoxify peroxynitrite and H_2O_2, as would occur normally. NAPQI also damages lysosomes, causing Fe^{2+} release into the cytosol and subsequent uptake into mitochondria via the MCU. Mitochondrial loading with Fe^{2+} facilitates nitrotyrosine protein adduct formation and Fenton chemistry with H_2O_2 to produce the highly reactive •OH. •OH, in turn, causes lipid peroxidation, the formation of toxic aldehydes, and induction of the MPT, ultimately leading to cell death. Accordingly, blocking pathways of iron movement into mitochondria via MCU, preventing iron-related mitochondrial •OH and $ONOO^-$ formation, and accelerating aldehyde metabolism are potential novel strategies to intervene against APAP hepatotoxicity in a clinical setting.

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No new data were created or analyzed. Data sharing is not applicable to this article.

Abbreviations

•OH	hydroxyl radical
ΔΨ	membrane potential
Alda-1	N-(1,3-benzodioxol-5-ylmethyl)-2,6-dichlorobenzamide

ALDH2	mitochondrial aldehyde dehydrogenase-2
ALF	acute liver failure
AM	acetoxymethylester
ANT	adenine nucleotide translocator
APAP	N-acetyl-para-aminophenol, acetaminophen
CsA	cyclosporin A
CypD	cyclophilin D
DMT1	divalent metal transporter 1
DPD	dipyridyl
DPPD' N	N'-diphenyl-p-phenylenediamine
FPN1	ferroportin 1
FTMT	mitochondrial ferritin
GAS1	growth arrest-specific 1
GSH	glutathione
HDM	hormonally defined medium
4-HNE	4-hydroxynonenal
HO-1	heme oxygenase 1
JNK	c-Jun N-terminal protein kinase
LIP	labile iron pool
L•	lipid radicals
LOO•	peroxyl radical
LOOH	lipid peroxide
MAPK	mitogen-activated protein kinase
MASLD	metabolic dysfunction-associated steatotic liver disease
MCU	mitochondrial calcium uniporter
MDA	malondialdehyde
MFF	mitoferrofluor
Mfrn	mitoferrin
MPT	mitochondrial permeability transition

NAC	N-acetylcysteine
NAPQI	N-acetyl-p-benzoquinone imine
NO	nitric oxide
NTBI	non- transferrin-bound iron
$ONOO^-$	peroxynitrite
p-JNK	phospho-JNK
PI	propidium iodide
PT	permeability transition
PTPN6	protein tyrosine phosphatase nonreceptor type 6
Rh123	rhodamine 123
ROS	reactive oxygen species
SAB	SH3 domain-binding protein that preferentially associates with Bruton's tyrosine kinase
SOD	superoxide dismutase
Tf	transferrin
TfR1	Tf receptor-1
TMHF	3,5,5-trimethyl-hexanoyl ferrocene
TMRM	tetramethylrhodamine methylester
VDAC	voltage-dependent anion channels
ZIPs	ZRT/IRT-like proteins

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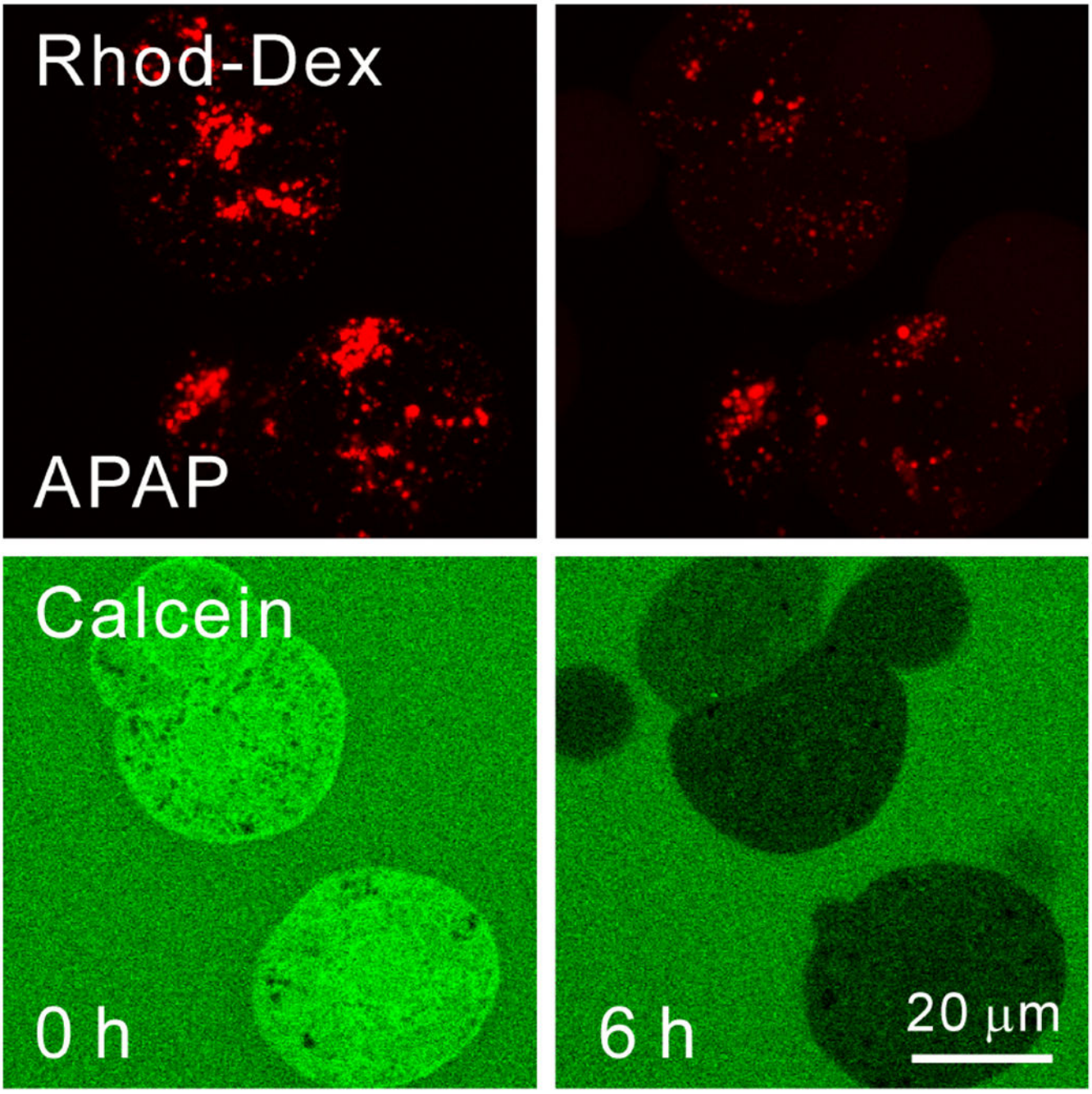

Figure 1.

Acetaminophen-dependent lysosomal permeabilization and release of Fe^{2+} into the cytosol. Wildtype mouse hepatocytes were isolated from mice injected with 70 kDa rhodamine-dextran and then loaded with 1 μM calcein-AM. Rhodamine-dextran labeled lysosomes, whereas calcein-AM was de-esterified to release calcein-free acid into the cytosol. In the presence of 20 mM of fructose plus 5 mM of glycine to prevent cell death after APAP-induced disruption of mitochondrial metabolism, hepatocytes were then exposed to acetaminophen (APAP, 10 mM). Before APAP (0 h), rhodamine-dextran-labeled lysosomes

were intact, and cytosolic calcein fluorescence was bright in comparison to the fluorescence of 300 μM of calcein-free acid placed in the extracelluar medium. At 4 h after APAP, many rhodamine-dextran-labeled lysosomes disappeared in parallel with the quenching of calcein fluorescence. This calcein quenching signified increased cytosolic chelatable Fe^{2+}. As lysosomes disappeared, diffuse red fluorescence appeared in the cytosol, signifying that acetaminophen permeabilized many lysosomes. After [116].

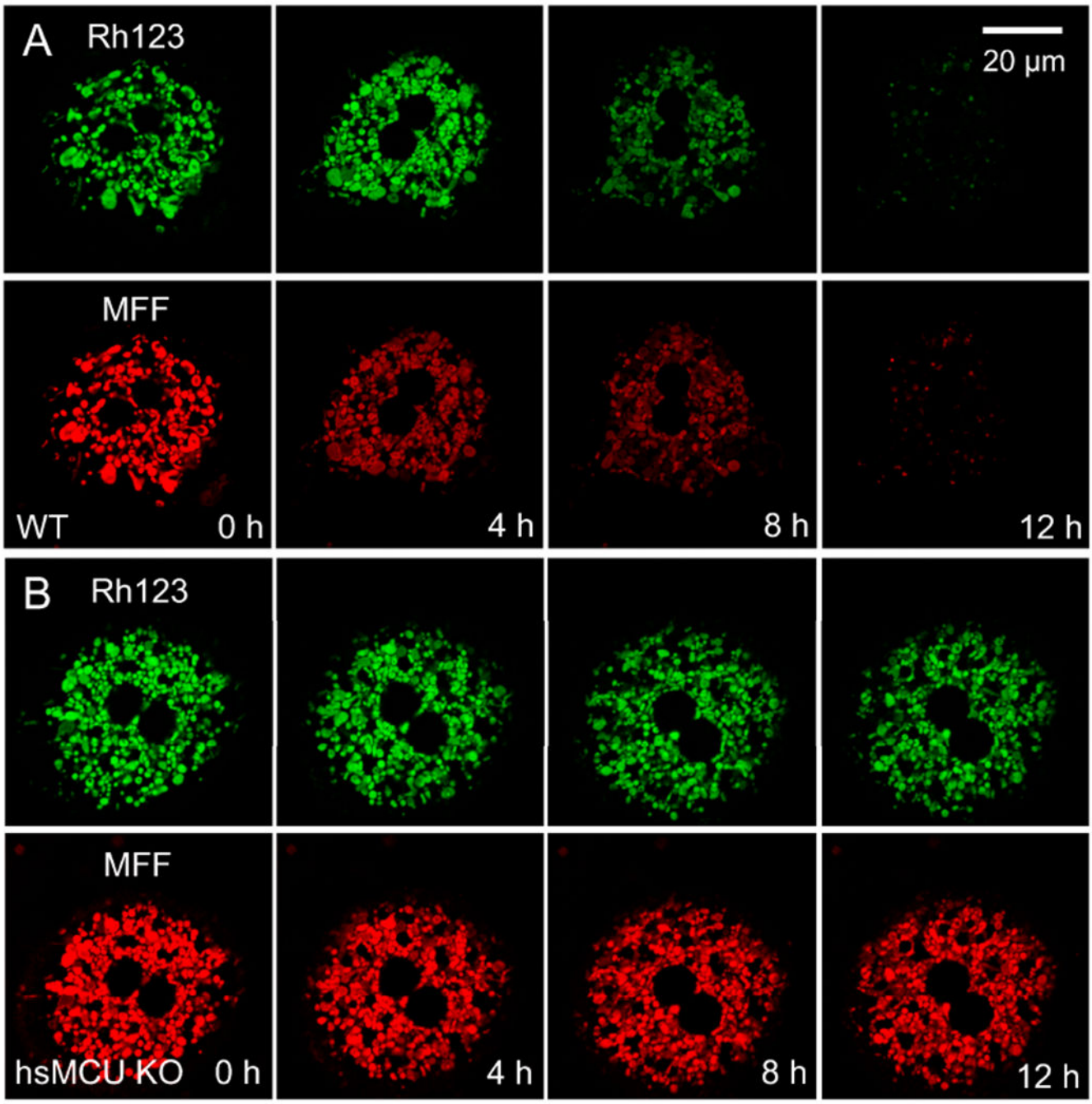

Figure 2.

Suppression of mitochondrial iron uptake and depolarization after acetaminophen treatment of hepatocytes deficient in the mitochondrial calcium uniporter. Wildtype and hsMCU KO hepatocytes were loaded with 300 nM of Rh123 plus 1 μM of MFF and exposed to 10 mM APAP in the presence of 20 mM of fructose plus 5 mM of glycine. Rh123 is a green-fluorescing indicator of mitochondrial $\Delta\Psi$. Mitoferrofluor (MFF) accumulates electrophoretically into mitochondria, binds covalently, and becomes quenched as mitochondrial Fe^{2+} increases. (**A**) In wildtype (WT) hepatocytes, red mitochondrial MFF

fluorescence was bright at 0 h but subsequently quenched progressively, beginning within 4 h and becoming virtually complete after 12 h (bottom row). Mitochondrial depolarization (loss of green Rh123 fluorescence) began to occur at 8 h and was complete after 12 h (top row). (**B**) In hsMCU KO hepatocytes, mitochondrial MFF quenching and mitochondrial depolarization were suppressed after APAP. After [126].

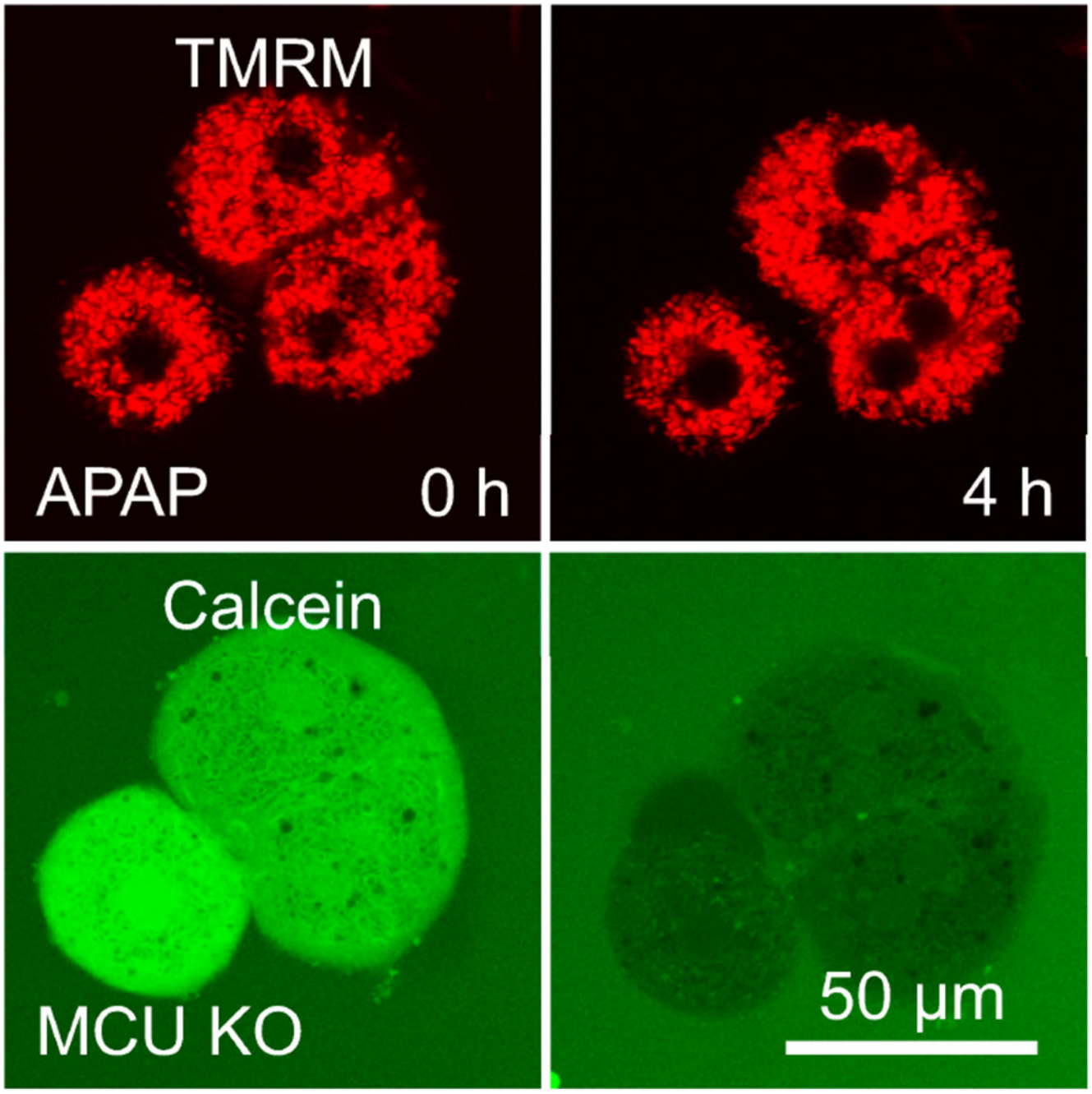

Figure 3.

Increased cytosolic Fe^{2+} in MCU-deficient hepatocytes after acetaminophen. Hepatocytes were loaded with 300 nM of TMRM plus 1 μM of calcein-AM and incubated with 300 μM of calcein-free before exposure to 10 mM APAP in the presence of 20 mM fructose plus 5 mM glycine. TMRM is a red-fluorescing indicator of mitochondrial ΔΨ. When MCU-deficient hepatocytes were exposed to 10 mM APAP, mitochondrial depolarization (loss of TMRM fluorescence) was suppressed. However, the green cytosolic calcein fluorescence

decreased substantially similarly to wildtype hepatocytes, signifying increased cytosolic chelatable Fe^{2+}. After [126].

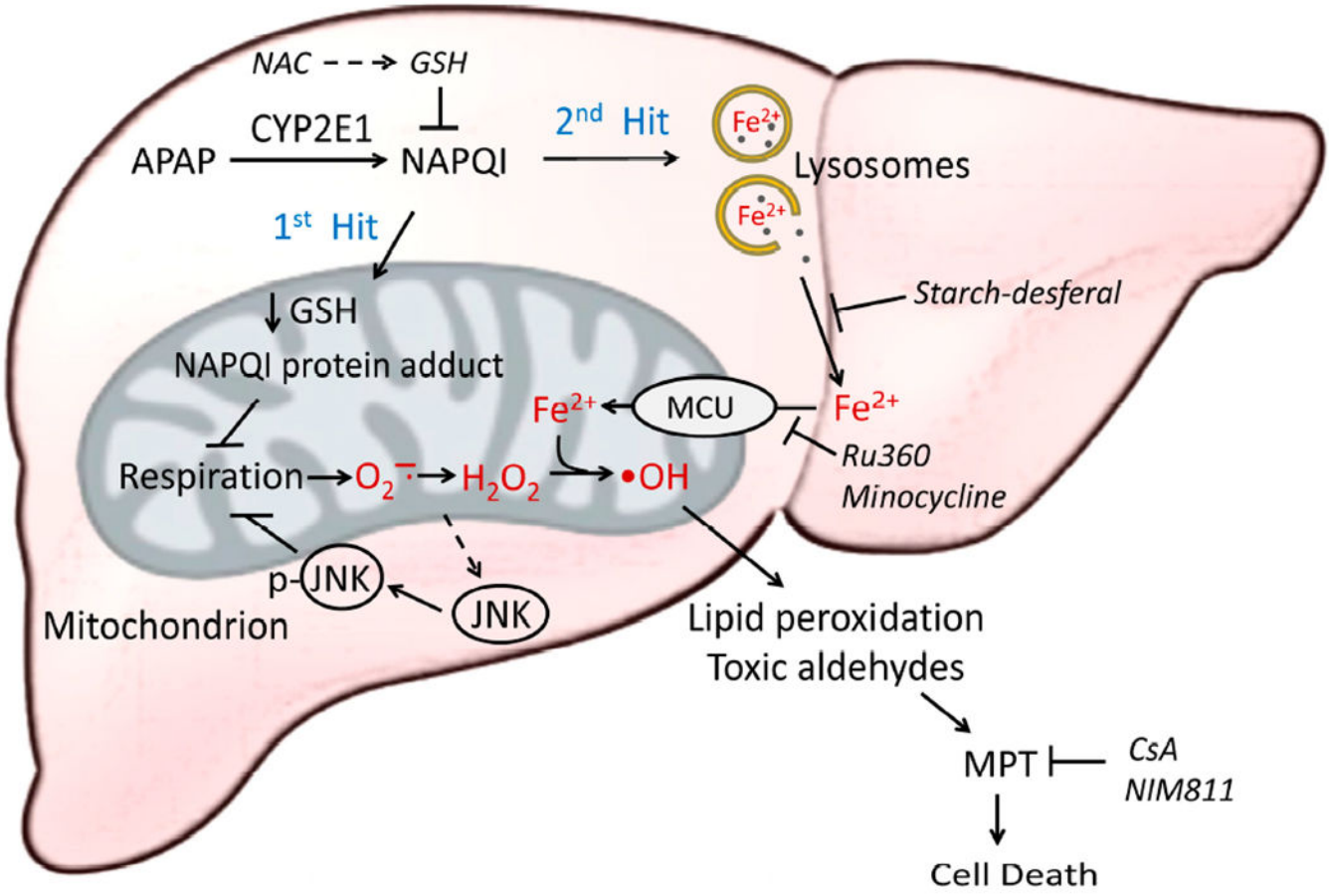

Figure 4.

Two-hit model of APAP hepatotoxicity. After an overdose of APAP, the first hit occurs when APAP causes GSH depletion, NAPQI protein adduct formation, and the inhibition of mitochondrial respiration, which induces $O_2^{\bullet-}$ and H_2O_2 formation. ROS-induced JNK phosphorylation and activation further enhance respiratory inhibition and mitochondrial ROS formation. The second hit occurs when NAPQI damages lysosomes and releases Fe^{2+} into the cytosol, which is then taken up into mitochondria via the electrogenic MCU to promote intramitochondrial •OH formation by the Fenton reaction. •OH, in turn, induces lipid peroxidation, the formation of toxic aldehydes, MPT onset, and mitochondrial bioenergetic failure, leading to the loss of cell viability. Starch-desferal chelates lysosomal iron to prevent the release of chelatable iron after lysosomal disruption and subsequent uptake into mitochondria to promote •OH formation. Ru360 and minocycline block mitochondrial iron uptake via MCU to also suppress iron-catalyzed •OH formation in the mitochondrial matrix. CsA and NIM811 inhibit MPT. Blocking either hit protects against APAP-induced hepatic injury.

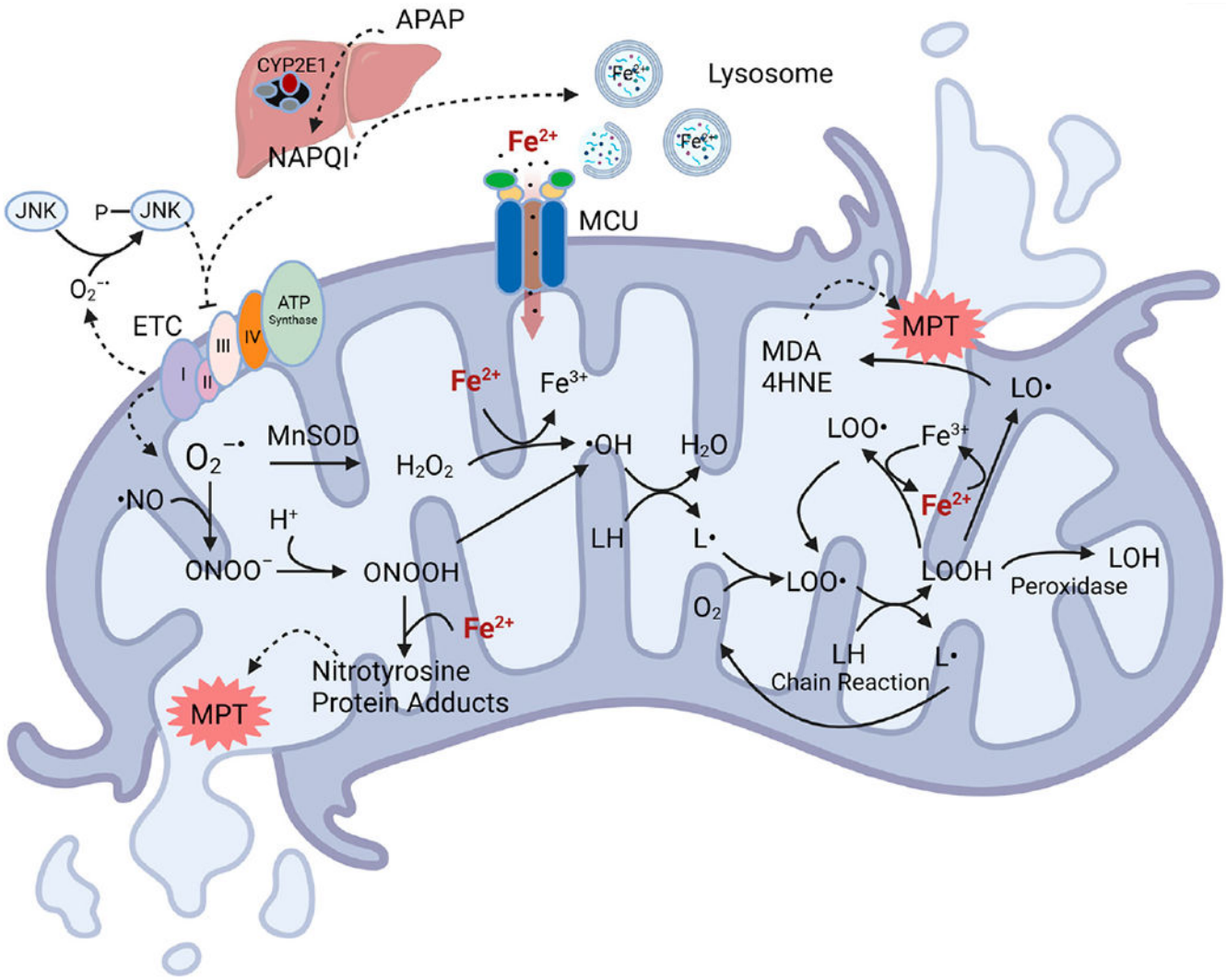

Figure 5.

Role of iron in oxidative stress in APAP-induced mitochondrial damage. After an overdose of APAP, NAPQI binds to mitochondrial proteins to inhibit mitochondrial respiration. Respiratory inhibition leads to increased levels of flavin semiquinones and ubisemiquinone, which react with oxygen to form $O_2•^-$. Such respiratory inhibition and ROS generation are further amplified through ROS-driven JNK activation. $O_2•^-$ reacts rapidly with NO to form $ONOO^-$. The iron influx into mitochondria facilitates the reaction of $ONOO^-$ with proteins to produce nitrotyrosine adducts, ultimately promoting the MPT. SOD2 in mitochondria also converts $O_2•^-$ to H_2O_2. Fe^{2+}, which is released from damaged lysosomes, is taken up into mitochondria via MCU and reacts with H_2O_2 to form the toxic •OH, which induces L• formation. L• then initiates an oxygen-dependent chain reaction generating peroxyl radicals (LOO•) and lipid peroxides (LOOHs). In the presence of Fe^{2+}, LOOH produces LO•. The beta scission of LO• then leads to the formation of reactive aldehydes like MDA and 4HNE, which also promote MPT onset. This figure was created with BioRender.com.