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Mitochondrial Damage and Biogenesis in Acetaminophen-induced Liver Injury

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Abstract

Liver injury and acute liver failure caused by acetaminophen (APAP) overdose is the clinically most important drug toxicity in western countries. Mechanistic investigations have revealed a central role of mitochondria in the pathophysiology. Excess formation of the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) after an overdose leads to hepatic glutathione depletion, mitochondrial protein adducts formation and an initial oxidant stress, which triggers the activation of mitogen activated protein (MAP) kinase cascade ultimately leading to c-jun N-terminal kinase (JNK) phosphorylation. Phospho-JNK translocates to the mitochondria and amplifies the oxidative and nitrosative stress eventually causing the mitochondrial membrane permeability transition pore opening and cessation of ATP synthesis. In addition, mitochondrial matrix swelling ruptures the outer membrane and releases endonucleases, which cause nuclear DNA fragmentation. Together, the nuclear DNA damage and the extensive mitochondrial dysfunction result in necrotic cell death. However, the pro-cell death signaling events are counteracted by adaptive responses such as autophagy and mitochondrial biogenesis. The improved mechanistic insight into the pathophysiology leads to better understanding of the mechanisms of action of the existing antidote N-acetylcysteine and justifies the clinical testing of novel therapeutics such as 4-methylpyrazole and calmagafodipir.

Keywords

Acetaminophen hepatotoxicity; mitochondrial dysfunction; biogenesis; oxidant stress; peroxynitrite; c-jun N-terminal kinase

1. Introduction

Acetaminophen (APAP) is one of the most widely used analgesic and anti-pyretic drugs. At therapeutically recommended doses, APAP is generally considered a safe drug,^{1,2} even in high risk populations such as alcoholics³ or patients with liver disease.⁴ More than 90% of a

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Authors' contributions

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Conflict of interest

The authors declare no conflict of interest.

therapeutic dose is directly glucuronidated or sulfated and rapidly excreted.⁵ Only a small fraction is metabolized by cytochrome P450 enzymes, especially Cyp2E, to form the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI), which is effectively detoxified by hepatocellular glutathione (GSH); the GSH conjugate is then excreted into bile or blood.⁶ The effectiveness of the protective system is documented by the very low levels of protein adducts formed after therapeutic doses in both humans and animals.⁷⁻⁹ However, due to the availability of APAP in numerous prescription and over-the-counter medications, intentional and unintentional overdosing is a significant clinical problem.¹⁰ APAP overdose can induce severe liver injury and even acute liver failure. In fact, APAP overdose is the most common cause of acute liver failure in most western countries.¹¹ After an overdose, the phase II conjugation reactions are either saturated (sulfation) or, despite a dramatic increase, the glucuronidation cannot keep up with the high levels of APAP.¹² This leads to enhanced NAPQI formation, which causes depletion of the cellular GSH stores and a dramatic increase in hepatic protein adducts.⁵ In the 1970s, when GSH depletion and protein adduct formation in response to APAP overdose in mice was first discovered, it was assumed that this is the main mechanism of cell death.^{13,14} However, over the years it became obvious that the early protein adduct formation is just an initiating event, which triggers complex cell death signaling pathways and adaptive reactions centered around mitochondria. The critical role of mitochondria in the pathophysiology will be discussed in this review.

2. Mitochondrial Dysfunction and Damage after APAP Overdose

2.1 Acetaminophen-induced generation of mitochondrial protein adducts

Although mitochondrial protein adducts after APAP overdose in mice were described during the early studies by Mitchell and coworkers,¹³ a functional relevance was not recognized. However, later studies by Sid Nelson's group comparing protein adducts and injury in mice after treatment with APAP (N-acetyl-para-aminophenol) versus its regional isomer AMAP (N-acetyl-meta-aminophenol) suggested significant differences between the two compounds. Although AMAP formed reactive metabolites and overall protein adducts in the liver similar to APAP,¹⁵ AMAP in contrast to APAP did not form adducts in mitochondria and did not cause liver injury.^{16,17} These observations suggested that mitochondrial protein adducts are relevant for the injury process. In addition, increased oxidant stress inside liver mitochondria in APAP- but not AMAP-treated animals implicated the mitochondrial protein adducts in the generation of a mitochondrial oxidant stress during APAP hepatotoxicity.¹⁸ More recent studies demonstrated that AMAP can cause cell injury in human liver slices¹⁹ and in primary human hepatocytes.²⁰ This susceptibility of human cells to AMAP compared to mouse hepatocytes correlated with the formation of mitochondrial protein adducts.²⁰ Furthermore, comparison between APAP treatment of rats and mice showed delayed formation and significantly less protein adducts in rat liver mitochondria, which may explain the lower susceptibility of rats to an APAP overdose.²¹ Together, these observations support the hypothesis that not the overall protein adduct levels in the cell but rather the protein adducts on the mitochondria are the most critical initiating events in the pathophysiology of APAP-induced cell death (Figure 1).

2.2 Mitochondrial oxidant stress-induced MAP kinase activation

The initial discovery of a mitochondrial oxidant stress was based on elevated glutathione disulfide (GSSG) levels inside mitochondria and the absence of relevant GSSG levels in the cytosol or GSSG excreted into bile.^{18,22} It is known that GSSG formed inside mitochondria can only be reduced to GSH but cannot be exported into the cytosol.²³ This suggested a selective formation of reactive oxygen in the matrix of mitochondria, which was later confirmed by the use of MitoSOX Red, a mitochondrial superoxide indicator.²⁴ In addition, the formation of peroxynitrite, a reaction product of superoxide and nitric oxide, was also located predominantly inside mitochondria.²⁵ Interestingly, other cellular sources of reactive oxygen such as cytochrome P450, as hypothesized earlier,²⁶ were excluded based on the fact that neither GSSG formation nor 2',7'-dichlorodihydrofluorescein diacetate fluorescence increased during the drug metabolism phase.^{27,28} Despite the convincing evidence of a mitochondrial oxidant stress and peroxynitrite formation during APAP hepatotoxicity,^{18,22,24,25} the oxidant and nitrosative stress appeared to be a late event that correlated closely with cell injury and not with the early mitochondrial protein adduct formation.⁸

The evidence for an early oxidant stress emerged after it was recognized that the mitogen activated protein (MAP) kinase c-jun N-terminal kinase (JNK) is involved in APAP-induced liver injury²⁹ and the JNK inhibitor SP600125 prevented the APAP-induced oxidant stress (Figure 1).³⁰ An upstream mediator of this JNK activation seems to be the redox-sensitive apoptosis signal-regulating kinase 1 (ASK1), a member of MAP kinase kinase kinase (MAP3K) family, which exists in the cytosol in a complex with thioredoxin and can be liberated and activated by oxidation of thioredoxin.³¹ Deficiency of ASK-1 and inhibitors of ASK-1 were shown to attenuate JNK activation and reduce APAP-induced liver injury.^{32,33} Other upstream redox-sensitive MAP3K involved in the process include mixed-lineage kinase 3 (MLK3)³⁴ and glycogen synthase kinase-3beta (GSK-3 β)³⁵ all of which inhibit JNK activation and reduce injury. These upstream kinases do not directly interact with JNK but act through the MAP2K MKK4, which then phosphorylates JNK (Figure 1).³⁶ JNK activation is counteracted by mitogen-activated protein kinase phosphatase (Mkp)-1, which inhibits APAP-induced liver injury.³⁷ Together these data suggest that a mitochondrial protein adduct-induced mild initial oxidant stress, which is not readily detected by even sensitive oxidant stress markers, activates redox-sensitive MAP3 kinases and triggers a kinase cascade resulting in the extensive activation of JNK. Importantly, this initial oxidant stress is insufficient to cause direct damage to mitochondria or trigger the mitochondrial membrane permeability transition (MPT).

2.3 Mitochondrial JNK translocation and amplification of the mitochondrial oxidant stress

After the importance of JNK activation in APAP toxicity was recognized,^{29,38,39} it became clear that JNK seems to regulate the main mitochondrial oxidant/nitrosative stress.³⁰ However, the mechanism by which JNK might amplify the initial oxidant stress remained unclear.⁴⁰ Follow-up studies from Neil Kaplowitz's laboratory shed light on this mystery. First, it was established that activated JNK (phospho-JNK) translocates from the cytosol to the mitochondria⁴¹ and binds to the anchor protein Sab on the outer mitochondrial membrane.⁴² The mitochondrial JNK translocation is responsible for the oxidative/nitrosative stress and prolonged JNK activation.⁴² The mechanism by which P-JNK binding

and phosphorylation of Sab enhances the superoxide formation involves the release of SHP1 (protein tyrosine phosphatase, nonreceptor type 6, PTPN6) from Sab on the inside of the mitochondrial outer membrane (Figure 1). This triggers its activation and transfer of P-SHP1 to the inner membrane, where it dephosphorylates P-Src (active).⁴³ This process requires docking protein 4 (DOK4) on the inner membrane. Src (inactive) further inhibits the electron transport chain and promotes release of superoxide, which gives rise to an amplified oxidative/nitrosative stress that is responsible for further JNK activation and cell death.⁴³

Since the first reports of a mitochondrial oxidant stress during APAP hepatotoxicity^{18,22} it was demonstrated that the reactive oxygen did not trigger extensive lipid peroxidation and loading the liver with vitamin E did not protect.⁴⁴ In contrast, superoxide derived from the electron transport chain reacts with nitric oxide to form the potent oxidant peroxynitrite,⁴⁵ which was recognized as the actual oxidant responsible for the injury (Figure 1).⁴⁶ The mitochondrial superoxide dismutase (SOD2, MnSOD), which accelerates the dismutation of superoxide to hydrogen peroxide and oxygen, is actually a protective factor as it limits peroxynitrite formation and promotes the detoxification of hydrogen peroxide by glutathione peroxidase.⁴⁷ Thus, partial deficiency of MnSOD enhanced peroxynitrite formation and aggravated APAP-induced liver injury.^{48,49} Unfortunately, during APAP toxicity, MnSOD is in part inactivated by peroxynitrite⁵⁰ and hepatic GSH, a direct scavenger of peroxynitrite, is severely depleted.⁴⁶ Thus, the accelerated recovery of hepatic GSH levels after treatment with antidotes like GSH or N-acetylcysteine restores mitochondrial GSH levels, which scavenge peroxynitrite and protect against APAP-induced liver injury.^{46,51–53} In addition, Mito-TEMPO, a mitochondrial-targeted SOD mimetic, eliminated nitro-tyrosine staining and effectively protected against APAP toxicity.^{54,55} Furthermore, it was shown that APAP causes a block of complex II of the electron transport chain (ETC), which triggers the oxidant stress and causes ATP depletion.⁵⁶ Methylene blue, a redox-active compound that can accumulate in mitochondria, was able to accept electrons from NAPQI-altered, succinate-energized complex II and transfer them to cytochrome c, thereby overcoming the block of the ETC.⁵⁶ Thus, methylene blue effectively prevented APAP hepatotoxicity without affecting metabolic activation.⁵⁶ In addition to the oxidant stress, lysosome-derived iron is taken up into mitochondria through the mitochondrial electrogenic Ca^{2+} , Fe^{2+} uniporter during APAP-induced cell death.^{57,58} Chelation of lysosomal iron and inhibition of mitochondrial iron uptake prevented mitochondrial dysfunction and cell death.⁵⁸ Taken together, there is extensive evidence for a critical role of a mitochondrial oxidant stress being responsible for mitochondrial dysfunction leading to the MPT pore opening, breakdown of the membrane potential and cessation of ATP synthesis.^{59,60} These events also lead to mitochondrial matrix swelling with rupture of the outer membrane, release of intermembrane proteins and nuclear translocation of endonuclease G and apoptosis-inducing factor (AIF), which cause nuclear DNA fragmentation as indicated by the TUNEL assay^{61–63} and nuclear DNA fragments of various sizes (Figure 1).⁶⁴ Other intermembrane proteins released from mitochondria into the cytosol include cytochrome c and second mitochondria-derived activator of caspase (Smac)^{62,65,66} but this does not trigger apoptotic cell death.⁶⁷

2.4 New therapeutic options for APAP toxicity

The only clinically approved antidote against APAP overdose is N-acetylcysteine (NAC), which was discovered based on the initial mechanistic insight of reactive metabolite formation, GSH depletion and protein adduct formation.^{13,14} When treated very early after the overdose, NAC protects mainly through scavenging of NAPQI and preventing protein adduct formation.⁶⁸ However, NAC does not react directly with NAPQI but acts through promoting hepatic GSH synthesis.⁶⁹ At later time points, the newly synthesized mitochondrial GSH scavenges peroxynitrite and assists in detoxification of hydrogen peroxide by glutathione peroxidase.^{46,51–53} In addition, any excess NAC not used for GSH synthesis will be converted to Krebs cycle intermediates, which support the mitochondrial bioenergetics and enhance ATP levels.⁵³ Thus, NAC has multiple modes of action over a significant therapeutic window. As a result, it was shown that NAC is most effective in human overdose patients during the first 8 h after APAP but is still partially effective up to 24 h.^{70,71}

Despite the effectiveness of NAC, there are some side-effects including anaphylactic reactions, that limit the dose that can be given to a patient. Therefore, additional antidotes with complementary modes of action are desirable. The clinically approved antidote for methanol and polyethylene glycol poisoning 4-methylpyrazole (4MP) is a potential candidate.⁷² Recent preclinical studies showed that 4MP co-treatment with APAP eliminated the oxidative metabolism through inhibition of P450 enzymes and completely prevented the injury in a murine model of APAP hepatotoxicity.⁷³ Interestingly, a delayed treatment, which did not affect oxidative metabolism and protein adduct formation, was also highly effective.⁷⁴ These studies demonstrated that 4MP can also inhibit the activation and mitochondrial translocation of JNK.⁷⁴ Furthermore, studies in human volunteers indicated that 4MP co-treatment with supratherapeutic doses of APAP effectively prevented any oxidative metabolism of APAP but had no adverse effects in humans.⁷⁵ Thus, 4MP can have complementary mechanisms of action compared to NAC. However, in contrast to NAC, 4MP acts directly, i.e. does not need to be metabolized to be effective and does not appear to have any relevant side-effects. A co-treatment with NAC and 4MP may be implicated in patients with extremely high overdoses of APAP.⁷²

Another antidote which is currently in clinical development is the mitochondrial SOD mimetic calmagafodipir.⁷⁶ As discussed above, SOD mimetics targeted to mitochondria are highly effective in preventing peroxynitrite formation and therefore are protective in the mouse APAP toxicity model.^{54,55} Calmagafodipir has recently been tested in APAP overdose patients.⁷⁷ Although these were early presenting patients receiving the standard of care NAC and therefore were at low risk of developing liver injury, treatment with calmagafodipir proved to be safe and without adverse effect.⁷⁷ In addition, using highly sensitive injury parameters, e.g. cytokeratin-18, showed a trend to less liver injury.⁷⁷ Thus, if efficacy in high overdose patients can be established, calmagafodipir may be a promising complementary antidote to NAC.

3. The role of mitochondrial biogenesis in APAP Hepatotoxicity

3.1. Mitochondrial biogenesis in cellular homeostasis

Mitochondrial biogenesis is a fundamental process in cell biology which is critical for normal cellular regeneration and homeostasis. It is essential for recovery of cellular function by facilitating controlled regeneration of mitochondria so that critical cellular processes such as respiration and metabolism can be maintained. Mitochondrial biogenesis is a complex coordinated process which ensures that new protein synthesis in the mitochondria is coupled to that from the nucleus and acts in concert with mitochondrial fission and fusion to ensure proper functioning of newly synthesized mitochondria (Figure 2). Since mitochondrial biogenesis is an energy intensive process requiring a large number of coordinated changes in gene expression and protein synthesis, it is a tightly controlled process which can be initiated by a number of stimuli indicating a shift in cellular homeostasis. These could include fasting, exercise and oxidative stress.⁷⁸ Despite the large variety of initiating signals, however, they all converge at peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC1 α), which is considered to be the central mediator for the process (Figure 2). PGC1 α controls a number of downstream targets including nuclear respiratory factor (NRF) 1 & 2 and Transcription Factor A, Mitochondrial (Tfam)⁷⁹ and while the complex transcriptional regulation of mitochondrial biogenesis is outside the scope of this review, it has been comprehensively examined.⁸⁰ Among the downstream targets of PGC1 α , the nuclear respiratory factors NRF-1 and NRF-2 were the first nuclear transcription factors implicated in induction of multiple facets of mitochondrial function.⁸⁰ While NRF-1 acts on genes encoding respiratory subunits as well as Tfam, in addition to other genes whose products regulate mitochondrial transcription and ribosome assembly, NRF-2 was initially identified as a transcriptional upregulator of the cytochrome oxidase subunit IV (COXIV) promoter.⁸⁰ Both factors cooperate in the expression of mitochondrial import proteins as well, and their coordinated control of mitochondrial related gene expression has been suggested to link expression of the respiratory chain complexes with the biogenesis of the organelle itself.⁸⁰ It should also be noted that the nuclear respiratory factor NRF-2 is distinct from the nuclear factor erythroid 2-related factor 2, which is also often abbreviated as Nrf2, and regulates the expression of antioxidant proteins. Tfam is a transcription factor which plays a central role in maintenance of the mitochondrial genome and biogenesis. Tfam was the first mammalian protein demonstrated to regulate mtDNA copy number *in vivo* and is essential for mitochondrial biogenesis and embryonic development.⁸¹ Tfam coats the mitochondrial genome and compacts it by inducing U turns on the DNA molecule⁸² and interactions between Tfam and mtDNA participate in regulation of mitochondrial biogenesis.⁸³ The essential role of Tfam in mitochondrial maintenance is illustrated by the fact that whole body disruption of Tfam is embryonically lethal,⁸¹ and animals with tissue specific knockout of Tfam in the heart and muscle are viable but develop a mosaic cardiac-specific progressive respiratory chain deficiency and functional deficits.⁸⁴

3.2 Mitochondrial biogenesis in recovery after APAP-induced liver injury

As discussed in the initial sections, mitochondria are critical organelles involved in APAP induced liver injury, though it is now becoming evident that mitochondrial biogenesis plays an important role in liver recovery and regeneration after an APAP overdose. While

mitochondrial biogenesis measured by amounts of mtDNA and proteins increased significantly in HepG2 cells exposed to low, non-cytotoxic concentrations of APAP, accompanied by upregulated expression of PGC-1 α , NRF-1 and TFAM,⁸⁵ these studies have important issues. The caveat with this study in HepG2 cells is that since APAP-induced liver injury requires metabolic activation to a reactive metabolite, the lack of metabolic competency of HepG2 cells deviates from the mechanism of injury in mice and humans and thus HepG2 cells are not a relevant model of APAP hepatotoxicity.⁸⁶ More relevant *in vivo* studies in the mouse clearly demonstrated that APAP overdose triggers unique zoned changes in the mouse liver, with necrosis around the central vein (zone 1), mitochondrial spheroid formation beyond that (zone 2), followed by autophagy (zone 3) and mitochondrial biogenesis in cells farthest from the central vein (zone 4).⁸⁷ Our earlier study in the mouse model demonstrated that both electron transport chain activity as well as mtDNA content, which decrease during the APAP-induced injury phase, begin to recover at later time points (12 h after a 300 mg/kg dose of APAP), suggesting that mitochondrial biogenesis occurs at late time points after APAP overdose (Figure 2).⁸⁸ Immunofluorescence staining for the mitochondrial outer membrane protein Tom20 in liver sections at various time points demonstrated that elevation in mitochondrial mass occurs selectively in hepatocytes surrounding necrotic areas.⁸⁸ This spatially selective increase in mitochondrial abundance was accompanied by induction of mitochondrial biogenesis signaling mediators including PGC1 α and NRF-1, with PGC1 α upregulation selectively in hepatocytes surrounding necrotic areas.⁸⁸ The importance of mitochondrial biogenesis for liver recovery after APAP induced injury was further illustrated by the enhanced recovery and regeneration after post-treatment with the known mitochondrial biogenesis inducer SRT1720.⁸⁸ Other interventions such as diphenyl diselenide treatment was also shown to enhance levels of PGC-1 α and help to restore NRF-1 levels associated with mitochondrial biogenesis in the context of APAP-induced liver injury.⁸⁹ One of the signaling mediators implicated in upregulation of mitochondrial biogenesis is fibroblast growth factor 21 (FGF21), which is a hepatocyte-secreted hormone with pleiotropic effects on glucose and lipid metabolism upregulated in response to APAP overdose.⁹⁰ FGF21 was shown to induce hepatic expression of PGC-1 α , thereby increasing the nuclear abundance of NRF-2 and FGF21 knockout mice showed aggravated liver damage after APAP overdose.⁹⁰ In addition to FGF21, sestrin 2, a stress inducible protein, was also shown to be a regulator of PGC1 α that confers survival and facilitates recovery of liver cancer cells from APAP-induced mitochondrial damage under glucose-starved conditions.⁹¹ These data clearly indicate that induction of mitochondrial biogenesis in the unique population of surviving cells around the necrotic area play a critical role in liver recovery and regeneration after acute APAP-induced liver injury (Figure 2).

4. Summary and Conclusions

Oxidative metabolism of APAP forms the reactive metabolite NAPQI, which depletes GSH and binds to cellular proteins. Mitochondrial dysfunction starts with early protein adducts on mitochondria triggering a mild oxidant stress, which activates a MAP kinase signaling cascade ultimately phosphorylating JNK. Phospho-JNK translocates to the mitochondria and amplifies the oxidative and nitrosative stress triggering the MPT pore opening. Mitochondrial dysfunction is also responsible for the release of mitochondrial

intermembrane endonucleases, which cause nuclear DNA fragmentation. Hence, mitochondrial dysfunction and damage is central to the pathophysiology of APAP-induced cell death. However, these events are counteracted by removal of damaged mitochondria and protein adducts by autophagy (reviewed recently⁹²) and replacement of these mitochondria by biogenesis. Thus, the ultimate outcome of cell death or survival is determined by the extent of pro-death signaling and the opposing effects of autophagy and biogenesis. This new mechanistic insight explains the protective effect of the existing antidote N-acetylcysteine and provides the rationale for the testing of novel therapeutics focusing on mitochondrial dysfunction.

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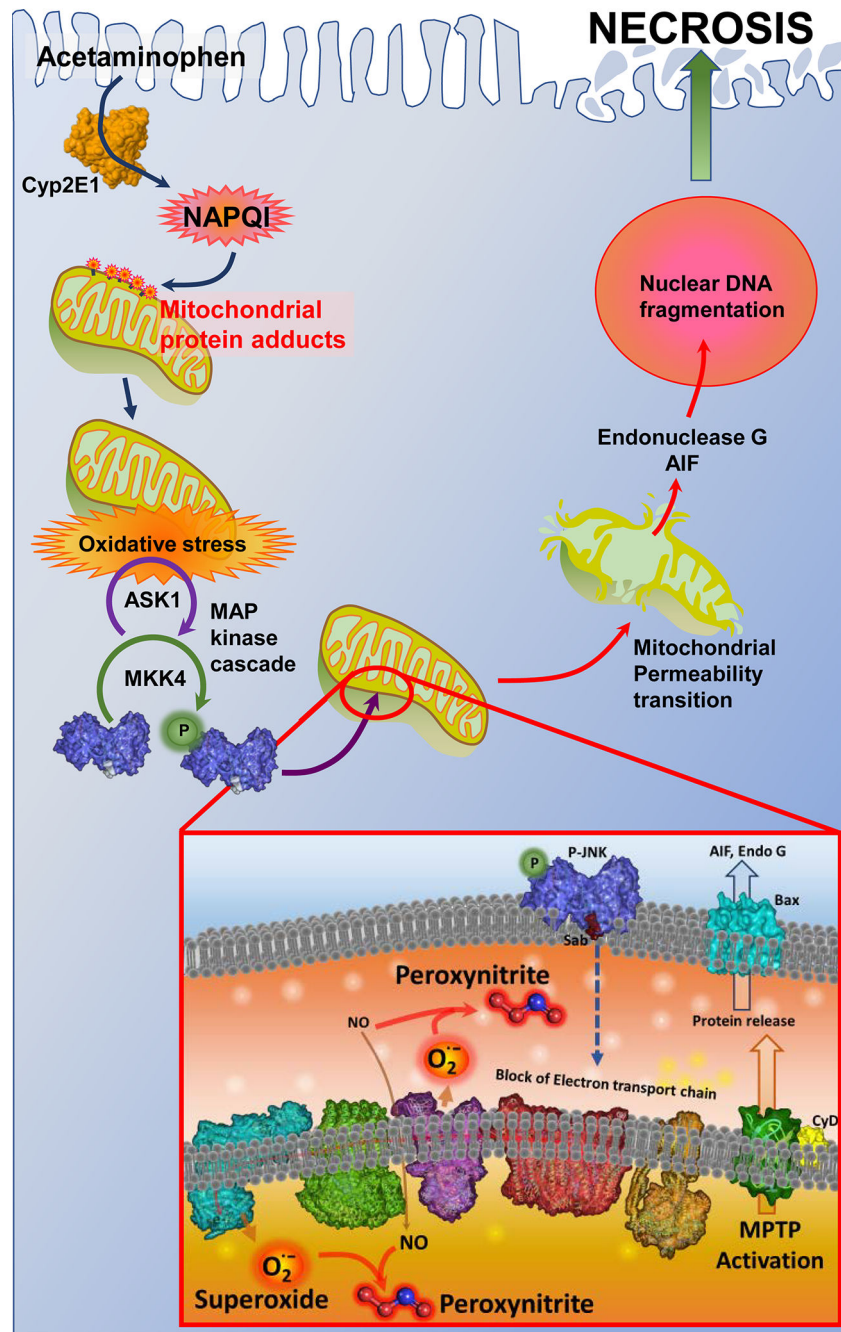


Figure 1: Mitochondria are an important target of the reactive metabolite of APAP. Metabolism of APAP by Cytochrome P450 2E1 (Cyp2E1) results in formation of NAPQI, which forms protein adducts on mitochondria and induces mitochondrial oxidant stress. This initiates a MAP kinase cascade, which ultimately results in phosphorylation and activation of JNK. JNK translocation to the outer mitochondrial membrane and binding to Sab then triggers inhibition of the mitochondrial respiratory chain. The subsequent reaction of mitochondrial derived superoxide with nitric oxide within mitochondria forms the reactive peroxynitrite, which ultimately induces the mitochondrial permeability transition with

release of mitochondrial proteins such as endonuclease G and apoptosis inducing factor (AIF). These then translocate to the nucleus and induce DNA fragmentation and ultimately hepatocyte necrosis (Adapted from reference⁹³).

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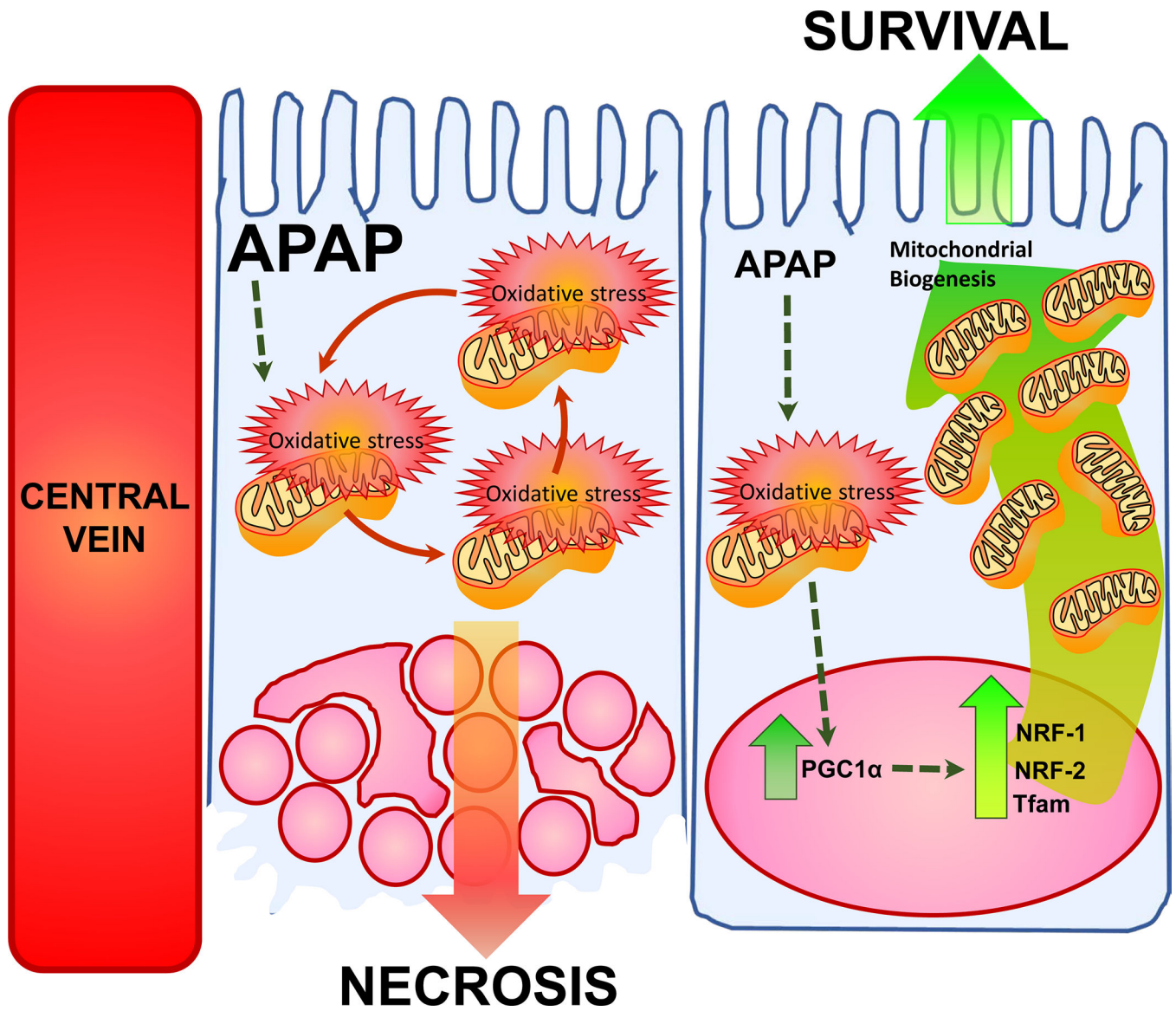


Figure 2: Mitochondrial biogenesis as an adaptive response to APAP-induced injury. APAP overdose induces a mitochondrial oxidant stress, which results in the amplification of mitochondrial defects to ultimately induce hepatocyte necrosis around the central vein. In surviving cells around this area of necrosis, the mitochondrial dysfunction induces upregulation of PGC1 α , the central regulator of mitochondrial biogenesis. This in turn enhances levels of important transcription factors such as NRF-1, NRF-2 and Tfam, which promote mitochondrial biogenesis to compensate for APAP induced mitochondrial defects and facilitate regeneration and recovery.