Gene Expression, Vol. 20, pp. 169–178
Printed in the USA.

1052-2166/21 $90.00 + .00
DOI: https://doi.org/10.3727/105221621X16104581979144
E-ISSN 1555-3884
www.cognizantcommunication.com

Review

Ferroptosis and Acetaminophen Hepatotoxicity: Are We Going Down Another Rabbit Hole?

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Acetaminophen (APAP) hepatotoxicity is the most frequent cause of acute liver failure in the US. The mechanisms of APAP-induced liver injury have been under extensive investigations for decades, and many key events of this necrotic cell death are known today. Initially, two opposing hypotheses for cell death were proposed: reactive metabolite and protein adduct formation versus reactive oxygen and lipid peroxidation (LPO). In the end, both mechanisms were reconciled, and it is now generally accepted that the toxicity starts with formation of reactive metabolites that, after glutathione depletion, bind to cellular proteins, especially on mitochondria. This results in a mitochondrial oxidant stress, which requires amplification through a mitogen-activated protein kinase cascade, leading ultimately to enough reactive oxygen and peroxynitrite formation to trigger the mitochondrial membrane permeability transition and cell death. However, the earlier rejected LPO hypothesis seems to make a comeback recently under a different name: ferroptosis. Therefore, the objective of this review was to critically evaluate the available information about intracellular signaling mechanisms of APAP-induced cell death and those of ferroptosis. Under pathophysiologically relevant conditions, there is no evidence for quantitatively enough LPO to cause cell death, and thus APAP hepatotoxicity is not caused by ferroptosis. However, the role of mitochondria-localized minor LPO remains to be further investigated.

Key words: Acetaminophen hepatotoxicity; Oncotic necrosis; Apoptosis; Ferroptosis; Lipid peroxidation; Fenton reaction; Glutathione peroxidase 4

INTRODUCTION

Acetaminophen (APAP), which is one of the most used analgesic drugs in the world, is generally safe when used within the therapeutic range. However, the extensive scientific and clinical interest in APAP comes from the fact that APAP is an intrinsic hepatotoxin, which reliably can cause liver injury after an overdose in animals and in humans[1,2]. Because of its clinical importance as the main cause of acute liver failure in many Western countries[3] and the development of a mouse model[4–6] that replicated many aspects of the human pathophysiology[7], significant progress was made in understanding the mechanisms of cell death and liver injury over the years[8–12]. In the early days, APAP-induced cell death was considered necrosis or oncotic necrosis because of cell and organelle swelling, release of cell contents, karyorrhexis, and karyolysis[4–6]. Only when it was recognized that there is a fundamentally different mode of cell death (i.e., apoptosis) did some studies claim that APAP-induced liver injury was caused in part by apoptotic cell death[13,14]. These conclusions were easily disputed because the morphological characteristics of apoptosis (cell shrinkage, chromatic margination and condensation, and apoptotic bodies) were not present, there was no relevant caspase activation, and highly potent caspase inhibitors did not protect[15,16]. However, during the last decade, the idea of apoptotic cell death in APAP hepatotoxicity resurfaced mainly based on unspecific parameters such as the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and bax and bcl-2 protein expression, among others[17]. This led to the situation that currently an ever-increasing number of articles are being published that claim without sound scientific evidence the presence of APAP-induced apoptosis in APAP hepatotoxicity[17,18].

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More recently, with the increased recognition of various forms of programmed necrosis[19], the hypothesis was brought up that ferroptosis contributes to or is even solely responsible for APAP-induced liver injury[20–22]. The objective of this review was to critically evaluate the validity of this new hypothesis based on our understanding of the pathophysiology of APAP hepatotoxicity.

WHAT IS FERROPTOSIS?

Ferroptosis is a more recently described regulated necrotic cell death mode with excessive lipid peroxidation (LPO) as the central mechanism[23] (Fig. 1). Living in an oxygen-containing environment comes with formation of reactive oxygen species in cells leading to oxidation of fatty acids, especially polyunsaturated fatty acids (PUFAs) in phospholipids. Because this process is dependent on the iron-dependent Fenton reaction, this form of cell death was termed ferroptosis[23]. In addition to the many antioxidant enzymes, which metabolize various reactive oxygen species, fatty acid peroxides are specifically reduced to the hydroxy fatty acids by the seleno-peroxidase glutathione peroxidase 4 (GPx4) using glutathione (GSH) as electron donor[24]. Thus, GPx4 and GSH are important defense mechanisms against the accumulation of these LPO products. However, inhibition of cysteine uptake or other ways of inhibiting GSH synthesis and inactivation of GPx4 are ways to trigger ferroptotic cell death[24,25]. Thus, ferroptotic cell death occurs when defense mechanisms are impaired and LPO is allowed to proceed uninhibited[24,25]. In the meantime, additional defense mechanisms against ferroptosis, besides GPx4/GSH, were identified[24,25]. Because of the critical dependence of ferroptosis on the availability of

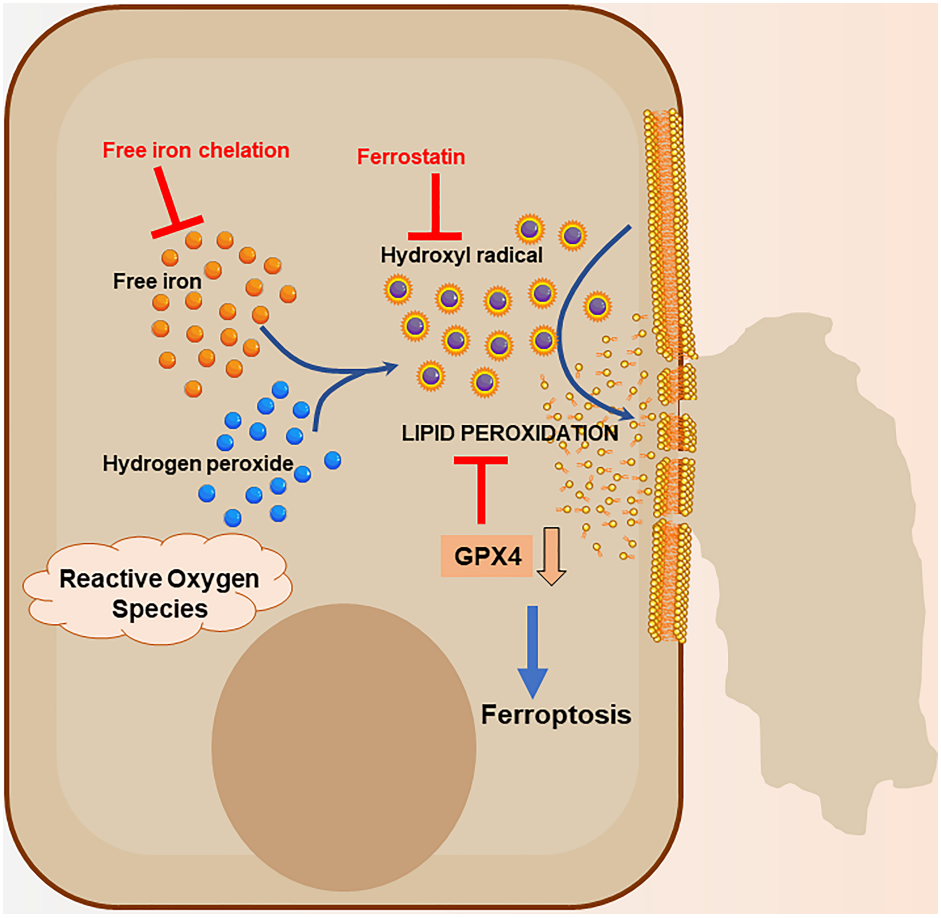

Figure 1. Ferroptosis. Ferroptosis is a mode of cell death mediated by lipid peroxidation and cellular free iron when protective mechanisms such as glutathione peroxidase activity have been compromised. Cellular generation of hydrogen peroxide derived from reactive oxygen species can react with cellular free iron through the Fenton reaction to generate the reactive hydroxyl radical. These reactive moieties can attack lipid membranes to induce lipid peroxidation and membrane instability, ultimately causing leakage of cellular material and cell death. These detrimental events are generally prevented by antioxidant enzymes such as glutathione peroxidase 4 (GPX4) and iron chelators that prevent accumulation of free iron. Synthetic antioxidants such as ferrostatin can also prevent lipid peroxidation and protect against ferroptosis. This figure includes templates from Servier Medical Art, which is licensed under a Creative Commons Attribution 3.0 generic license (https://smart.servier.com).

free iron (labile Fe^{2+} pool), high levels of the Fe(III) storage protein ferritin in the cell confer resistance to ferroptosis[26]. Another way to reduce iron levels in the cell is by promoting the exosomal export of ferritin[27]. On the other hand, if ferritin is removed by autophagy resulting in the lysosomal release of free iron, the cell's susceptibility to ferroptosis is increased[28]. Due to the fact that PUFAs are the main target during LPO, the levels of PUFAs in membrane phospholipids are a critical measure of the susceptibility to LPO and ferroptosis. Feeding animals a diet high in soybean oil resulted in high levels of arachidonic acid (20:4) and docosahexaenoic acid (22:6) in cell membranes and dramatically increased the susceptibility to LPO with the primary loss of these PUFAs during allyl alcohol hepatotoxicity[29]. As LPO is a free radical mechanism, chain-breaking antioxidants are a critical defense mechanism against excess LPO. Vitamin E (tocopherol) and coenzyme Q_{10} are potent, lipid-soluble antioxidants that can prevent LPO and thus protect against ferroptosis[24,25]. As a result, the dietary intake of vitamin E and the modulation of various steps of the synthesis pathway of coenzyme Q_{10} can impact the susceptibility of cells to ferroptosis[25]. Taken together, the continuous generation of oxygen free radicals in an aerobic environment and the availability of free iron can lead to a free radical chain reaction (LPO) involving mainly PUFAs of membrane phospholipids[24]. If this process is unchecked, the excessive LPO causes necrotic cell death, termed ferroptosis. However, cells have multiple layers of complementary and redundant defense mechanisms[30], which effectively prevent this process. Thus, for a cell to die by ferroptosis, several events have to occur simultaneously, including, in general, more severe oxidant stress, potential mobilization of free iron, and the impairment of several of these aforementioned defense mechanisms.

OXIDANT STRESS AND APAP HEPATOTOXICITY

Formation of reactive oxygen species and other oxidants during APAP hepatotoxicity was evaluated in detail over the years[31] (Fig. 2). After the early focus on protein adduct formation[4–6], the idea of an oxidant stress-mediated injury was introduced by Wendel and co-workers[32]. Based on the modulation of LPO with inhibitors and inducers of cytochrome P450 enzymes, it was hypothesized that leakage of electrons from these enzymes during APAP metabolism was the source of the oxidant stress[33]. However, the validity of this idea was challenged when no increased formation of intracellular glutathione disulfide (GSSG), a direct indicator of hydrogen peroxide detoxification, was detected during APAP metabolism in rats or mice[34,35]. Similarly, an increase in 2′,7′-dichlorofluorescein (DCF) fluorescence as indicator of an intracellular oxidant stress was only observed after but not during the metabolism phase of APAP in primary mouse hepatocytes[36]. The main location of reactive oxygen formation was shown to be in the mitochondria as indicated by the selective formation and accumulation of GSSG and the loss of protein sulfhydryl groups inside of the mitochondria[37–39], which was confirmed by Mitosox fluorescence, a selective indicator of mitochondrial oxidant stress[40]. Importantly, the mitochondrial oxidant stress was only observed in animals treated with APAP but not with its nonhepatotoxic regioisomer 3′-hydroxyacetanilide (AMAP), which causes protein adducts only in extra mitochondrial compartments of hepatocytes[38,39]. Together, these data provided convincing evidence that APAP triggers reactive oxygen formation in the mitochondria. More recent studies showed that the initial, limited oxidant stress is triggered by APAP protein adduct formation in the mitochondria[41]. This induces the activation of various redox-sensitive mitogen-activated protein kinases in the cytosol, ultimately leading to the phosphorylation of c-jun N-terminal kinase (P-JNK), which then translocates to the mitochondria[42]. As a consequence of this mitochondrial P-JNK translocation, the original mitochondrial oxidant stress is amplified[43], which triggers the opening of the mitochondrial membrane permeability transition pores and cell death[44].

Despite the increased acceptance of an oxidant stress being involved in APAP-induced liver injury, the actual oxidant responsible for the injury remained unclear. However, Hinson and co-workers reported evidence for peroxynitrite formation (staining for nitro-tyrosine protein adducts) in the area of necrosis[45]. This was followed up by documentation that peroxynitrite (a reaction product between superoxide and nitric oxide) occurred only inside the mitochondria[46]. Time-dependent scavenging experiments with GSH provided the first evidence for the pathophysiological relevance of peroxynitrite in APAP hepatotoxicity[47]. However, the most convincing support for the importance of peroxynitrite came from experiments showing that partial deficiency of the mitochondrial MnSOD (SOD2) dramatically aggravated APAP-induced liver injury and nitrotyrosine staining[48], and treatment with a mitochondria-targeted SOD mimetic, Mito-Tempo, can virtually eliminate peroxynitrite formation and APAP hepatotoxicity[49,50]. In addition, gene deficiency of neuronal nitric oxide synthetase (nNOS) and an inhibitor of nNOS reduced APAP-induced liver injury and nitrotyrosine staining[51,52]. Together, these data suggest that the water-soluble oxidant peroxynitrite generated in the mitochondria is a critical oxidant in the pathophysiology of APAP hepatotoxicity, which causes damage to mitochondrial proteins such as MnSOD[53] and mtDNA[46] but does not induce relevant LPO[54].

LPO AND APAP HEPATOTOXICITY

LPO is a hallmark of ferroptosis[23–26]. Interestingly, the role of LPO in APAP-induced liver injury was already

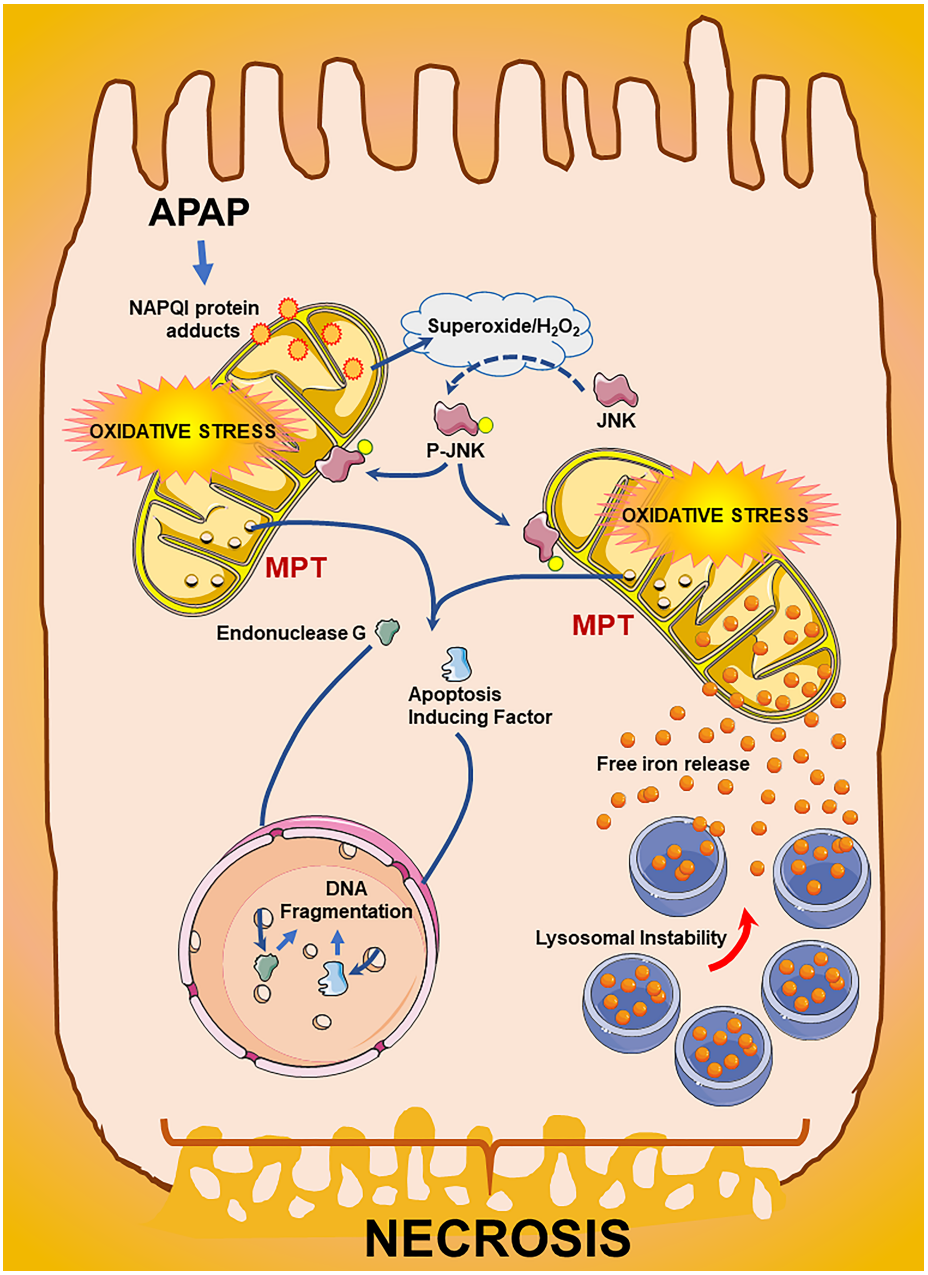

Figure 2. Iron and acetaminophen hepatotoxicity. Acetaminophen (APAP)-mediated hepatocyte cell death is initiated by the cytochrome P450-mediated generation of NAPQI, which forms mitochondrial protein adducts and induces enhanced superoxide and hydrogen peroxide generation into the cytosol from the mitochondria. This activates the mitogen-activated protein kinase c-jun N-terminal kinase (JNK), which translocates onto the mitochondria and amplifies the mitochondrial oxidant stress. These changes are accompanied by lysosomal instability, which releases free iron into the cytosol, which is taken up by mitochondria. Increased JNK-mediated mitochondrial oxidant stress as well as elevated mitochondrial free iron then induce the mitochondrial permeability transition (MPT), which results in the release of mitochondrial proteins such as endonuclease G and apoptosis-inducing factor (AIF) into the cytosol, from where they translocate into the nucleus and induce nuclear DNA fragmentation. All these detrimental events ultimately result in hepatocyte necrosis. This figure includes templates from Servier Medical Art, which is licensed under a Creative Commons Attribution 3.0 generic license (https://smart.servier.com).

controversially discussed 40 years ago. Wendel and co-workers reported a dramatic (30- to 50-fold) increase in ethane and pentane exhalation as specific markers of LPO, and severe liver injury within 2–4 h after treatment with APAP or allyl alcohol in mice[29,32,33,55–57]. LPO and liver injury in these models could be modulated by inducers and inhibitors of cytochrome P450 enzymes[33] and inhibited by pretreatment with vitamin E and iron chelators[29,56]. However, these effects were only observed in animals fed a diet deficient in vitamin E and enriched in soybean oil,

which led to much higher levels of PUFAs, especially 20:4 and 22:6, in the lipid membranes of the liver[29]. These PUFAs were also preferentially depleted during LPO[29]. These data clearly demonstrate the extent of LPO necessary to cause liver injury and can serve as a reference. However, in contrast to the LPO in vitamin E-deficient and PUFA-sensitized animals, APAP caused only minimal LPO in animals on a regular diet[58]. Although this minor LPO after APAP overdose could be reduced by iron chelation[58] and was aggravated by cotreatment with Fe^{2+} [59], APAP-induced liver injury was not affected by the manipulation of LPO in these animals[58,59]. This suggested that LPO is not a relevant mechanism of injury after APAP in mice on a normal diet but may be more of a secondary effect of cell death. However, over the years, there were conflicting reports with some claiming an effect of LPO on APAP-induced liver injury and a partial protective effect of vitamin E[60]. In contrast, we did not find evidence of extensive LPO during APAP hepatotoxicity in mice on a regular diet, and a sevenfold elevation of membrane vitamin E levels had no effect on the injury[54]. However, in a positive control of LPO (allyl alcohol + Fe^{2+}), vitamin E pretreatment reduced LPO and liver injury.[54] Overall, these results are consistent with numerous studies with APAP overdose in the literature that showed a very limited (two- to threefold) increase in LPO parameters. Although many claim interventions that are alleged antioxidants reduced liver injury and LPO as evidence to support the conclusion that LPO causes the liver injury in APAP hepatotoxicity, these are generally only correlations, and the lower LPO is likely a secondary effect of the reduced liver injury[31]. Furthermore, there is generally limited evidence that most compounds can accumulate in liver membranes in high enough concentrations to effectively enhance the already high antioxidant capacity of vitamin E in vivo. However, even if liver levels of coenzyme Q_{10} can be increased above physiological levels, the hepatoprotective effect against APAP overdose was not caused by reduced LPO but by enhanced mitophagy[61], which is known to limit APAP-induced cell death[62].

A similar discussion occurred in the field of hepatic ischemia–reperfusion injury regarding the relevance of LPO for liver cell death[63]. During reperfusion after prolonged hepatic ischemia, there is severe injury indicated by plasma ALT activities of >5,000 U/L, which correlate with a two- to threefold increase in LPO[64]. In order to assess the fundamental question, quantitively how much LPO is necessary to directly cause cell injury in the liver, LPO was induced by continuous infusion of t-butyl hydroperoxide into the portal vein, and LPO was specifically measured by gas chromatographic-mass spectrometric analysis of hydroxy-eicosatetraenoic acids (HETES), and F2-isoprostanes[64]. Exposure to this oxidant stress for 45 min triggered a two- to fourfold increase in LPO parameters but no liver injury; in contrast, prolonged exposure caused severe LPO with parameters elevated by 12- to 30-fold triggered some liver injury[64]. These results confirmed the data of APAP-induced LPO and toxicity in vitamin E-deficient and PUFA-enriched livers, that is, for LPO to cause direct cell damage, LPO has to be quantitatively an order of magnitude higher than generally observed during the pathophysiology of APAP-induced liver injury. Thus, LPO is not a relevant contributor to the cell injury mechanism of most pathophysiologies.

IRON AND APAP HEPATOTOXICITY

LPO is generally initiated by the Fenton reaction, which is the reductive cleavage of hydrogen peroxide to form hydroxyl radicals. This reaction is catalyzed by the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}). Because of the critical role of iron in these highly dangerous processes, the levels of free iron that could participate in these reactions are kept very low in all cells by effective chelation in proteins like ferritin[30]. Under conditions of vitamin E deficiency, APAP- or allyl alcohol-dependent LPO and liver injury in vivo can be prevented or aggravated by treatment with the iron chelator deferoxamine (DFO) or Fe^{2+}-sulfate, respectively[29,57]. In animals on a regular diet, similar effects on the low levels of LPO can be observed but without effects on the injury[54,58,59,65,66]. However, over the years, many conflicting results of the effect of iron chelation with DFO were published in addition to the no-effect reports[58,59,65], and studies demonstrated some delay in injury[67] and protection in vivo in rats[68] and in cultured rat or mouse hepatocytes[69–71]. Caveats of some of these studies are the use of hepatocytes cultured under hyperoxic conditions (room air), which leads to enhanced reactive oxygen formation that could mobilize iron[72] and the use of rats as suboptimal models[73]. These findings indicate that any form of LPO in the liver depends on the availability of iron; the degree of iron-dependent LPO determines whether this has an effect on the injury.

More recently, new evidence emerged for the involvement of iron in APAP hepatotoxicity (Fig. 2). It was recognized that APAP triggers lysosomal instability[74], which causes the release of iron from this compartment and enhances the levels of cytosolic ferrous iron[75]. The iron is taken up into the mitochondria by the mitochondrial electrogenic Ca^{2+}, Fe^{2+} uniporter (MCFU). Inside the mitochondria, iron is promoting the MPTP opening and cell death as indicated by beneficial effects of a lysosomal iron chelator and MCFU inhibitors[76]. These events were also confirmed in vivo[77]. In addition, activation of mitochondrial aldehyde dehydrogenase reduced hydroxynonenal levels and in part attenuated the MPTP opening and cell death[78]. Together, these findings raise the possibility of a more subtle impact of iron mobilization

and very localized LPO inside the mitochondria on the pathophysiology of APAP-induced cell death. However, how can these mechanisms be reconciled with the prominent role of mitochondrial peroxynitrite and the profound protection of MnSOD mimetics in APAP toxicity[47–52]? One possible explanation could be that peroxynitrite and the limited iron-dependent LPO need to act together to effectively trigger the MPTP opening. In this scenario, the formation of peroxynitrite inactivates mitochondrial proteins such as MnSOD[53] as well as mitochondrial DNA[46], and also effectively scavenges any remaining or newly imported GSH inside the mitochondria, thus rendering mitochondria highly susceptible to any additional stress (i.e., even very limited iron-dependent LPO). This mechanism could explain the profound protection of interventions that prevent peroxynitrite formation[46–52], but also drugs that strongly promote GSH synthesis (i.e., N-acetylcysteine)[79]. More investigations are needed into the potential cooperation between peroxynitrite formation and a localized iron-dependent LPO specifically in the mitochondria to induce the MPTP[80].

FERROPTOSIS AND APAP HEPATOTOXICITY

Although the first beneficial effect of ferrostatin in APAP-induced cell death was shown in cultured hepatocytes[20], the first in vivo evidence for a protection of ferrostatin and also vitamin E and DFO was reported in APAP-treated mice[21]. Parallel to the reduced liver reduction in LPO as measured by the levels of HETEs[21], and consistent with previous data[29], this also prevented mainly the loss of PUFAs[21]. On first glance, the conclusion that APAP triggers ferroptosis seems to be justified. However, a closer look raised serious concerns regarding these experiments (Figs. 1 and 2).

The authors used fasted male C57BL/6J mice treated with 200 mg/kg APAP (intraperitoneally)[21]. These animals developed massive liver injury within 3 h (ALT activities >6,000 U/L) and significant centrilobular necrosis, and all animals died within 24–48 h[21]. However, this extremely rapid and severe injury was accompanied by only a 50% increase in MDA levels and a 100%–300% increase in HETEs levels[21]. This is an extremely unusual result for animals on a regular diet treated with this low dose of APAP. In our hands, 200 mg/kg APAP caused much less injury at 6 h (ALT: 800 U/L in B6129SF2/J mice[81] and 1,500 U/L in C57BL/6J mice) (Adelusi et al., unpublished) with limited necrosis and 100% survival and recovery. Under these conditions, we did not observe an increase in MDA levels (Adelusi et al., unpublished). However, the sensitivity of Yamada's mice was similar to what we observed with 400 mg/kg APAP in vitamin E-deficient, high-PUFA animals[32,33,55], with the important difference that this accelerated injury correlated with a 30- to 50-fold elevation of LPO parameters[32,33,55].

Yamada and co-workers pretreated animals with the ferroptosis inhibitor ferrostatin and observed a close to 100% protection; likewise, pretreatment with the iron chelator DFO for 7 days or a single dose of vitamin E 1 h before APAP also protected close to 100%[21]. Interestingly, each of these interventions prevented LPO and strongly reduced the depletion of hepatic GSH levels[21]. As has been established, an overdose of APAP depletes hepatic GSH content by 90% within 30 min[82]. The recovery of the GSH levels is dependent on the dose and requires at least 5–6 h for complete recovery for a dose of 200 mg/kg[82]. Thus, the elevated hepatic GSH levels in the ferrostatin, DFO, and vitamin E groups reflect most likely an inhibition of GSH depletion (i.e., reduced NAPQI formation, rather than improved recovery). This is supported by the fact that Yamada et al. reported that posttreatment with ferrostatin (subsequent to APAP metabolism) did not show any protection against hepatotoxicity[21]. In addition, we were not able to reproduce the complete protection with ferrostatin under the conditions described by Yamada et al. (Adelusi et al., unpublished), and the DFO and vitamin E data are inconsistent with the previous literature[31,54,58,59,65]. Together, these data contradict most of the published literature in terms of the time course and degree of the injury with a very low overdose of APAP and the close to 100% protection with interventions like ferrostatin, DFO, and vitamin E. Overall, these data make little sense unless these animals were highly sensitized to LPO, and even then, there is a clear internal contradiction as to the limited LPO versus the extremely aggressive progression of the liver injury. Thus, these inconsistencies need to be resolved before data of such an outlier study with its conclusions that contradict most of the literature over the last 40 years can be considered any further.

SUMMARY AND CONCLUSIONS

Cells have enormously effective, multilayered, and redundant defense systems against oxidant stress and LPO[30]. This includes, among others, several SODs and GSH peroxidases, catalase, thioredoxin, and peroxiredoxin together with vitamin E and coenzyme Q_{10} in lipid membranes and effective iron chelation. Because of the multiple levels of antioxidant defense mechanisms, it is very difficult to acutely overcome these systems, especially in hepatocytes. Thus, it would be very challenging to execute the ferroptosis pathways without prior severe impairment of several of these defense systems. As we have argued many years ago, just increasing reactive oxygen formation, even beyond pathophysiological relevant levels, is not sufficient to cause cell death[83]. As the early studies by Wendel and co-workers clearly demonstrated, animals with vitamin E deficiency in combination with elevated PUFA levels are highly sensitive to APAP or AA, which cause, in addition, massive depletion of GSH and

an oxidant stress (APAP) or NADH-dependent reductive iron mobilization (allyl alcohol)[29,32,33,55–57]. Hence, in these animals, both APAP and allyl alcohol trigger massive LPO and very acute injury. However, under normal circumstances, with normal membrane vitamin E levels, chelated iron, and without artificially increased PUFA levels, it is almost impossible to trigger high enough LPO that would directly cause necrotic cell death. In addition, APAP overdose generates peroxynitrite, which is unlikely to have a biologically relevant effect on lipid membranes. Furthermore, the highly effective protection against APAP hepatotoxicity by SOD mimetics, despite generation of the Fenton reaction substrate H_2O_2 while preventing peroxynitrite formation, is another indication of the lack of biological relevance of LPO in APAP-induced liver injury. Thus, there is no credible evidence to support the hypothesis that under clinically relevant conditions APAP induces ferroptotic cell death in hepatocytes. Importantly, if somebody wants to put forward new evidence for such a mechanism, it should not just be experiments with ferrostatin or other ferroptosis inhibitors, vitamin E, DFO, etc., with some of the results opposite to previous publications[21]. The authors also have the obligation to make a serious effort to explain the differences to the previous literature and not just lead us down yet another rabbit hole as it is happening currently with apoptosis. It also needs to be considered that most cell death pathways have features that are overlapping[18]. For example, mitochondrial bax translocation, cytochrome c release, and nuclear DNA fragmentation are detectable during APAP hepatotoxicity, but the lack of caspase activation and morphological evidence argue against apoptosis as a relevant cell death mechanism[17,18]. Furthermore, receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and RIPK3 have been implicated in APAP-induced cell death[84,85], but the lack of mixed-lineage kinase domain-like protein (MLKL) involvement[84], which is considered the final critical mediator of the necroptotic signaling pathway[86], argues against labeling this form of cell death as necroptosis[19]. Likewise, GSH depletion and quantitively minor iron-dependent LPO in the presence of severe mitochondrial dysfunction caused by protein adducts and peroxynitrite formation, which triggers the irreversible DNA fragmentation[10–12], cannot be simply labeled as ferroptosis, an LPO-dominated cell death[23,24]. Therefore, assigning cell death mechanisms in APAP-induced liver injury to a certain category such as apoptosis, necroptosis, or ferroptosis based on a few individual parameters or alleged specific inhibitors that may fit the assumed signaling mechanism does not appear useful or productive. In order to make real progress in our understanding of the mechanisms of APAP hepatotoxicity and identify new therapeutic targets that have clinical relevance[87], unbiased investigations into the cell death signaling mechanisms are needed.

ACKNOWLEDGMENTS: This work was supported by the National Institutes of Health grant R01 DK102142 and the National Institute of General Medical Sciences (P20 GM103549 and P30 GM118247) from the National Institutes of Health to H.J. The authors declare no conflicts of interest.

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