Published in final edited form as:

Adv Pharmacol. 2019 ; 85: 195–219. doi:10.1016/bs.apha.2019.01.007.

Acetaminophen Hepatotoxicity - A Mitochondrial Perspective

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Keywords

Liver; Drug toxicity; Hepatocyte; Superoxide; Nitric oxide; Necrosis

Introduction

Acetaminophen (APAP) is the most consumed drug for pain and fever in the United States and Europe and is very effective at therapeutic doses. In addition to its availability as a single drug, APAP is also extensively marketed in combination with a wide variety of other drugs, increasing its consumption in the general population. While very safe at therapeutic doses, APAP unfortunately can produce severe hepatotoxicity as an overdose and is the most common cause of acute liver failure in the United States. In addition to the instances of attempted self-harm with APAP, an unintentional overdose is also common due to the plethora of combination products containing APAP. Decades of research have provided significant insight into the mechanisms of APAP-induced hepatotoxicity, which is initiated by its cytochrome P450 mediated metabolism into a reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI). Hepatic glutathione depletion and subsequent mitochondrial dysfunction then sets the stage for induction of a characteristic centrilobular hepatocyte necrosis, which ultimately can lead to acute liver failure in the absence of supportive treatment. The standard of care currently for an APAP overdose is administration of N-acetylcysteine (NAC), which replenishes hepatic glutathione stores and supports recovery of mitochondrial function. However, NAC has a short therapeutic window for maximum benefit and there is an ongoing search for additional therapeutics with alternate targets, which could complement NAC and increase the therapeutic window. The central role of mitochondria in APAP-induced liver injury make this an attractive target and this review will focus on the organelle and delve into the various facets of mitochondrial biology in the context of APAP-induced cell signaling.

Mitochondria in APAP overdose

As mentioned earlier, APAP-induced cell death is initiated by the cytochrome P450-mediated generation of NAPQI. While the role of the reactive metabolite NAPQI in

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Conflict of interest statement: The authors have no conflict.

mediating APAP-induced liver injury was recognized relatively early, with identification of protein adducts within various cellular compartments, the recognition of the important role of the mitochondria came later. Since cytochrome P450 is localized to the endoplasmic reticulum (ER), studies using radioactive APAP demonstrated highest levels of covalent binding of radioactive material to the ER and cytosolic fractions, with smaller amounts in the mitochondria (Jollow et al., 1973). Subsequently, changes in mitochondrial ultrastructure after APAP were appreciated on electron microscopy in the late 1970's (Petersen et al., 1979) and further studies on mitochondrial respiration and ultrastructure after APAP indicated the organelle's role in APAP-induced cell signaling (Myers et al., 1986; Placke et al., 1987). Functional deficits in mitochondria were also recognized (Andersson et al., 1990; Donnelly et al., 1994), though whether these were causes or consequence of the APAP-induced injury was not very clear. The role of mitochondrial free radicals as well as oxidative stress in mediating APAP-induced injury *in vivo* was first described in the early 1990s (Jaeschke, 1990) and subsequently confirmed by others *in vitro* (Burcham et al., 1991). Further studies reported evidence for peroxynitrite formation during APAP hepatotoxicity (Hinson et al., 1998), which was also located inside of mitochondria (Cover et al., 2005). The importance of mitochondrial peroxynitrite over general oxidant stress was also documented (Knight et al., 2002). Further studies in the mid to late 2000's uncovered the role of translocation of cytosolic proteins such as Bax (Bajt et al., 2008) and the MAP kinase JNK (Gunawan et al., 2006) onto mitochondria, which amplifies the oxidant stress and ultimately induces the mitochondrial permeability transition pore opening. These various aspects will be examined in detail in the subsequent sections.

Mitochondrial protein adducts in APAP hepatotoxicity

Studies using the regioisomer of APAP, 3'-hydroxyacetanilide (AMAP), which has been shown to be non-toxic in mice, hamsters and several cell lines, indicated that the main difference between the two was formation of mitochondrial protein adducts with APAP but not AMAP (Matthews et al., 1997; Myers et al., 1995; Tirmenstein et al., 1989). Interestingly it was found that AMAP was actually toxic in human hepatocytes, where it did induce the formation of mitochondrial protein adducts (Xie et al., 2015), again confirming the importance of these mitochondrial adducts in APAP-induced liver injury. Mitochondrial protein adducts also seem to be responsible for the APAP-induced mitochondrial dysfunction (Hu et al., 2016). But why is it that these comparatively lower amounts of mitochondrial protein adducts are directly involved in mediating downstream effects rather than the more abundant adducts in ER and microsomes? While a definitive answer is still lacking, several inferences can be made based on the information from proteomic studies examining specific mitochondrial targets, as well as the functions of the mitochondria. The mitochondrial electron transport chain (ETC) normally transfers electrons to cytochrome c oxidase (Complex IV) to generate the proton gradient used by ATP synthase (Complex V) for ATP synthesis. However, even during physiological respiration, leakage of electrons from the ETC results in generation of free radicals such as the superoxide anion. Thus, any alteration in ETC components, which increase leak of electrons could presumably elevate generation of superoxide and result in oxidant stress. This does not occur physiologically since the robust anti-oxidant systems within the mitochondria, including manganese

superoxide dismutase (MnSOD), copper zinc superoxide dismutase (CuZnSOD) within the intermembrane space (Okado-Matsumoto et al., 2001), glutathione peroxidase and thioredoxin function to scavenge these radicals and keep oxidant stress under control. This is all the more important with the presence of nitric oxide (NO) generating systems within the mitochondria, since reaction of superoxide with NO can generate the potent oxidant peroxynitrite. While the source of NO within the mitochondria is not well characterized, it seems that one possibility could be the presence of the alpha isoform of neuronal NOS within mitochondria (Lacza et al., 2009). Additionally, it has also been shown that endothelial NOS (eNOS) could be associated with VDAC2 (Alvira et al., 2012), an outer mitochondrial membrane protein, and could thus produce NO close to the mitochondria. Thus, formation of APAP adducts on mitochondrial proteins would need to hit multiple targets, including components of the ETC, mitochondrial anti-oxidants and modulate nitric oxide to ultimately result in mitochondrial oxidative and nitrosative stress.

Though the direct connections between formation of NAPQI-mitochondrial protein adducts and increased superoxide and peroxynitrite formation within the mitochondria are still nebulous, a number of proteomic analysis examining adducted proteins within mitochondria enable the generation of hypothesis which could be tested. Proteomic analysis of whole liver homogenates after exposure of mice to APAP *in vivo* have identified a number of mitochondrial proteins which were adducted, including the α -subunit of ATP synthetase (Qiu et al., 1998). Since ATP synthase is central for mitochondrial ATP generation, formation of APAP adducts on the protein could be expected to prevent ATP synthesis, which had been shown very early in studies on APAP hepatotoxicity (Andersson et al., 1990; Jaeschke, 1990) and decreases in ATP synthase enzyme activity have also been demonstrated (Parmar et al., 1995), albeit in a rat model. The other prominent target identified was glutathione peroxidase, which would again be relevant since this is an important mitochondrial anti-oxidant enzyme and its inactivation by APAP-adduct formation could compromise free radical scavenging within mitochondria and aggravate mitochondrial oxidant stress. Interestingly however, deficiency of glutathione peroxidase did not make mice more sensitive to APAP compared to wild type mice (Knight et al., 2002), indicating that adducts on glutathione peroxidase are probably a general effect rather than causative of downstream events. Another recent proteomic study identified additional mitochondrial proteins modified by NAPQI in 3D cultures of human hepatocytes and nonparenchymal cells (Bruderer et al., 2015). These included antioxidant enzymes such as peroxiredoxin 6 and the redox sensitive chaperone PARK7, as well as the voltage gated ion channel VDAC2 present on the mitochondrial outer membrane (Bruderer et al., 2015). Since it has been shown that NAPQI binding to mitochondrial proteins correlates with APAP toxicity (Jaeschke et al., 2003) the finding of APAP-adducts on ATP synthase, coupled with the preponderance of anti-oxidant targets being adducted after an APAP overdose lends credence to the hypothesis that the initial source of enhanced superoxide within mitochondria after an APAP overdose is the ETC. This probably occurs due to the over-reduction of the coenzyme Q pool and the higher membrane potential due to block of ATP synthase, which results in reverse electron transport and enhanced generation of superoxide. Coupled with the compromised anti-oxidant systems, this would then result in the subsequent mitochondrial oxidant stress (Figure 1).

APAP induced mitochondrial oxidative and nitrosative stress

Whether mitochondrial adduct formation is directly responsible for it or not, APAP overdose causes substantial elevations in mitochondrial superoxide levels (Yan et al., 2010), as well as peroxynitrite (Cover et al., 2005). As alluded to earlier, the scavenging capacity of the mitochondria for these free radicals seems to be a critical feature of the injury process, since mice with a partial deficiency of the main superoxide scavenging mitochondrial enzyme, manganese superoxide dismutase show significant exacerbation of APAP induced liver injury (Fujimoto et al., 2009; Ramachandran et al., 2011). This is further corroborated by the protective effect of the mitochondria-targeted SOD-mimetic - Mito-Tempo, which provides significant protection against APAP hepatotoxicity (Du et al., 2017; Du et al., 2018). Further proof that the ETC is the likely source of these free radicals comes from early studies on permeabilized hepatocytes, which demonstrated that exposure to APAP resulted in the loss of mitochondrial respiratory function, accompanied by a decrease in ATP levels, which was preceded by a loss of reduced glutathione (Burcham et al., 1991). Complex II in particular was found to be more sensitive to inhibition by direct exposure to NAPQI, when compared to complex I (Burcham et al., 1991; Lee et al., 2015; Ramsay et al., 1989). However, these direct effects of NAPQI were demonstrated *in vitro* and hence their relevance *in vivo* is not known. Acetaminophen overdose in vivo in mice was shown to significantly inhibit complex I activity, which may contribute to the oxidative stress (Du et al., 2016). It was also demonstrated that APAP interferes with the formation of mitochondrial respiratory super complexes via the mitochondrial negative regulator MCJ, which leads to decreased production of ATP and increased generation of ROS (Barbier-Torres et al., 2017). In any case, in the context of APAP-induced mitochondrial oxidant stress, it seems that reaction of the generated superoxide with nitric oxide to produce peroxynitrite is more relevant (Cover et al., 2005; Knight et al., 2001) than superoxide generation *per se*. Interestingly, nitro-tyrosine staining used as a marker of protein modification induced by peroxynitrite revealed early staining in sinusoidal endothelial cells prior to hepatocytes after APAP (Knight et al., 2001). Endothelial cell staining was evident at 1h post APAP, and staining in hepatocytes was only visible by 4h, the next time point tested (Knight et al., 2001). This time course tallies with scanning electron microscopy studies which showed morphological changes in the sinusoidal endothelium prior to alterations in hepatocytes. One of the earliest changes appreciated was formation of large pores of 0.5 to 3µm diameter in the sinusoidal endothelial cell lining at 1.5 hours after APAP (Walker et al., 1983). Since this occurs subsequent to peroxynitrite staining in endothelial cells, it is possible that APAP initially causes sinusoidal endothelial cell (SEC) dysfunction prior to affecting hepatocytes. In fact, SEC have been shown to be susceptible to APAP-induced injury *in vitro* (DeLeve et al., 1997). SEC damage during APAP hepatotoxicity varies among mouse strains depending on the P450 content of the SEC (Yin et al., 2010). Thus, the evidence of early peroxynitrite formation, coupled with morphological changes suggest that the earliest target of APAP-induced injury are SEC (Ito et al., 2003) and it is likely that endothelial mitochondrial oxidant stress and peroxynitrite formation play a critical role in this process (Knight et al., 2004). However, the role SEC mitochondrial dysfunction plays in subsequent hepatocyte injury is not well characterized and needs further study. This is all the more relevant considering the role of nitric oxide in the process. While studies have been conducted on the

role of NO in mediating APAP induced SEC damage (Ito et al., 2004), they lacked a mitochondrial focus. This is relevant since nitric oxide would have to be produced close to the location where superoxide is being produced for efficient reaction to peroxynitrite. It has been shown that pharmacological inhibition of nNOS, the putative mitochondrial NOS, protects hepatocytes against APAP-induced injury *in vitro* (Banerjee et al., 2015). This was corroborated *in vivo* too, where nNOS deficient mice were protected against APAP-induced hepatotoxicity (Agarwal et al., 2012). Interestingly, one of the targets for modification by peroxynitrite is mitochondrial manganese superoxide dismutase (Agarwal et al., 2011), which would further corrode the cells superoxide scavenging ability. The elevated peroxynitrite also contributes to mitochondrial DNA damage and loss after APAP (Cover et al., 2005), which has significant consequences later during resolution and recovery from APAP induced injury, which will be discussed in the last paragraph. The important role of peroxynitrite in mediating APAP-induced liver injury is clear by the fact that its direct scavenging either by mitochondrial glutathione (Knight et al., 2002; Saito et al., 2010) or resveratrol (Du et al., 2015), prevented APAP hepatotoxicity. Since most recent studies on APAP hepatotoxicity focus on hepatocytes, the role played by nitric oxide in modulation of mitochondrial function in sinusoidal endothelial cells is not well understood. This is relevant since NO can have significant effects on endothelial mitochondrial function (Ramachandran et al., 2004) and one study has shown endothelial-derived NO affecting APAP hepatotoxicity *in vivo* (Salhanick et al., 2006). However, SEC damage appears to be mouse strain-dependent and may not be particularly relevant for the human pathophysiology (Maes et al., 2016). Even if the SEC are the primary target for induction of mitochondrial oxidative and nitrosative stress, the functional consequences of APAP overdose are mediated by mitochondrial dysfunction in hepatocytes and this will be elaborated next.

Consequences of mitochondrial oxidative/nitrosative stress in the cytosol

It could be argued that a cell such as the hepatocyte, with large number of mitochondria, would be expected to have significant reserve mitochondrial capacity to overcome dysfunction of few mitochondria due to oxidative damage. This could be the case if the consequences of NAPQI adduct formation and subsequent steps were restricted to only the organelle. In APAP overdose however, hepatocyte mitochondrial oxidative/nitrosative stress initiates signaling events within the cytosol, which subsequently amplify the mitochondrial damage and perpetuate the injury process. Though the half-life of peroxynitrite is short, in the range of 10–20 ms, this is sufficient to cross biological membranes, and diffuse one to two cell diameters (Pacher et al., 2007) and it has been shown that peroxynitrite can cross erythrocyte membranes either through an anion channel in its anionic form or by passive diffusion in the protonated form (Denicola et al., 1998). This could mean that APAP-induced peroxynitrite generated within mitochondria could either passively diffuse out or be transported through mitochondrial anion channels such as VDAC, which have been shown to control the release of the superoxide anion from mitochondria to cytosol (Han et al., 2003). The presence of peroxynitrite in the cytosol then enables activation of a MAP kinase cascade ultimately resulting in activation of the c-Jun N-terminal kinase (JNK), which ultimately amplifies the mitochondrial dysfunction.

Though the exact stepwise mechanism of JNK activation by mitochondrial peroxynitrite is not well characterized, the process is likely initiated by oxidation of the protein thioredoxin, which has been shown to be a target both within the mitochondria as well as in the cytosol. Oxidation of thioredoxin 2 within mitochondria has been shown after APAP overdose (Ramachandran et al., 2015) and both mitochondrial thioredoxin 2 as well as cytosolic thioredoxin 1 are susceptible to direct modification by NAPQI (Jan et al., 2014). Administration of recombinant thioredoxin-1 fused to albumin in a delayed fashion, 4 hours after APAP was also shown to be protective in mice (Tanaka et al., 2014). Thioredoxin 1 in the cytosol is typically associated with its binding partner apoptosis signal-regulating kinase 1 (ASK1) and oxidation of thioredoxin 1 after APAP results in its dissociation from ASK1, and activation of ASK1 by phosphorylation (Nakagawa et al., 2008). The role of peroxynitrite in this process of activation of JNK is all the more probably since it has been shown that peroxynitrite can induce ASK1-thioredoxin dissociation in post ischemic myocardial infarction (Tao et al., 2006), which could also be occurring in the liver after APAP overdose. Other components of the APAP induced MAP kinase cascade include Mixed-lineage kinase 3 (MLK3), which is a ubiquitously expressed mammalian mitogen-activated protein kinase kinase kinase (MAP3K), which is suggested to control a positive feedback loop titrating proliferative or cell death signaling in response to ROS (Lee et al., 2014). It was found that low concentrations of ROS induced activating phosphorylation of ERKs and proliferation, whereas high concentrations of ROS activated phosphorylation of JNKs and cell death (Lee et al., 2014). These studies suggested that ASK1 may be involved in the ROS-induced activation of the MLK3-mediated positive feedback loop (Lee et al., 2014). MLK3 has also been shown to be activated during APAP hepatotoxicity (Sharma et al., 2012), and ultimately, activated ASK1 and MLK3 phosphorylate MKK4 (Zhang et al., 2017), which then phosphorylates JNK (Hanawa et al., 2008; Saito et al., 2010). The role of ASK1 in the process is further illustrated by the protection against APAP induced liver injury afforded by pharmacological inhibition of ASK1 (Xie et al., 2015) or inhibition of JNK activation in mice lacking ASK1 (Nakagawa et al., 2008).

While activated JNK translocates to the mitochondria to amplify mitochondrial dysfunction, it also facilitates movement of other proteins such as Bax to the mitochondria (Bajt et al., 2008; El-Hassan et al., 2003). Bax is typically tethered to the 14-3-3 protein in the cytosol and JNK has been shown to promote Bax translocation to mitochondria through phosphorylation of 14-3-3 proteins (Tsuruta et al., 2004). Bax can also be phosphorylated by activated glycogen synthase kinase-3 β (GSK-3 β), which also induces its translocation to the mitochondria, at least in cancer cells (Chiara et al., 2013). However, silencing of GSK-3 β did not have any effect on Bax translocation in the liver (Shinohara et al., 2010), rendering this connection tenuous. However, APAP treatment in mice was shown to cause GSK-3 β activation and translocation to mitochondria during the initial phase of APAP-induced liver, and animals lacking GSK-3 β showed significant protection against APAP hepatotoxicity (Shinohara et al., 2010). Thus, GSK-3 β does play a role in the early activation of mitochondrial dysfunction after APAP, though the exact mechanisms behind its role are not very clear. Subsequent to these changes in the cytosol, which result in JNK, Bax and GSK-3 β activation, all three proteins translocate to the mitochondria to amplify organelle dysfunction.

Mitochondrial amplification of injury

APAP-induced phosphorylation of JNK in the cytosol results in its translocation to the mitochondria (Hanawa et al., 2008), where it binds to the scaffold protein Sab on the outer mitochondrial membrane (Win et al., 2011). In case of GSK-3 β , though direct evidence after APAP overdose is lacking, experiments in cardiomyoblasts have indicated that GSK-3 β translocation to the mitochondria occurs in a kinase activity dependent manner where the N-terminal domain of GSK-3 β may function as a mitochondrial targeting sequence, with the protein binding to VDAC2 on the mitochondrial outer membrane (Tanno et al., 2014). Though the exact mechanism of activated Bax translocation is still being worked out, there is a consensus that the protein directly inserts into the membrane (Westphal et al., 2011) rather than requiring a ligand to dock unlike the case for JNK. While it is known that mitochondrial membrane lipids such as cardiolipin are susceptible to oxidation in various pathologies (Yin et al., 2012), and APAP has been shown to remodel cardiolipin in mitochondria to affect mitochondrial respiration (Vergeade et al., 2016), those studies were unfortunately carried out in myeloma cells, and their relevance to hepatocytes is unknown. Binding of JNK to Sab on the mitochondrial outer membrane initiates inhibition of the mitochondrial electron transport chain in a Src dependent manner, resulting in amplification of oxidative stress (Win et al., 2011) and peroxynitrite generation (Saito, et al., 2010). The critical role of phospho-JNK translocation to the mitochondria in mediating APAP induced hepatotoxicity is illustrated by the protection afforded when JNK translocation to mitochondria is blocked (Huo et al., 2017). The effect of Bax translocation to mitochondria after APAP is a bit more ambiguous, since it was found that while mice deficient in Bax were protected initially against APAP hepatotoxicity, they showed both liver necrosis as well as peroxynitrite generation similar to wild type mice at later time periods (Bajt et al., 2008), suggesting that cells could adapt to the lack of Bax and pursue alternate necrotic signaling in response to APAP. However, Bax, along with its homologue Bak have been suggested to form pores on the mitochondrial outer membrane to facilitate mitochondrial membrane permeability, which will be the focus of the next section.

The End Game - Mitochondrial permeability transition and subsequent cellular necrosis

The above mentioned translocation of JNK and Bax to the mitochondria further compromises mitochondrial function through inhibition of the mitochondrial ETC and subsequent amplification of superoxide and peroxynitrite production (Figure 2). Though this ultimately results in activation of the mitochondrial permeability transition pore and collapse of mitochondrial membrane potential (Kon et al., 2004; Masubuchi et al., 2005; Ramachandran et al., 2011), these events are titrated to the dose of APAP and are reversible at lower doses of APAP as will be elaborated below. The mitochondrial permeability transition pore (MPTP) forms within the inner membrane allowing movement of molecules of less than 1.5kDa (Halestrap, 2009), and while its exact molecular composition is still being determined, one of its well defined components is cyclophilin D, which regulates it from within the mitochondrial matrix (Baines et al., 2005; Karch et al., 2015). Thus, the role of the mitochondrial MPTP in APAP hepatotoxicity was implicated by protection offered by

pharmacological blockade of cyclophilin D *in vitro* (Kon et al., 2004). However, this protection was transient, and lost at later time points similar to the effect seen *in vivo* with Bax deficient mice. Interestingly a dose-dependent effect was seen *in vivo*, where mice deficient in cyclophilin D were protected against APAP-induced liver injury at a dose of 200mg/kg (Ramachandran et al., 2011), while animals subjected to a higher 600mg/kg dose showed no protection (LoGuidice et al., 2011). Also, animals treated with APAP doses of 150mg/kg showed reversible induction of MPTP accompanied by transient JNK activation, while those receiving 300mg/kg had irreversible MPTP (Hu et al., 2016). In addition to the various proteins translocating to the mitochondria in response to APAP-induced cytosolic oxidant stress, translocation of lysosomal iron has also been shown to occur in mouse hepatocytes after treatment with APAP, with induction of the MPTP (Kon et al., 2010). This translocation of iron occurs through the calcium uniporter (Hu et al., 2016) and is probably due to lysosomal instability, which has also been demonstrated after APAP overdose (Woolbright et al., 2012).

While the components of the MPTP on the inner membrane other than cyclophilin D are still being characterized, it is well recognized that the outer membrane components are composed of Bax, along with the protein Bak on the mitochondrial outer membrane (Karch et al., 2013; Karch et al., 2014). Though these outer membrane components function during the MPTP, they are not essential for loss of mitochondrial membrane potential since it has been shown that the inner membrane portion of the MPTP was functional even in the absence of Bax and Bak, and able to induce opening that resulted in loss of membrane potential (Karch et al., 2013). This could be another explanation for lack of protection in Bax deficient mice, where the sustained production of peroxynitrite by upstream events would ultimately cause activation of the inner membrane components of the MPTP, loss of mitochondrial membrane potential and subsequent dysfunction. Another layer of complexity is added to this process by the fact that mitochondria are dynamic organelles undergoing continuous fusion and fission reactions for maintenance of cellular homeostasis. The fact that APAP overdose affects mitochondrial dynamics was first recognized by the identification of significant elevations in the mitochondrial fission protein Drp1 and its translocation to mitochondria after APAP overdose (Ramachandran et al., 2013), which was subsequently confirmed by independent studies (Dara et al., 2015), indicating that mitochondrial dynamics could influence cellular signaling after APAP overdose. This is relevant for induction of the MPTP, since spatial and temporal interactions between Drp1 and Bax have been identified (Karbowski et al., 2002), and Bax and Drp1 have both been shown to regulate each other in a reciprocal manner (Wang et al., 2015; Wu et al., 2011). Thus, these interactions between Bax and Drp1, could influence activation of the MPTP after APAP and subsequent mitochondrial dysfunction.

Ultimately as a consequence of the MPTP, critical mitochondrial proteins such as cytochrome c, endonuclease G, apoptosis inducing factor and Smac are released into the cytosol to initiate DNA fragmentation and cell necrosis. While loss of cytochrome c compromises mitochondrial electron transport and shuts down ATP synthesis, apoptosis inducing factor and endonuclease G have nuclear localization signals (Norberg et al., 2010), which allow their translocation to the nucleus (Bajt et al., 2006). The importance of AIF in APAP induced hepatotoxicity is indicated by protection offered in mice with a partial

deficiency of AIF (Bajt et al., 2011). In the nucleus, AIF causes chromatin condensation and DNA fragmentation (Boujrad et al., 2007), while endonuclease G cleaves nuclear DNA resulting in DNA fragmentation (Cover et al., 2005).

Mitochondria in adaptation and recovery after APAP overdose

In addition to the well characterized role of mitochondria in APAP-induced injury detailed above, more recent studies highlight the role of the organelle in adaptive responses to APAP as well as in recovery after APAP-induced injury. An important adaptive feature within the liver after APAP-induced formation of protein adducts is induction of a form of autophagy, called mitophagy, which enables removal of damaged mitochondria (Ni et al., 2012; Ni et al., 2013). The importance of this is illustrated by the aggravation of APAP hepatotoxicity by prevention of mitophagy by cholesterol accumulation (Baulies et al., 2015). Mitophagy would also be beneficial from a spatial perspective, where cells at the border of the necrotic area may be spared induction of cell death by timely removal of damaged mitochondria (Ni et al., 2013). The cells at the border of the necrotic area are also important from the perspective of recovery after an APAP overdose and recent evidence shows spatially defined activation of mitochondrial biogenesis in these cell populations, which plays a critical role in recovery (Du et al., 2017). Induction of mitochondrial biogenesis was also shown to facilitate recovery and regeneration after APAP-induced liver injury (Du et al., 2017).

Role of mitochondria-derived damage-associated molecular patterns in injury and recovery

Because of the extensive mitochondrial damage and the resulting severe necrosis after APAP overdose, mitochondrial components are released into the cytosol and ultimately into the circulation. Some of the macromolecules detected in plasma include mtDNA, the matrix enzymes glutamate dehydrogenase (GLDH) and carbamoyl phosphate synthetase-1 and long-chain acylcarnitines (Bhattacharyya et al., 2014; Chen et al., 2009; McGill et al., 2014; McGill et al., 2012; Weerasinghe et al., 2014). All these compounds can be used as mechanistic biomarkers to detect mitochondrial damage in the pathophysiology of APAP hepatotoxicity and potentially predict outcome in patients (McGill et al., 2018). However, some of these mitochondria-derived molecules, e.g. mtDNA, are potent damage-associated molecular patterns (DAMPs), which bind to toll like receptors on macrophages, e.g. TLR9, and transcriptionally activate cytokine and chemokine formation (Kubes et al., 2012; Woolbright et al., 2017). The resulting activation and hepatic recruitment of neutrophils and monocyte-derived macrophages has the potential to further aggravate the injury (Kubes et al., 2012). However, the preponderance of experimental evidence suggest that these inflammatory cells are mainly recruited to clear necrotic cell debris and promote regeneration (Woolbright et al., 2017). Importantly, the pro-regenerative function of neutrophils and monocytes is also supported by human studies (Antoniades et al., 2012; Williams et al., 2014). Thus, mitochondria-derived and other DAMPs prepare the damaged tissue for recovery.

Relevance of APAP-induced mitochondrial dysfunction to humans

While most findings on mitochondrial involvement in APAP-induced hepatotoxicity have been determined by investigations in the mouse model, the organelle's involvement in APAP induced hepatotoxicity in humans has also been extensively documented. As mentioned earlier, formation of mitochondrial protein adducts have been recognized in human hepatocytes treated with AMAP (Xie et al., 2015) and studies on freshly isolated human hepatocytes exposed to APAP overdose demonstrated formation of mitochondrial protein adducts as well as a time dependent activation and mitochondrial translocation of phospho-JNK to the mitochondria, followed by loss of mitochondrial membrane potential (Xie et al., 2014). A number of mitochondrial proteins modified by NAPQI have also been identified in 3D cultures of human hepatocytes and nonparenchymal cells (Bruderer et al., 2015). Human HepaRG cells exposed to APAP show mitochondrial oxidant stress and peroxynitrite formation (McGill et al., 2011) as in the mouse, indicating that similar mechanisms are operative in human hepatocytes subsequent to APAP overdose. This is corroborated by direct evidence from human patients, where biomarkers of mitochondrial damage such as circulating mitochondrial DNA and glutamate dehydrogenase were also elevated in APAP overdose patients with liver injury (McGill et al., 2012). Patients with more mitochondrial damage are also less likely to survive, since the levels of these mitochondrial biomarkers were also found to be significantly higher in non-surviving patients when compared to survivors (McGill et al., 2014). The relevance of APAP-induced mitochondrial dysfunction in human patients with APAP overdose is further re-iterated by the introduction into clinical trials of a SOD mimetic, which will be discussed below.

Conclusion

While the mitochondria are a critical target during APAP-induced hepatotoxicity and act as an integration center for various cellular signaling components of necrotic cell death, the organelle also plays very important roles in adaptation and regeneration in a spatially defined manner to enable robust recovery of cells further away from the necrotic area. While extensive experimentation has provided significant mechanistic insight into the role of hepatocyte mitochondria in APAP-induced liver injury, they have also uncovered tantalizing clues towards the other roles the organelle plays, especially in recovery and regeneration after APAP overdose. Studies to better understand these roles, such as the influence of mitochondrial dynamics and biogenesis in mediating recovery and regeneration are ongoing with the ultimate hope to develop mitochondrial targeted therapeutics to prevent APAP-induced acute liver failure. Our unpublished data indicate that drugs such as 4-methylpyrazole, which provides protection against APAP-induced injury (Akakpo et al., 2018), inhibits not only cytochrome P450 enzymes but also prevents JNK activation and its mitochondrial translocation and could thus be a useful therapeutic with similar potency as the current standard of care *N*-acetylcysteine. The SOD mimetics Calmangafodipir and Mito-TEMPO were also shown to have beneficial effects in APAP overdose in mice (Bedda et al., 2003; Du et al., 2017; Du et al., 2018) and Calmangafodipir is currently in clinical trials (Dear et al., 2017). Methylene blue, a redox-active compound used clinically to treat methemoglobinemia has also been shown to protect against APAP-induced mitochondrial injury by circumventing the compromised complexes I/II of the electron transport chain (Lee

et al., 2015). In addition, inducers of mitochondrial biogenesis such as SRT1720 have also been shown to be beneficial (Du et al., 2017) and could be developed as therapeutics. Overall, while research over the previous decades have provided significant insight into the role of mitochondria in APAP-induced injury, the various facets of the organelle in recovery and regeneration, which probably have more therapeutic potential, are poised to be discovered in the future.

Acknowledgements

Work in the authors' laboratory was supported by a grant from McNeil Consumer Health Care, Inc., 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.

Abbreviations

AIF	Apoptosis Inducing Factor
AMAP	3'-hydroxyacetanilide
APAP	Acetaminophen
ASK1	Apoptosis Signaling Kinase 1
CuZnSOD	Copper Zinc Superoxide Dismutase
DAMP	Damage Associated Molecular Pattern
eNOS	Endothelial Nitric Oxide Synthase
ER	Endoplasmic Reticulum
ERK	Extracellular signal Regulated Kinase
ETC	Electron Transport Chain
GLDH	Glutamate Dehydrogenase
GSK-3β	Glycogen Synthase Kinase-3 β
JNK	c-Jun N-terminal Kinase
MAP	Mitogen Activated Protein
MKK4	Mitogen-Activated protein Kinase Kinase 4
MLK3	Mixed-Lineage Kinase 3
MnSOD	Manganese Superoxide Dismutase
MPTP	Mitochondrial Permeability Transition Pore
NAC	N-Acetyl Cysteine
NAPQI	N-Acetyl-p-benzoquinone imine

nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
PARK7	Parkinsonism Associated Deglycase
ROS	Reactive Oxygen Species
SEC	Sinusoidal Endothelial Cell
VDAC	Voltage Dependent Anion Channel

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Fig 1–. Peroxynitrite formation is critical for initial mitochondrial dysfunction and signaling to the cytosol:

The mitochondrial electron transport chain (ETC) comprised of 4 proteins transfer electrons from complex I to complex 4, while pumping protons to generate a proton gradient which is utilized by ATP synthase (complex V) to generate ATP. Though free radicals such as superoxide are generated during electron transport, superoxide dismutases (SOD) within the mitochondria scavenge them efficiently. Thus, reaction of superoxide with nitric oxide (NO) generated from neuronal NOS and probably eNOS on mitochondria is minimal. When mitochondria are exposed to NAPQI, adduct formation on mitochondrial proteins such as

ATP synthase could result in reverse electron transport within the ETC, which elevates superoxide generation. Protein adduct formation on anti-oxidant enzymes prevents efficient scavenging of superoxide, which enables its reaction with nitric oxide to form peroxynitrite radicals. These in turn can modify enzymes such as SOD as well as spill out of the mitochondria into the cytosol, probably through outer membrane protein such as VDAC.

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Fig 2— JNK and Bax translocation to mitochondria amplify mitochondrial dysfunction: Phosphorylated JNK as well as Bax translocate to the mitochondria and JNK binds to its ligand Sab, on the outer mitochondrial membrane. This binding initiates a signaling cascade which inhibits the ETC and further amplifies superoxide and peroxynitrite generation. This in turn activates the mitochondrial permeability transition pore on the inner membrane, of which cyclophilin D is a component. This, along with the formation of Bax pores on the outer membrane allow release of mitochondrial proteins such as endonuclease G and apoptosis inducing factor (AIF) into the cytosol.