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The Development and Hepatotoxicity of Acetaminophen. Reviewing Over a Century of Progress.

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Abstract

Acetaminophen (APAP) was first synthesized in the 1800s, and came on the market approximately 65 years ago. Since then, it has become one of the most used drugs in the world. However, it is also a major cause of acute liver failure. Early investigations of the mechanisms of toxicity revealed that cytochrome P450 enzymes catalyze formation of a reactive metabolite in the liver that depletes glutathione and covalently binds to proteins. That work led to the introduction of *N*-acetylcysteine (NAC) as an antidote for APAP overdose. Subsequent studies identified the reactive metabolite N-acetyl-p-benzoquinone imine, specific P450 enzymes involved, the mechanism of P450-mediated oxidation, and major adducted proteins. The mechanisms downstream of metabolism remain poorly understood but several events appear critical. These events include development of an initial oxidative stress, reactive nitrogen formation, altered calcium flux, JNK activation and mitochondrial translocation, inhibition of mitochondrial respiration, the mitochondrial permeability transition, and nuclear DNA fragmentation. Additional research is necessary to fill remaining gaps in our knowledge of the toxicity, such as the source of the initial oxidative stress, and to improve our understanding liver regeneration after APAP overdose. A better understanding of these mechanisms may lead to additional treatment options. Even though NAC is an excellent antidote, its effectiveness is limited to the first 16 hours following overdose.

Keywords

Acetaminophen overdose; APAP-protein adducts; biomarkers; drug-induced liver injury; drug metabolism; liver injury; liver regeneration; mitochondria; oxidative stress; reactive nitrogen

Introduction

Acetaminophen (*N*-acetyl-p-aminophenol; APAP), or paracetamol as it is known in Europe, is a popular over-the-counter drug with analgesic and antipyretic properties similar to

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aspirin. However, unlike aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs), it has only weak anti-inflammatory effects. At therapeutic doses, it is believed to be safe. In fact, it is one of the most commonly used drugs in the United States (Kaufman et al. 2002). Unfortunately, overdose can cause severe liver injury.

The development of APAP as a drug began with a happy accident. In the 1880's, two medical interns working under German physician Adolf Kussmaul at the University of Strassburg were given the wrong compound by a pharmacist who thought he was dispensing naphthalene to treat a patient's intestinal worms. While the compound was ineffective against the parasite, it did reduce the patient's fever (Cahn and Hepp 1886). Chemists soon identified the new drug as acetanilide and it was soon marketed by the German company Kalle under the brand name Antifebrin (Cahn and Hepp 1886). The analgesic effects of acetanilide were initially attributed to the acetyl moiety that distinguished it from its precursor aniline, resulting in an acetylation craze that likely led to the development of phenacetin (*N*-acetyl-p-ethoxyacetanilide), the immediate precursor of APAP. Bayer chemists seeking a use for the unwanted by-product of dye synthesis, *p*-aminophenol, decided to acetylate it. Like acetanilide, the product phenacetin exhibited strong analgesic and antipyretic effects and quickly became the analgesic of choice. However, acetanilide and phenacetin both produced serious adverse effects, leading to the search for a safer alternative. In 1893, clinical pharmacologist Joseph Baron von Mering was the first to administer APAP to humans (Von Mering 1893). However, Von Mering claimed that APAP produced side effects similar to acetanilide and phenacetin, most notably methemoglobinemia. APAP was subsequently discarded in favor of phenacetin, which was taken either alone or in combination with aspirin and caffeine. Phenacetin remained the preferred mild analgesic until approximately 1970.

Only in the past 50 years has APAP become the analgesic drug of choice. This occurred for a few major reasons. First, Greenberg and Lester demonstrated that APAP is a metabolite of acetanilide, and that the methemoglobinemia observed by Von Mering was probably not due to APAP itself (Greenberg and Lester 1947). Shortly thereafter, Brodie and Axelrod found that APAP is also the major metabolite of phenacetin (Brodie and Axelrod 1949), and indeed that it is the pharmacologically active product (Flinn and Brodie 1948). Second, phenacetin was acutely and chronically nephrotoxic. Long-term abuse of phenacetin was associated with kidney damage, renal carcinoma, and tumors of the bladder. It was also carcinogenic in long-term feeding studies in both mice and rats and induced tumors of the kidney and lower urinary tract. Thus, the US FDA removed it from the market in 1983. Although phenacetin is to APAP, nephrotoxicity was not associated with APAP early on. Seeing an opportunity, McNeil Laboratories introduced APAP to the market in a barbiturate combination called Algoson, then later as a stand-alone product under the brand name Tylenol. Although the NSAID aspirin was also available as an analgesic by this time, APAP became more popular than aspirin due to the absence of GI toxicity. Aspirin causes GI bleeding and has also been associated with Reye's syndrome (Schror 2007), a rare condition that may occur in children that causes swelling of the liver and brain. Furthermore, in contrast to aspirin, APAP does not prevent blood from clotting, so it can be used in patients who have concerns with blood coagulation such as during and following surgery.

First Reports of Hepatoxicity and Description of Pathology

The first reports that APAP is hepatotoxic were published by Boyd and Bereczky (Boyd and Bereczky 1966) and by Davidson and Eastham (Davidson and Eastham 1966) in 1966. Boyd and Bereczky found that acute doses of APAP in rats produced hepatotoxicity. Davidson and Eastham reported on two individuals who developed hepatotoxicity following overdose. Both individuals died on the third day following the overdose. Microscopic examination of sections from their livers revealed fulminating hepatic necrosis. The necrosis was primarily in the centrilobular areas but extended through the midzonal areas to the periportal areas. High power examination revealed eosinophilic degeneration of the cells with pyknosis of the nuclear material within the centrilobular areas. Vacuolization and early degenerative changes were observed in the more peripheral cells surrounding the portal tracts. A mild polymorphonuclear leukocytic infiltration had also occurred in both cases. These features indicated an acute fulminating hepatic necrosis of the centrilobular type.

Subsequently there were a number of reports of APAP toxicity in overdose patients (Thomson and Prescott 1966; Rose 1969; Proudfoot and Wright 1970; Boyer and Rouff 1971; Prescott et al. 1971). The clinical features of the toxicity were described by Boyer and Rouff (Boyer and Rouff 1971): nausea and vomiting within two to three hours followed by abdominal pain, chiefly in the upper right quadrant. Liver dysfunction is present within approximately 24 hours and peaks at four days after the overdose. The clinical biochemistry was summarized by Prescott et al. (Prescott et al. 1971): dramatic increases of circulating alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH); mild hyperbilirubinaemia; increases in prothrombin time; and increased APAP half-life to greater than 4 hours. When the half-life was greater than 12 hours, hepatic coma frequently occurred.

Dixon et al. (Dixon et al. 1971) subsequently described the pathology of APAP in more detail. There was a high incidence of lethality. In rats surviving at 24 hours there was hepatic congestion, dilation of central veins and surrounding sinusoids, and accumulation of red blood cells. Necrosis of the hepatocytes was observed. The nuclei of the necrotic cells showed characteristic signs of pyknosis and karyorrhexis. The necrotic zones contained neutrophils but no inflammatory cell reactions were observed. The endothelial and Kupffer cells were primarily in the necrotic zones.

Researchers later discovered that mice are much more sensitive to APAP than rats, and develop hepatotoxicity at doses that are quite similar to humans. As a result, mice have become the primary animal to investigate mechanisms of APAP hepatotoxicity. In mice, toxicity can occur at doses as low as 200 mg APAP per kg bodyweight (mg/kg), or an order of magnitude lower than that which occurs in the rat (Mitchell et al. 1973, 1973a). Histologically, glycogen depletion and vacuolization of centrilobular hepatocytes are observed by 2 hours, resulting in a clear demarcation of the hepatic centrilobular areas. By 3 hours, nuclear changes are observed in hepatocytes of the centrilobular areas with single cell necrosis and pyknotic cells showing eosinophilic degeneration. By 6 hours, gross necrosis of the centrilobular zones is evidenced by nuclear pyknosis, karyorrhexis, karyolysis and eosinophilia of the necrotic hepatocytes. A more detailed description of the pathological

changes in the liver over time with photographs was later published in the American Journal of Pathology in a manuscript by Roberts et al. (Roberts et al. 1991), with similar results. A representative image of APAP-induced liver necrosis is shown in figure 1.

Walker et al. performed scanning electron microscopic analysis of the liver of APAP-treated mice (Walker et al. 1983). They reported endocytic vacuolation at lateral and sinusoidal margins of the centrilobular hepatocytes, sparing periportal areas. Associated with the changes in the centrilobular areas, there was loss of microvilli, Space of Disse enlargement with the presence of erythrocytes, dilation of bile canaliculi, and alterations of cell surfaces. Congestion abated by 24 hours.

In humans, APAP-induced hepatotoxicity occurs with hepatic congestion in overdose victims (Thompson et al. 1972). Hepatic congestion was also observed in rodents (Dixon et al. 1971; Walker et al. 1980; Walker et al. 1985). In mice, hepatic congestion occurs early and before necrosis. Walker et al. (Walker et al. 1980; Walker et al. 1983, 1985) also reported accumulation of erythrocytes within endocytic vacuoles and the Space of Disse with collapse of the sinusoidal lumens. At 1.5 hours after administration of a toxic dose of APAP to mice, liver weights significantly increased and doubled by 6 hours. At 24 hours, the weights had decreased. Increased liver weights occurred with a very large increase in hepatic hemoglobin levels (4-fold at 6 hours) and a subsequent decrease by 24 hours. The decrease in liver weights has been attributed to hepatocyte lysis and leakage with large increases in serum ALT and AST as a result (Roberts et al. 1991).

In 1995, Lim and coworkers (Lim et al. 1995), using a vascular casting technique, reported that APAP toxicity in the centrilobular areas of the rat occurred with microvascular injury. Subsequently, McCuskey and coworkers examined APAP-induced microvascular injury in more detail in the mouse (Ito et al. 2003; McCuskey 2006). They reported hepatocellular injury to the sinusoidal endothelial cells, preceding hepatocellular injury. Swelling of the endothelial cells and penetration of erythrocytes into the extrasinusoidal Space of Disse was observed (Ito et al. 2003). Blood flow in the sinusoids by 2 h was significantly decreased (Ito et al. 2003). The functional integrity of the endothelial cells was impaired in the centrilobular regions but not in the periportal regions. It was postulated that endothelial damage may play a role in the reduced sinusoidal perfusion (Ito et al. 2003).

Metabolism in APAP Toxicity: Early Research Findings

In a series of four manuscripts, Mitchell, Jollow, Potter, Gillette, and Brodie examined the role of metabolism in the hepatotoxicity of APAP (Jollow et al. 1973; Mitchell et al. 1973; Potter et al. 1973; Mitchell et al. 1973a). These are now classic, highly cited manuscripts that have contributed significantly to our understanding mechanisms of APAP-induced hepatotoxicity.

The first manuscript described the toxicity and showed the importance of drug metabolism (Mitchell et al. 1973). Importantly, the group found that mice are more susceptible to APAP hepatotoxicity than rats. Whereas a dose of 300 mg/kg was toxic to the mouse, a dose eight to ten times higher was required to produce a similar hepatotoxic effect in the

rat. Moreover, they observed frequent lethality at these very high doses in the rat. They demonstrated the role of drug metabolism by pre-treating mice with inducers and inhibitors of drug metabolizing enzymes and then histologically evaluating the incidence of hepatic necrosis at 24 hours. Thus, following a dose of 375 mg/kg of APAP, saline pre-treated mice had a 46% incidence of hepatic necrosis, whereas pre-treatment of mice with the cytochrome P450 inducer phenobarbital increased the incidence of hepatic necrosis to 90%. In contrast, pre-treatment with piperonyl butoxide or cobaltous chloride, two inhibitors of drug metabolizing enzymes, decreased the incidence of hepatic necrosis to 0%. These results pointed to a cytochrome P450-dependent mechanism of hepatotoxicity.

In the second manuscript, following toxic doses of radiolabeled APAP to mice, radiolabel was associated with proteins (covalent binding) (Jollow et al. 1973). Pre-treatment of mice with phenobarbital increased both the incidence of hepatic necrosis and the amount of radiolabel associated with proteins, while pre-treatment with the inhibitors of drug metabolism greatly decreased both. Importantly, the relative amount of radiolabel associated with protein preceded hepatic necrosis and was dose dependent. Autoradiographs also indicated that the radiolabel was localized in the necrotic hepatocytes. The nature of the association was then investigated. The radiolabel was firmly attached to proteins and could not be removed by extraction with organic solvents. Moreover, treatment of the protein with pronase released a product with the characteristics of an amino acid. These data were interpreted to indicate that APAP had been metabolized to a reactive metabolite (metabolic activation) that had covalently bound to proteins (protein adduct formation). Important to this mechanism is that APAP does not act through hepatic lipid peroxidation (Knight et al. 2003), a mechanism of hepatotoxicity believed to be mechanistically important in carbon tetrachloride-induced liver injury (Koster et al. 1977).

In the third manuscript, they studied the covalent binding of APAP to proteins *in vitro* in microsomal incubation mixtures (Potter et al. 1973). Microsomes from mice pre-treated with phenobarbital had increased velocities of covalent binding whereas microsomes from mice pre-treated with inhibitors of drug metabolism had decreased velocities of covalent binding. Covalent binding was shown to require NADPH and oxygen, and was inhibited in an oxygen/carbon monoxide atmosphere. These data were consistent with the involvement of cytochrome P450 enzymes in the metabolism to a reactive metabolite. N-Hydroxylation was postulated to be the metabolic step.

Finally, the fourth manuscript reported the importance of glutathione (GSH) in preventing the APAP-induced hepatotoxicity (Mitchell et al. 1973). Thus, a dose of 375 mg/kg of APAP produced approximately a 50% incidence of hepatotoxicity. Pre-treatment with the nontoxic compound diethylmaleate to deplete hepatic GSH increased the incidence to 100%. Pre-treatment with cysteine decreased the incidence of hepatotoxicity in a dose dependent manner. Hepatic GSH was depleted following a toxic dose of APAP in a time and dose dependent manner. By 2 hours following a very hepatotoxic dose of APAP (400 mg/kg), total hepatic GSH was depleted by more than 80%. Pre-treatments that altered drug metabolism and toxicity (increased or decreased) similarly altered hepatic GSH depletion. Importantly, GSH depletion preceded covalent binding and, as discussed above, covalent binding preceded the development of hepatotoxicity (Jollow et al. 1973).

These data indicated that GSH is important to prevent covalent binding and development of hepatotoxicity.

Overall, these manuscripts showed that APAP is converted by drug metabolizing enzymes to a reactive metabolite that covalently binds to proteins. At nontoxic doses, the metabolite is efficiently detoxified by GSH, forming an APAP-GS conjugate. A small amount binds proteins, but it is not sufficient to cause toxicity (McGill et al. 2013). However, at toxic doses, the metabolite depletes hepatic GSH by as much as 80-90% and binds extensively to protein. Finally, the amount of protein binding correlates with the relative hepatotoxicity.

Introduction of N-acetylcysteine as an Antidote

In the 1970's, there was an intense effort by various clinical research groups to develop an effective antidote that could be used in APAP overdose patients. Three major drugs were considered: *N*-acetylcysteine (NAC), methionine, and cysteamine. All three are sulfur-containing drugs. NAC may be deacetylated to cysteine and cysteine may then be metabolized to GSH. Thus, the reactive metabolite may be detoxified by either NAC, cysteine, or GSH. Methionine may also be metabolized to cysteine. On the other hand, cysteamine (2-methylthioethylamine) is a small molecular thiol derivative which was envisioned to directly detoxify the reactive metabolite (Mitchell et al. 1974).

Ultimately, NAC was determined to be the antidote of choice (Rumack and Peterson 1978; Prescott et al. 1979; Smilkstein et al. 1988; Bateman and Dear 2019). Most importantly, it was found to be more effective than cysteamine or methionine and was already FDA approved to treat bronchitis. Moreover, it was free from serious adverse effects, whereas cysteamine causes distressing off-target effects and methionine may itself be toxic as some doses (Prescott et al. 1979). However, for superior antidotal properties, NAC treatment should begin within approximately 10 hours following APAP ingestion (Prescott et al. 1979).

The currently used decision for utilization of NAC in APAP overdose is based on a graph of APAP concentration versus time, the so called Rumack-Matthew Nomogram (Smilkstein et al. 1988; Rumack 2002) (Figure 2). The nomogram plots a line at times from 4 to 24 hours. If the APAP plasma concentration is above this line at a given time after overdose, then NAC treatment is recommended. If the APAP concentration is below this line, NAC treatment is not recommended.

Lauterburg and coworkers (Lauterburg et al. 1983) examined the question if NAC detoxified the reactive metabolite of APAP directly or via increased GSH synthesis, and therefore if increased GSH was responsible for the hepatoprotective effects of NAC. Utilizing [^{35}S]-NAC, it was shown that following a toxic dose of APAP (1 g/kg) and radiolabeled NAC (1.2 g/kg) to the rat, the specific activity of the resulting NAC-APAP in the urine averaged 76% of the specific activity of the APAP-GS conjugate in the bile. These data were interpreted to indicate that virtually all of the NAC-APAP originated from the APAP-GS conjugate. Moreover, following APAP treatment to rats, NAC increased GSH synthesis. These data

suggest that the mechanism by which NAC prevents APAP hepatotoxicity is by an increase in GSH synthesis and efficient detoxification of the toxic metabolite.

Studies on the Mechanism of Metabolic Activation

In the initial research on mechanisms, metabolic activation by N-hydroxylation was postulated to be the mechanism of activation of APAP to a reactive toxic metabolite (Potter et al. 1973). N-Hydroxylation was known to be important in the hepatocarcinogenesis of 2-acetylaminofluorene, another *N*-acetyl aromatic amine (Miller JA and Miller 1975). Subsequently, both p-chloroacetanilide and phenacetin (p-ethoxyacetanilide), two other APAP analogs, were shown to be N-hydroxylated (Hinson and Mitchell 1976; Hinson et al. 1976).

This mechanism was subsequently modified following reports by Dr. Ian Calder's laboratory in Australia that N-acetyl-p-benzoquinone imine (NAPQI) was an electrophilic species (Calder et al. 1973; Calder et al. 1974; Calder et al. 1981). They were able to synthesize it by oxidation with lead tetra-acetate, but it rapidly decomposed. Identification was indirect as a Diels-Alder adduct (Calder et al. 1973). Based on their findings that it was a reactive species, Mitchell and coworkers subsequently postulated that APAP was initially N-hydroxylated by cytochrome P450 to form N-hydroxy-APAP, which then spontaneously dehydrated to form NAPQI (Fig. 3). Thus, the reactive toxic metabolite that covalently bound to protein was hypothesized to be NAPQI, and the binding site in proteins was postulated to be cysteine (Hinson 1980; Streeter et al. 1984).

Dr. Calder's group was able to synthesize N-hydroxy-APAP and test that hypothesis (Calder et al. 1981). They found that it spontaneously dehydrated to NAPQI with a half-life of 80 minutes. Hinson *et al.* obtained a sample of the N-hydroxy-APAP from Dr. Calder and using that sample they developed a HPLC assay to determine if NAPQI was a metabolite of APAP (Hinson, Pohl, Gillette 1980). In a microsomal incubation mixture, they showed that N-hydroxy-APAP was a metabolite of APAP. However, N-hydroxyphenacetin (N-hydroxy-p-ethoxyacetanilide) was metabolized to N-hydroxy-APAP (a deethylation reaction) (Hinson, Pohl, et al. 1979), but there was more covalent binding of radiolabeled APAP to protein than covalent binding of radiolabeled N-hydroxyphenacetin to protein (Hinson, Pohl, et al. 1979). Also, Dr. Nelson obtained a sample of the proposed metabolite from Dr. Calder and using an isotope dilution approach they were unable to find N-hydroxy-APAP as a metabolite. Thus, N-hydroxy-APAP is apparently not a metabolite of APAP. However, as reported by Corcoran and coworkers, N-hydroxy-APAP led to a reactive metabolite that had metabolic properties similar to the reactive metabolite of APAP: both formed conjugates with cysteine and were reducible by ascorbate (Corcoran et al. 1980). Taken as a whole, these data indicated that APAP is converted directly to NAPQI by a two electron oxidation. However, this mechanism had not yet been described as a P450 mechanism. Thus, at that time, the mechanism of APAP metabolic activation to NAPQI remained unclear.

APAP epoxidation had been suggested by a number of investigators as another possible toxic mechanism. Epoxidation was known to be important in the carcinogenicity of polycyclic aromatic hydrocarbons (Nebert et al. 1976) and the hepatotoxicity of

bromobenzene (Jollow et al. 1974). Thus, the potential 3,4-epoxidation of APAP was evaluated. By this mechanism, the 3,4-epoxide could react directly with a nucleophile, or the ring could open to yield the hydrated NAPQI and dehydration would yield NAPQI. This mechanism was investigated by incubating APAP in a microsomal incubation mixture in the presence of GSH and an $^{18}O_2$ atmosphere. If 3,4-epoxidation were the mechanism of metabolic activation of APAP to yield a GSH conjugate, then ^{18}O would be incorporated into the APAP-GS metabolite at a 50% level. Isolation of the APAP-GS metabolite from this experiment and analysis by mass spectrometry indicated no incorporation of ^{18}O (Hinson et al. 1977). Also, the p-^{18}O-APAP was synthesized and incubated with GSH in a microsomal incubation mixture and ^{18}O was not lost in the isolated ^{18}O-APAP-GS metabolite. Finally, p-^{18}O-APAP was administered to hamsters and the urinary APAP mercapturate (N-acetylcysteine-APAP) metabolite isolated (Hinson, Nelson, et al. 1979). The ^{18}O content in the mercapturate was the same as that in the administered p-^{18}O-APAP. Thus, 3,4-epoxidation was not the mechanism of metabolic activation of APAP to a reactive metabolite (Figure 3B).

2,3-Epoxidation was also investigated as a mechanism of APAP metabolic activation (Hinson, Pohl, Monks, et al. 1980). Since epoxides may break down to phenols, it seemed possible that 3-hydroxy-APAP may be a metabolite. In a microsomal incubation, the 3-hydroxy-APAP was found to be a significant metabolite. However, addition of epoxide hydrolase to the microsomal incubation mixture did not affect the rate of its formation. Moreover, addition of GSH to the incubation did not alter the amount of the 3-hydroxy metabolite. Also, it was shown that ascorbic acid almost completely inhibited covalent binding but had no effect on the formation of the 3-hydroxy-APAP formation. Finally, the finding that ascorbic acid inhibited covalent binding was consistent with the postulation that a readily reducible product such as NAPQI was the reactive metabolite (Hinson 1980). Subsequent studies showed that in mice the 3-hydroxy-APAP was not a major *in vivo* metabolite and also was not very toxic (Forte et al. 1984).

Even though the data of Calder and co-workers implicated NAPQI as a reactive metabolite, they were unable to purify it and thus identified it by an indirect technique, as a Diels-Alder adduct. Since they were unable purify it they were not able to perform detailed studies on its chemical properties (Calder et al. 1973). Nelson and co-workers were able to synthesize pure NAPQI by oxidation of APAP with silver oxide (AgO) in chloroform and study its chemical reactivity (Dahlin and Nelson 1982). The compound was unstable in aqueous buffer with a half-life of approximately 11 minutes. It decomposed to a number of products including hydrolysis to benzoquinone. In isolated liver cells, it was about ten times more toxic than APAP and was also toxic *in vivo* (Dahlin and Nelson 1982). Subsequently, using purified cytochromes P450, radiolabeled APAP and cumene hydroxide HPLC analysis indicated a radiolabeled metabolite with the retention time of NAPQI. In the presence of NADPH and NADPH-cytochrome P450 reductase, steady-state levels of NAPQI were below their detection limits of 6.7 X 10-8 M. Thus, they were forced to use cumene hydroperoxide in the incubation mixture. In the presence of GSH, an APAP-glutathione conjugate was formed (Dahlin et al. 1984). Overall, these data support the hypothesis that NAPQI is the major reactive metabolite of APAP (Figure 3).

Studies on APAP Peroxidation

N-Acetyl-p-benzosemiquinone imine (NAPSQI) has also been evaluated as a reactive toxic metabolite of APAP. This is the free radical of APAP that may be formed by one electron oxidation, a peroxidation reaction. Nelson and coworkers (Nelson et al. 1981) showed that incubation of APAP with protein, horseradish peroxidase, hydrogen peroxide, and radiolabeled APAP resulted in covalent binding of APAP to protein. Using a spin trap technique, further evidence was found for peroxidation of APAP to a phenoxy free radical (Nelson et al. 1981; West et al. 1984). The free radical and covalent binding were eliminated by addition of ascorbic acid. These data indicated that APAP was indeed metabolized to the free radical NAPSQI. Moreover, this metabolic pathway led to a reactive metabolite that covalently bound to protein.

Potter et al. (Potter et al. 1985) examined metabolic products formed by incubation of APAP with horse radish peroxidase and hydrogen peroxide. They identified a number of polymerization products: two dimers, three trimers, and a tetramer. The primary product was a 3,3’-dimer where the carbon ortho to the hydroxy group was bonded to the same carbon of the other APAP group in the dimer. Also, there was a 3,3’,3’’-trimer, a 3,3’’,3’’’,3’’’’-tetramer, a dimer bonded from the 3 position of one APAP to the nitrogen of another APAP, a trimer containing a N- to 3 linkage, and a tetramer containing a N- to 3 linkage. Dimers were the major products at high APAP concentrations or low horseradish concentrations, whereas trimers and tetramers predominated at low APAP concentrations or high horseradish peroxidase concentrations. The reaction was very fast. Approximately one mole of hydrogen peroxide was utilized per two moles of free radical, a result consistent with a free radical termination reaction. 99% of polymerization products were quenched by 2 mM ascorbate (Potter et al. 1985).

In subsequent work, it was shown that addition of GSH to an incubation mixture containing APAP, horseradish peroxidase and hydrogen peroxide resulted in the formation of an APAP-GS conjugate and a GSH conjugate of the 3,3’-dimer (Potter and Hinson 1987b). Increasing concentrations of GSH decreased polymer formation and formation of the GSH conjugates. Ascorbate and NADPH were extremely effective in decreasing both polymers and GSH conjugates. NAPQI was detected in the horseradish peroxidase, APAP, and hydroperoxide incubation mixtures. Maximal NAPQI formation was at 15 seconds followed by a decrease with a half-life of approximately 1 minute. The NAPQI could be trapped by addition of GSH with formation of an APAP-GS conjugate. These data indicated that APAP was metabolized by peroxidation to form NAPSQI. This species can react with another free radical to form a dimer or higher polymers, or disproportionate to form NAPQI plus APAP. The NAPQI rapidly conjugated with GSH to form an APAP-GSH conjugate. The NAPQSI was very easily reduced by ascorbate, GSH, or NADPH but there was no evidence it could conjugate with GSH (Potter and Hinson 1987b).

Polymer formation was not observed when rat liver microsomes from phenobarbital treated rats were incubated with APAP and NADPH (Potter and Hinson 1987b). When GSH was added, the APAP-GS conjugate was the major product. Incubation of the microsomes with APAP and cumene hydroperoxide resulted in polymer formation, and addition of GSH

resulted in formation of an APAP-GS conjugate and a decrease in polymer formation. These data indicate that P450 enzymes metabolize APAP by both one and two electron oxidation. Two electron oxidation results in formation of NAPQI, which rapidly reacts with GSH to form an APAP-GS conjugate or covalently bind to protein. One electron oxidation forms NAPQSI which polymerizes or disproportionates to form polymers or NAPQI and APAP, respectively. It is easily reduced by GSH or NADPH back to APAP (Potter DW and Hinson 1987b).

APAP metabolism studies were also performed using purified prostaglandin H synthase (Potter and Hinson 1987a). In the presence of arachidonic acid, APAP was metabolized to polymers. Metabolism was not observed with hydrogen peroxide. Addition of GSH resulted in formation of an APAP-GS conjugate and a decrease in polymerization. Addition of GSH plus ascorbic acid, or NADPH, only slightly decreased APAP-GS formation, but almost completely inhibited polymer formation. It was concluded that prostaglandin H synthase metabolized APAP by both one electron oxidation leading to NAPSQI and formation of polymers, and two electron oxidation to NAPQI which conjugated with GSH (Potter and Hinson 1987a).

Overall, by the mid-1980s, it was firmly established that the reactive metabolite that covalently bound to protein was NAPQI. The mechanism was postulated to be a direct two-electron oxidation of APAP, a previously unrecognized mechanism for cytochrome P450 enzymes (Figure 3C). This mechanism has been postulated to occur by interaction of APAP with P450 by an initial one-electron abstraction from the amide nitrogen, followed by a rapid recombination of the radical pair to form a P450 heme bound N-hydroxy-Fe intermediate. As a result of a p-phenolic group on the APAP moiety, the enzyme-substrate intermediate dehydrates to generate NAPQI and a ferric heme (Harvison et al. 1988).

Mechanisms of NAPQI Detoxification

Structural identification of the APAP-GS conjugate was determined by NMR spectrometry on a biliary metabolite isolated from the bile of APAP treated rats. These data firmly established that the sulfur of GSH was attached to the ring of APAP at the carbon 3 position (3-glutathion-S-ylAPAP), ortho to the phenolic group (Hinson et al. 1982). Subsequently, Nelson and coworkers isolated and identified an additional GSH conjugate, a thiohemiketal ipso adduct (Chen et al. 1999). The sulfur of this adduct was attached to APAP at the carbon 1 position. Once formed, the ipso adduct was reversible to regenerate NAPQI and GSH, or it could react with another GSH to yield APAP and GSSG. Thus, a mechanism for how GSH reduced NAPQI was found. It was postulated that the ipso adduct could migrate and subsequently rearrange to yield NAPQI at a point distant from its formation. In the presence of acid, the ipso adduct decomposed to a GSH conjugate attached to the APAP ring in the carbon 2 position. The mechanism of reaction of GSH with NAPQI to yield 3-cys-APAP was postulated to be an initial attack on the carbonyl carbon in the 4 position to yield a conjugate which rearranged with sulfur migration to the 3 position to form 3-cys-APAP (Chen WQ et al. 1999). However, Bessems and Vermeulen have postulated a more traditional Michael Addition reaction where the sulfur attacks carbon 3 of NAPQI (Bessems and Vermeulen 2001).

The reaction of GSH with NAPQI was determined to be very rapid. The spontaneous reaction was first order in NAPQI and GSH, and inversely proportional to the H^+ ion concentration. At pH 7.0 and 25°C, the second-order rate constant was 3.2 X 10^4 M^{-1} s^{-1} (Coles et al. 1988). Generation of NAPQI in a microsomal incubation mixture and using a crude GSH transferase preparation revealed that the metabolite was enzymatically detoxified (Buckpitt et al. 1979). Determination of the conjugation of various purified human glutathione-S-transferases (GST) showed that GST-Pi was a major detoxification enzyme, but the reaction could be catalyzed by GST delta and mu as well (Coles et al. 1988). Surprisingly, GST-Pi knockout mice were not as susceptible to APAP hepatotoxicity as wildtype mice (Henderson CJ et al. 2000). However, depletion of hepatic GSH was significantly greater at 0.5, 1 and 5 hours in the wildtype mice than in the knockout mice post dosing. At 1 hour, hepatic GSH depletion was approximately 95% in the wildtype mice compared to approximately 70% in the knockout mice (Henderson et al. 2000). These data suggest that toxicity may be more complex than the initially proposed in the covalent binding hypothesis, where covalent binding to a critical protein is postulated to kill the hepatocyte.

NAPQI is readily detoxified not only by GSH but by ascorbic acid. Whereas the detoxification of NAPQI is by conjugation to form 3-glutathion-S-ylAPAP, the detoxification by ascorbic acid is by reduction back to APAP. Thus, if GSH is a critical detoxification mechanism *in vivo*, it could be asked how important ascorbic acid is *in vivo*. Miller and Jollow examined the effect of this compound on APAP hepatotoxicity in the hamster. A large dose of ascorbic acid (400 mg/kg, ip), which doubled hepatic ascorbic acid levels, did not alter the hepatotoxicity of APAP or decrease APAP covalent binding to protein (Miller and Jollow 1984). These data are consistent with the finding that the conjugation of NAPQI with GSH is much more rapid than the reduction of NAPQI by ascorbic acid or other reducing agents (Potter and Hinson 1987b). Thus, NAPQI preferentially reacts with GSH or cysteine groups in proteins.

Cytochrome P450 Enzymes in NAPQI Formation

In the third of the initial manuscripts by Mitchell, Jollow and co-workers on APAP metabolism described above, the importance of a reactive metabolite in APAP toxicity and covalent binding of APAP to proteins was studied in microsomal incubation mixtures (Potter et al. 1973). NADPH and oxygen were necessary for covalent binding. Pre-treatments which were known to alter P450 levels similarly altered *in vitro* covalent binding. From these data, it was concluded that APAP was metabolically activated to the reactive metabolite by P450.

Purification of the cytochromes P450 led to the determination of specific isoforms important in the metabolic activation of APAP. In 1983, Morgan et al. (Morgan et al. 1983) examined the ability of six purified rabbit P450s to oxidize APAP to the reactive metabolite (trapped as a GSH conjugate). They found that Cyp2e1, Cyp1a2 and Cyp1a1 readily activated APAP (Morgan et al. 1983). Subsequently, Harvison and coworkers reported that rat Cyp1a1, Cyp1a2 and Cyp2c11 metabolically activated APAP (Harvison et al. 1988). Using isolated human P450s, Patten and coworkers (Patten et al. 1993) found that the major isoforms that metabolically activated were CYP2E1, CYP1A2, and CYP3A4 (Patten et al. 1993; Sinclair

et al. 1998). CYP2E1 is the ethanol inducible form, CYP1A2 is inducible by polycyclic aromatic hydrocarbons, and CYP3A4 is not inducible but is the major constitutive P450 isoform important in drug metabolism. Moreover, animals pre-treated with ethanol to induce Cyp2e1 are more susceptible to APAP hepatotoxicity (McClain et al. 1980; Peterson et al. 1980). Interestingly, animals that are treated with ethanol at the same time as APAP are less susceptible because both are metabolized by Cyp2e1 (Lee et al. 1999).

Cyp2e1 is of major interest in human toxicity because of the consumption of alcoholic beverages. In 1986, Seef and coworkers (Seeff et al. 1986) reported on six alcoholics who developed toxicity after taking APAP and reviewed nineteen similar case reports. In 1995, Zimmerman and Maddrey (Zimmerman and Maddrey 1995) reported on an additional 67 alcoholic patients who developed liver toxicity after taking moderate doses of APAP. The increased toxicity of APAP was attributed to induction of CYP2E1 in alcoholics and increased production of the toxic metabolite as a result (Zimmerman and Maddrey 1995). However, the possibility that APAP may be toxic in alcoholics at moderate doses has been disputed by Prescott (Prescott 2000) and by Rumack (Rumack 2002, 2004). The problems in obtaining proper controls makes it difficult to know if alcoholics are more susceptible to APAP hepatotoxicity. Nevertheless, it is considered a risk factor when decisions are made whether to treat with NAC (Schmidt et al. 2002).

The importance of Cyp2e1 and Cyp1a2 in hepatotoxicity of APAP was clearly shown in knockout mice. In 1996, Lee and coworkers found that serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were significantly increased in wildtype but not knockout mice at an APAP dose of 200 mg/kg. However, serum ALT and AST did increase in the knockouts at a dose of 600 mg/kg (Lee et al. 1996), demonstrating that other P450 enzymes may compensate for loss of Cyp2e1 at very large doses. APAP hepatotoxicity was subsequently studied in mice where both Cyp2e1 and Cyp1a2 had been deleted. In the wildtype control mice, serum ALT levels increased at doses of 400 mg/kg, 600 mg/kg and 800 mg/kg in a dose-responsive manner. Even following doses a high as 1200 mg/kg, serum ALT levels did not increase in the double knockout mice (Zaher et al. 1998). These data demonstrate that Cyp1a2 becomes important for APAP bioactivation at very high doses that may saturate Cyp2e1. Altogether, it appears that the major P450 enzymes involved in APAP metabolism of CYP2E1, CYP1A2, and CYP3A4.

APAP-Protein Adducts

In the mid-1980s, two research groups raised antibodies in rabbits that were used to investigate APAP covalently bound to proteins (Bartolone et al. 1987; Roberts et al. 1987). Whereas the antiserum developed by Roberts and coworkers was specific for APAP covalently bound to cysteine (Roberts et al. 1987), the antiserum developed by Cohen and coworkers was specific for APAP itself (Bartolone et al. 1987). Nevertheless, the results obtained by the two groups were comparable. Three different immunochemical approaches were utilized: immunohistochemical analyses to determine specific hepatocyte targets (Roberts et al. 1991; Hart et al. 1995), competitive ELISA to quantify adducts in subcellular fractions (Pumford et al. 1989), and immunoblot for analysis of protein targets of

NAPQI (Bartolone et al. 1987; Bartolone et al. 1988; Pumford et al. 1990; Matthews et al. 1996). These data have been reviewed (Hinson et al. 1995; Cohen and Khairallah 1997).

APAP-protein adduct formation is specific for proteins in liver. Adduct formation occurs before toxicity and a number of specific proteins are primary targets. Figure 4 is an immunoblot of the liver cytosolic fraction from a mouse 2 h after a hepatotoxic dose of APAP, taken from the first study to identify a specific target of APAP binding (Cohen and Khairallah 1997). Note a major protein adduct at approximately 58 kDa. This protein was isolated and identified as the Selenium Binding Protein (Pumford et al. 1992) (Hoivik et al. 1996), which had been previously shown to associate with selenium following a tracer dose of the metal. A large number of other proteins have been subsequently identified, but the relevance of APAP binding of the Selenium Binding Protein or any other protein relative to toxicity has never been defined (James, Mayeux, et al. 2003).

Although the significance of covalent binding to specific proteins is unknown, it is clear that the mitochondrion is a critical target. The regioisomer of APAP, m-aminophenol (AMAP), is toxic to primary human hepatocytes but not to primary mouse hepatocytes, and it has been demonstrated that AMAP binds to mitochondrial proteins in the former but not the latter (Xie, McGill, et al. 2015). Furthermore, APAP-protein adduct levels are lower in mitochondria from rat liver than mouse liver after treatment with APAP despite prolonged GSH depletion in rats, and it is thought that this may partially explain the resistance of rats to APAP toxicity (McGill, Williams, et al. 2012).

APAP-protein adduct formation is an excellent biomarker of toxicity. Immunohistochemical analysis for APAP protein binding in histological sections of livers from mice treated with APAP shows protein binding occurs only in the necrotic centrilobular hepatocytes. Moreover, binding and toxicity co-localized in hepatocytes high in cyp2e1 (Hart et al. 1995). This is additional evidence of the importance of metabolic activation in toxicity and the role of Cyp2e1. Figure 5A is a time course for the immunohistochemical detection of APAP-protein adducts in liver sections of mice treated with a hepatotoxic dose of APAP (400 mg/kg) (Roberts et al. 1991). Adducts are stained red. By 1 and 4 hours after APAP, adducts are present in the centrilobular area, the site of the ensuing necrosis. Adducts are not detectable in the periportal areas. By 6 hours, hepatic necrosis is maximal, with loss of adducts from the centrilobular areas as a result of hepatocyte lysis. These data clearly indicate the importance of metabolic activation in APAP toxicity and that APAP-protein adducts are an excellent biomarker of toxicity. Although it cannot be concluded that adducts *per se* caused the toxicity, necrosis was not observed in any hepatocytes that did not contain adducts. This strongly argues against a mechanism proposed by Mehendale (Mehendale 2012) where lysing cells release proteolytic enzymes which cause necrosis in surrounding cells, a phenomenon called toxic expansion. If this mechanism occurred with APAP, there would not be a 100% correlation between protein adduct formation and necrosis. Furthermore, it is inconsistent with the hypothesis that sterile inflammation is a significant contributor to APAP-induced liver injury.

A time course for APAP-protein adduct formation was quantified by competitive ELISA (Pumford et al. 1989). Mice were treated with a hepatotoxic dose of APAP and were

subsequently sacrificed at various times. Serum levels of ALT and AST peaked at 4 hours and remained high for the duration (Figure 5B). APAP-protein adducts in liver peaked at 2 hours and subsequently decreased with a concomitant rise in adducts in serum. Thus, APAP-protein adducts accumulate in liver between 0 and 2 hours. By 4 hours, adducts were released into the blood along with ALT and AST as a result of hepatocyte lysis. Interestingly, experiments with primary mouse hepatocytes have demonstrated that APAP-protein adducts are released in part by mechanisms other than necrosis (McGill et al. 2013). However, it is likely that the dominant mechanism *in vivo* is passive release due to hepatocyte death because induction of hepatocyte necrosis by ischemia-reperfusion after a nontoxic dose of APAP dramatically increased APAP-protein adduct levels in serum compared to sham surgery (McGill et al. 2013). Altogether, these data provide support for the importance of metabolic activation in APAP toxicity and show that adducts in serum are a biomarker of hepatotoxicity. This was further demonstrated by showing APAP-protein adducts in serum from APAP overdose patients (Hinson et al. 1990).

In 2002, a new chromatographic assay was developed to quantify APAP-protein adducts (Muldrew et al. 2002). In this assay, a protein sample is first gel filtered to remove any low molecular weight metabolites and then hydrolyzed with protease to release the APAP-cysteine conjugate (3-cys-APAP). The 3-cys-APAP is then separated by HPLC and quantified by electrochemical detection (HPLC-EC). The assay has proven to be excellent for analyzing serum samples of APAP overdose victims (Davern et al. 2006; James et al. 2007). The APAP-protein adducts are long-lived in serum, with a half-life of approximately 2 days (James et al. 2009). Of particular note, in 2006 Davern and coworkers analyzed 36 patients who had severe liver toxicity of indeterminate cause. Seven of these patients (19%) had APAP protein adducts in their sera (Davern et al. 2006). Thus, detection of APAP-protein adducts in serum may be invaluable in helping identify APAP as a cause of liver toxicity.

Nitration of Proteins

Whereas APAP-protein adducts have proven to be an excellent biomarker of toxicity, more recent data have indicated that toxicity is much more complex than simply NAPQI covalent binding to a critical protein which then loses activity or function, resulting in cell death. Immunohistochemical analysis of liver sections from APAP-intoxicated mice using an anti-3-nitrotyrosine antibody indicated tyrosine nitration in the proteins of the centrilobular cells (Hinson et al. 1998). Tyrosine nitration has been shown to occur by peroxynitrite, a reactive intermediate formed by an extremely rapid reaction of nitric oxide and superoxide (Beckman and Koppenol 1996; Reiter et al. 2000; Beckman 2009). Analysis of liver sections using an APAP antiserum indicated the centrilobular cells also contained APAP-protein adducts (Hinson et al. 1998) (Figure 6). Peroxynitrite is an intermediate that is detoxified by GSH (Radi et al. 1991; Lizasoain et al. 1996), which is depleted in APAP toxicity (Radi et al. 1991). Importantly, blocking protein nitration by peroxynitrite protects against APAP-induced liver injury. Knight et al. (Knight et al. 2002) demonstrated that 1.5 and 2.25 hour post-treatment with exogenous glutathione after APAP overdose does not affect reactive metabolite formation or glutathione oxidation, but does reduce nitrotyrosine formation and liver injury. They also found that it can enhance liver regeneration (Bajt

et al. 2003). Altogether, these data are consistent with APAP metabolic activation leading to increased synthesis of nitric oxide and superoxide and to peroxynitrite as an important intermediate in the toxicity.

Immunoblot analysis using anti-nitrotyrosine antibody indicated nitration affected a number of specific proteins (Hinson et al. 2000) and later subcellular fractionation revealed that nitration occurs primarily on mitochondrial proteins (Cover et al. 2005). Since nitration had been reported to occur specifically on the mitochondrial protein manganese superoxide dismutase (MnSOD) in renal cells (MacMillan-Crow et al. 1996), the possible nitration of this protein in the liver was investigated in mice treated with APAP (Agarwal, MacMillan-Crow, et al. 2010). MnSOD is a critical mitochondrial antioxidant enzyme that detoxifies superoxide and thus prevents peroxynitrite formation within the mitochondria (Macmillan-Crow and Cruthirds 2001). A dose-responsive decrease in MnSOD activity was observed after treatment with APAP at 100, 200, and 300 mg/kg. Immunoprecipitation of MnSOD from livers of APAP-treated mice followed by Western blot analysis revealed nitrated MnSOD. APAP-MnSOD adducts were not detected (Agarwal, MacMillan-Crow, et al. 2010). Thus, a vital enzyme preventing peroxynitrite formation in APAP toxicity was inhibited. In addition to MnSOD, other nitrated mitochondrial and cytosolic proteins have been more recently identified: mitochondrial aldehyde dehydrogenase, glutathione peroxidase, ATP synthase, and 3-ketoacyl-CoA thiolase (Abdelmegeed et al. 2013).

A critical role was ascribed to neuronal nitric oxide synthase (nNOS, NOS1). Knockout of one isoform of this enzyme decreased toxicity but did not eliminate it (Agarwal et al. 2012). Subsequently, two different inhibitors of nNOS were studied in freshly isolated mouse hepatocytes: NANT (10 μM)(N-[(4S)-4-amino-5-[(2-aminoethyl)amino]pentyl]-N'-nitroguanidinetris-trifluoroacetate) (Banerjee et al. 2015) and Trifluoperazine (10 μM) (Banerjee et al. 2017). Incubation of freshly isolated hepatocytes with APAP (1 mM) resulted in toxicity (release of lactate dehydrogenase into the media). Both nNOS inhibitors eliminated the toxicity without altering the depletion of GSH or covalent binding of APAP to proteins. These data suggest that a nNOS species is important in APAP toxicity in the hepatocytes. Moreover, trifluoperazine inhibited APAP toxicity in the mouse (Chaudhuri et al. 2012). Importantly, trifluoperazine inhibits nNOS by associating with the calcium binding protein calmodulin, which is required for nNOS activation (Vandonselaar et al. 1994). Thus, it appears that altered calcium homeostasis and peroxynitrite may play mechanistic roles in APAP hepatotoxicity.

Mitochondrial Dysfunction

A number of studies have examined the importance of mitochondrial dysfunction in APAP toxicity. As early as 1980, electron microscopic examination of livers from APAP treated mice revealed alterations in mitochondrial morphology (Walker et al. 1980). Jollow et al. reported that mitochondria were a covalent adduct target (Jollow et al. 1973) and a number of APAP-protein adducts have been reported in mitochondria (Pumford et al. 1990; Bulera et al. 1996). Calcium alterations and oxidative stress in mitochondria have also been reported (Tirmenstein and Nelson 1989; Jaeschke 1990). Moreover, inhibition of activity at complexes I and II, but not at complex III, were reported in isolated rat

hepatocytes (Burcham and Harman 1991) and *in vivo* (Donnelly et al. 1994). Also, ATP levels decrease *in vivo* and in treated hepatocytes (Burcham and Harman 1991; Halmes et al. 1995; Vendemiale et al. 1996; Banerjee et al. 2015; Banerjee et al. 2017). Similar changes have been shown by adding NAPQI to hepatocytes (Andersson et al. 1990). Also, in isolated rat liver hepatocytes NAPQI caused release of sequestered calcium (Weis et al. 1992; Weis et al. 1994).

The mitochondrial permeability transition (MPT) may be an important mechanism in APAP toxicity (Kon et al. 2004; Masubuchi et al. 2005; Reid et al. 2005). The MPT occurs with depolarization of the inner mitochondrial membrane, uncoupling of oxidative phosphorylation, release of intra-mitochondrial ions and metabolic intermediates, mitochondrial swelling, and decreased ATP synthesis. Blockade of APAP toxicity both *in vitro* and *in vivo* by MPT inhibitors has been reported. Kon and coworkers (Kon et al. 2004; Kon et al. 2007) showed that APAP toxicity in cultured mouse hepatocytes was inhibited by cyclosporine A and by the non-immunosuppressive Cyclosporine A analog NM811. Cyclosporine A did not alter APAP-induced glutathione depletion, which suggests that the prevention of toxicity did not occur by inhibition of metabolism of APAP to NAPQI. Reid et al. (Reid et al. 2005) examined the effect of MPT inhibitors in freshly isolated mouse hepatocytes using the approach of Boobis (Boobis et al. 1986; Tee et al. 1986). In these studies, APAP toxicity was inhibited by the MPT inhibitors Cyclosporine A, trifluoperazine, or dithiothreitol. Subsequently, Banerjee and coworkers (Banerjee et al. 2017) examined APAP toxicity and the effect of trifluoperazine in freshly isolated hepatocytes in greater detail. They reported that toxicity occurs with increased NO and superoxide formation, increased protein nitration, loss of mitochondrial membrane potential, decreased ATP levels, decreased oxygen consumption rate, and decreased NADH levels. All of these parameters were reversed in the presence of trifluoperazine without altering GSH depletion or APAP protein adduct formation. Finally, Ramachandran et al. (Ramachandran et al. 2011) reported that mice deficient for cyclophilin D (CypD), thought by some to be a component of the MPT pore, are less susceptible to APAP hepatotoxicity than wildtype mice. Together, these data have been interpreted as demonstrating that the MPT is a critical step in APAP hepatotoxicity, though the exact composition of the MPT pore and the role of CypD remain highly controversial (Baines and Gutiérrez-Aguilar 2018).

Reactive nitrogen may be important in the APAP-induced MPT. Cover and coworkers found that nitration, a biomarker of peroxynitrite, was predominantly in mitochondria of APAP-treated mice (Cover et al. 2005). LoGuidice and Boelsterli (LoGuidice and Boelsterli 2011) reported that CypD knock out and CypD inhibitors did not protect against a very large dose of APAP (600 mg/kg), but a peroxynitrite decomposition catalyst did protect. These data indicate that peroxynitrite may facilitiate MPT pore-independent mitochondrial depolarization and damage at especially large doses of APAP. In addition, iron may promote the oxidative stress and protein nitration that leads to the MPT. Dr. Lemasters's group has demonstrated that iron chelation prevents loss of mitochondrial membrane potential and reduces toxicity due to APAP in primary hepatocytes, but does not prevent lysosomal iron release (Kon et al. 2010; Hu et al. 2016). These data indicate that iron translocation from lysosomes to mitochondria, where ferrous iron can contribute to ROS formation, may

precipitate the MPT. Importantly, recent *in vivo* data also support that conclusion (Hu and Lemasters 2020).

Furthermore, Lizasoain and coworkers (Lizasoain et al. 1996) examined the effect of nitric oxide and peroxynitrite on respiration by brain submitochondrial particles. They found that nitric oxide potently inhibited respiration reversibly at cytochrome oxidase. Peroxynitrite inhibited NADH-dependent respiration and succinate dependent respiration. Thus, the transfer of electrons from Complex 1 to complex 3 (NADH dependent) and from Complex 2 to Complex 3 (succinate dependent) was inhibited. These electron transfers depend on the ubiquinone derivatives. Addition of GSH, which detoxifies peroxynitrite, prevented the inhibition of respiration. Importantly, mitochondrial GSH is depleted in APAP hepatotoxicity (Zhao and Slattery 2002). Schopfer and coworkers have shown that peroxynitrite will oxidize ubihydroquinone to the semiquinone. The semiquinone may subsequently react with molecular oxygen to produce superoxide followed by reaction of the superoxide with additional nitric oxide to regenerate peroxynitrite. The net result is oxidation of ubihydroquinone to ubiquinone with production of no apparent biomarker (Schopfer et al. 2000). If this occurred in APAP toxicity, it would explain why determining the mechanism of toxicity has been so elusive.

Calcium Flux in APAP Toxicity

The role of altered calcium flux has been postulated to be important in various drug-induced toxicities (Kaplowitz et al. 1986; Nicotera et al. 1992). A number of manuscripts have described altered calcium metabolism in APAP toxicity (Corcoran et al. 1987; Burcham and Harman 1988; Tirmenstein and Nelson 1989; Tsokos 1989). Increased mitochondrial calcium levels following a toxic dose of APAP were reported by Burcham and Harmon (Burcham and Harman 1988). They found no significant increases in cytosolic or microsomal calcium levels. Timerstein and Nelson (Tirmenstein and Nelson 1989) found that following a toxic dose of APAP to mice there was approximately a two-fold increase in mitochondrial calcium at 2 hours and a four-fold increase at 6 hours. In addition, a large decrease in plasma membrane calcium-ATPase activity has been reported (Tsokos-Kuhn et al. 1988; Tirmenstein and Nelson 1989). This enzyme is important in control of calcium levels in the cell, and inhibition of the enzyme results in calcium accumulation (Strehler and Zacharias 2001). It was suggested that protein adduction was responsible for the altered activity (Tsokos-Kuhn et al. 1988; Tirmenstein and Nelson 1989).

Boobis and coworkers examined APAP toxicity in freshly isolated hamster hepatocytes. They found that toxicity occurred in two phases (Boobis et al. 1986). In the first phase, GSH depletion occurred; in the second phase, toxicity occurred. Addition of the cell-permeable Ca^{2+} binder Quin 2-AM in the second phase prevented the loss of viability (Boobis et al. 1990). Subsequently, however, they reported that the alteration in calcium accompanied the loss of viability and suggested that it may not be mechanistically important (Hardwick et al. 1992).

Corcoran and coworkers reported nuclear DNA damage in APAP toxicity. Following a toxic dose of APAP to mice, there was an increase in hepatic nuclear calcium levels that coincided

in time with the development of toxicity. Electrophoretic analyses indicated a decline in large DNA accompanied by accumulation of small DNA fragments. It was suggested that calcium-activated endonuclease digestion of the DNA had occurred (Ray et al. 1990; Ray et al. 1993). Similar results were obtained using cultured hepatocytes (Shen et al. 1992). The calcium ion chelator EGTA blocked toxic cell death which suggested that altered calcium metabolism was important in the cell death. These data suggest a role for extracellular calcium in APAP toxicity since EGTA is ionized and does not enter the cells (Shen et al. 1992). The effects of various other calcium antagonists on APAP induced hepatotoxicity have been examined in experimental animals by a number of other investigators (Yamamoto 1990; Ray et al. 1993; Dimova et al. 1995). Generally, these drugs inhibit APAP toxicity.

It has been recently reported that Transient Receptor Potential Melastatin 2 (TRPM2) channels play a critical role in APAP toxicity (Ehsan Kheradpezhouha 2014). The TRPM2 channels are an important calcium ion (Ca^{2+}) signaling mechanism in a variety of cells, contributing to cellular functions. Importantly, TRPM2 channels may be activated by oxidative stress, leading to influx of calcium ions into the cell (Sumoza-Toledo and Penner 2011) (Miller BA and Cheung 2016). Kheradpezhouha and coworkers used patch clamp methods to show that APAP treatment of rat hepatocytes resulted in activation of a cation current similar to that observed with hydrogen peroxide. siRNA knockdown of TRPM2 in hepatocytes inhibits activation of the current by either APAP or hydrogen peroxide. In TRPM2 knockout mice, APAP-induced liver toxicity, as assessed by the blood concentrations of liver enzymes and liver histology, was significantly decreased compared with wildtype mice (Ehsan Kheradpezhouha 2014). These data suggest that TRPM2 channels are critical for development of hepatotoxicity by APAP. In subsequent work, Kheradpezhouha and coworkers found that curcumin, a natural plant obtained polyphenol from tumeric was an inhibitor of the TRPM2 channel. It prevented the hydrogen peroxide or APAP induced rise in calcium in rat hepatocytes (Kheradpezhouh et al. 2016). However, Curcumin has been reported to inhibit Cyp3a4, an enzyme that activates APAP, but it did not inhibit Cyp1a2 or Cyp2e1, other enzymes important in APAP metabolic activation (Volak et al. 2008). Thus, even the curcumin data are encourging, though additional work is needed to fully understand any antidotal potential that curcumin may have.

The involvement of TRPM2 channels in APAP toxicity would be consistent with a mechanism where metabolic activation by P450 enzymes to NAPQI results in GSH depletion which causes increased oxidative stress and, as a result, TRPM2 channels are activated, leading to an influx of calcium ions. The increased calcium ions could then activate NOS1 via a mechanism involving calmodulin. Increased NOS1 activity could then lead to increased NO which then reacts with the superoxide, occurring as a result of increased oxidative stress, to form peroxynitrite. Peroxynitrite may then nitrate MnSOD, resulting in its inhibition which leads to augmented levels of superoxide. The increased peroxynitrite could also react at Complex 1 and Complex 2 to disrupt mitochondrial electron flow with mitochondrial dysfunction and cell death. This proposed mechanism when applied to APAP toxicity pieces together large amounts of what previously appeared to be disparate data. However, more research is needed to explore that model.

Role of the c-Jun N-terminal Kinases

There is strong evidence that mitogen-activated protein kinases (MAPKs) are also important mediators of APAP-induced liver injury. Phosphorylation of the c-Jun N-terminal kinase (JNK) during APAP hepatotoxicity in mice was first reported in 2003 (Elsby et al. 2003). Shortly thereafter, Neil Kaplowitz's research group used multiple approaches to demonstrate that blocking JNK protects against the injury. Using both mice and primary mouse hepatocytes, they found that the JNK inhibitor SP600125, antisense oligonucleotides against JNK2, and JNK2 knockout all reduced APAP hepatotoxicity (Gunawan et al. 2006). Although the specific isoform of JNK of greatest significance was initially controversial, with multiple other groups reporting conflicting data on the role of JNK2 (Bourdi et al. 2008; Saito et al. 2010), that issue was resolved when it was discovered that some investigators had accidentally used $JNK2^{-/-}$ mice on a different background strain than their control groups (Bourdi et al. 2011). More recent studies using JNK1 knockout and double JNK1/2 knockout mice provided further conflicting data, as the double knockout mice had much worse injury (Cubero et al. 2016). Furthermore, they found that SP600125 was still protective in the JNK1/2 knockouts. However, it appears that those investigators may have used the same problematic JNK2 knockout mice from the previous conflicting studies to generate their double knockout, and it is not clear if the results were properly controlled using mice of the correct genetic background. Furthermore, there was a clear pattern of reduced serum ALT in the JNK1 knockout mice after APAP overdose. Although the differences between wildtype and JNK1 knockout mice were apparently not statistically significant, it is not obvious in the methods description whether or not the experiments were powered to detect those differences (Cubero et al. 2016). Finally, even if SP600125 has off-target effects, many other JNK inhibitors have also been shown to reduce liver injury after APAP overdose in mice, including leflunomide and D-JNKI1 (Henderson et al. 2007; Latchoumycandane et al. 2007), and blockade of apoptosis signal-regulating kinase 1 (ASK1), mixed-lineage kinase 3 (MLK3) or protein kinase C (PKC) upstream of JNK is also protective (Nakagawa et al. 2008; Sharma et al. 2012; Xie, Ramachandran, et al. 2015). Overall, most investigators agree that JNK contributes to APAP hepatotoxicity (Du et al. 2015). Importantly, the mechanism of JNK activation likely involves oxidative stress, as ASK1 is normally sequestered in an inactive state by thioredoxin, but dissociates after APAP overdose (Nakagawa et al. 2008).

The mechanism by which JNK exacerbates APAP toxicity is now coming into focus. We know that JNK can translocate into mitochondria after APAP overdose, and experiments with mitochondria isolated from livers of APAP-treated mice revealed that JNK can directly inhibit respiration and cause the MPT (Hanawa et al. 2008). These effects require the SH3 domain-binding protein (Sab), as knockdown of Sab expression with adenoviral shRNA and Sab knockout both prevent the JNK translocation and later injury (Win et al. 2011; Win et al. 2016). Furthermore, female mice have reduced Sab expression compared to males and are also less susceptible to APAP hepatotoxicity (Win et al. 2019). Additional studies have also revealed that JNK works through other mediators in addition to Sab to ultimately inactivate mitochondrial Src kinase (Win et al. 2016), which normally promotes electron transport chain activity. The end result is that JNK inhibits mitochondrial respiration and

increases ROS production, which could lead to a self-sustaining feed-forward loop wherein JNK activation is maintained by the additional oxidative stress.

Cell Death Mechanisms

The first reports of APAP hepatotoxicity in humans and rodents included descriptions of histopathological features characteristic of oncotic necrosis, including eosinophilic degeneration and pyknosis (Davidson and Eastham 1966). However, the later discovery that hepatocyte nuclear DNA is extensively fragmented after APAP overdose in mice (Ray et al. 1991; Ray et al. 1993) led to the hypothesis that apoptosis may be a major mode of cell death (Ray et al. 1996). At the time, DNA fragmentation with laddering in gel electrophoresis was thought to be specific for apoptotic cell death. However, rigorous studies by Gujral and Jaeschke in which they quantified apoptotic hepatocytes by morphology and using biochemical methods such as caspase 3 activity at multiple time points after APAP overdose revealed that apoptosis contributes very little to APAP hepatotoxicity (Gujral et al. 2002). Instead, the DNA fragmentation has been attributed to release of endonucleases from mitochondria due to the mitochondrial damage. Endonuclease G (Endo G) and apoptosis-inducing factor (AIF) are normally present within mitochondrial intermembrane space. Immunofluorescence staining of primary mouse hepatocytes treated with APAP revealed that both EndoG and AIF translocate to the nucleus during APAP toxicity (Bajt et al. 2006), and AIF-deficient mice were found to have reduced liver injury as a result of both lower oxidative stress and reduced DNA fragmentation (Bajt et al. 2011). There is also evidence that DNase I is released by necrotic hepatocytes and promotes the DNA fragmentation in neighboring cells (Napirei et al. 2006), though, as stated above, the evidence for propagation of injury by toxic expansion is questionable.

For many years, the lack of apoptosis during APAP toxicity despite release of Smac/DIABLO and cytochrome c from the damaged mitochondria, was assumed to be due to loss of ATP, which is necessary for activation of the apoptosome. However, Ramachandran et al. (Ramachandran et al. 2013) proposed a new explanation. They suggested that the major mode of cell death in APAP-induced liver injury is necroptosis, a specific type of programmed necrosis. Consistent with that, they found that the receptor-interacting protein kinase 3 (RIP3), a critical mediator of necroptosis, was increased in the liver after APAP overdose in mice, and that blocking RIP3 by knockdown with morpholinos or genetic knockout is protective (Ramachandran et al. 2013). It was also reported that the inhibitor of RIP1, necrostatin-1, is protective in primary mouse hepatocytes and in mice *in vivo* (Sharma et al. 2012; Ramachandran et al. 2013; Zhang et al. 2014). However, other groups quickly provided conflicting data on the involvement of both RIP1 and RIP3 in APAP hepatotoxicity using their own genetic and pharmacologic approaches (Dara et al. 2015; Deutsch et al. 2015). Currently, most researchers accept that necroptosis has a role in APAP hepatotoxicity, but the controversy regarding specific mediators is unresolved. Moreover, the overall progression of APAP toxicity described in this review, beginning with protein binding, progressing through mitochondrial damage and JNK activation, and ending with nuclear DNA fragmentation and cell death, has itself been referred to as a form of programmed necrosis (Jaeschke et al. 2019). Thus, programmed necrosis is certainly important in APAP hepatotoxicity, despite some confusion concerning the details.

Endogenous Protective Mechanisms and Liver Regeneration

Over the last several years, it has become apparent that APAP overdose also results in activation of endogenous protective mechanisms in order to limit the extent of injury. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a major regulator of antioxidant response genes in the liver. Normally, Nrf2 is held inactive in the liver by Kelch like-ECH-associated protein 1 (KEAP1). However, ROS can oxidize sulfhydryl groups in KEAP1, resulting in release of Nrf2. The latter then translocates to the nucleus and upregulates expression of antioxidant defense enzymes. Thus, Nrf2 is an important endogenous means to limit oxidative stress. It has been demonstrated that Nrf2 knockout mice are highly susceptible to APAP hepatotoxicity (Chan et al. 2001; Enomoto et al. 2001). Nrf2 KO mice have dramatically higher plasma ALT activity and increased mortality compared to WT mice. Measurement of non-protein sulfhydryls and anti-APAP reactivity in tissue indicated that this was likely due to reduced glutathione synthesis. Indeed, Nrf2 KO mice consistently have lower expression of glutamate-cysteine ligase and other enzymes involved in glutathione synthesis, and therefore greater APAP-protein binding and increased oxidative stress (Chan et al. 2001; Enomoto et al. 2001; Goldring et al. 2004). Similarly, mice with constitutive Nrf2 activation due to KEAP1 deficiency are resistant to APAP hepatotoxicity (Okawa et al. 2006). It is generally thought that Nrf2 activation after APAP overdose in mice is due to oxidation of cysteine sulfhydryl groups on KEAP1. However, there is also evidence that the reactive metabolite of APAP can directly modify KEAP1. Copple et al. (Copple et al. 2008) reported that treatment with NAPQI increases nuclear translocation of Nrf2 and Nrf2 transcriptional activity in a liver cell line, and further demonstrated that NAPQI can alkylate several cysteine residues in KEAP1 in cell lines. Since then, numerous studies have demonstrated that any stimulus that activates Nrf2 can protect against APAP-induced liver injury. For example, some genetic knockout models display off-target constitutive activation of Nrf2 and similar pre-conditioning effects, leading to protection (Ni, Boggess, et al. 2012; Williams et al. 2013). Interestingly, compelling recent data have demonstrated that the protective effects of Nrf2 are limited by JNK. JNK appears to phosphorylate Nrf2 and reduce its activity after APAP overdose in mice, and that inhibiting JNK may protect in part by reducing that effect (Chen Y et al. 2020). However, it should be noted that these data have recently been challenged due to the delayed effects of Nrf2 phosphorylation on target genes in the paper and the fact that JNK inhibitors protect even when administered after the time point at which Nrf2 phosphorylation is observed (Jaeschke and Ramachandran 2020). Nevertheless, overall, it is quite clear that Nrf2 is an important mediator of the endogenous protective response in APAP hepatotoxicity.

Autophagy is also thought to be critical to limit APAP-induced liver injury. Ni et al. (Ni, Bockus, et al. 2012) reported that autophagic flux increases in hepatocytes after APAP treatment, and that inhibition of autophagy enhances APAP-induced injury. Around the same time, Igusa et al. (Igusa et al. 2012) reported that Atg7-deficient mice have greater APAP hepatotoxicity than control animals. Extending these observations, Ni et al. recently proposed a novel mechanism whereby APAP-protein binding contributes to APAP hepatotoxicity. They treated primary mouse hepatocytes with 5 mM APAP and observed colocalization of immunofluorescence-labeled APAP-protein adducts with markers of

autophagosomes and lysosomes (Ni et al. 2016). They also reported that blocking autophagy using both pharmacologic and genetic approaches increases APAP-protein adducts in serum, while stimulation of autophagy results in lower serum adducts and less injury (Ni et al. 2016). Based on these observations, the researchers proposed that APAP-protein binding leads to accumulation of damaged proteins that must be removed through either autophagy or transport out to circulation in order to minimize cell damage. The same authors have further demonstrated that combined deletion of two proteins necessary for autophagic degradation of damaged mitochondria (mitophagy) also worsens APAP hepatotoxicity (Wang et al. 2019). Thus, autophagy appears to protect against APAP-induced liver injury by removing NAPQI-modified proteins and damaged mitochondria.

There is strong evidence that the outcome of liver injury depends upon a balance of the damage and repair (Mehendale 2005). Furthermore, the liver has an incredible capacity for regeneration, and acute liver failure patients with greater expression of regeneration biomarkers like proliferating cell nuclear antigen (PCNA) in tissue (Kayano et al. 1992) and alpha-fetoprotein in circulation are more likely to survive (Schmidt and Dalhoff 2005). However, until recently, little effort was made to explore the mechanisms and importance of liver regeneration and repair after APAP hepatotoxicity. Many researchers have assumed that the mechanisms resemble those seen in the partial hepatectomy model, but recent data have challenged that notion. For example, a recent transcriptomics study revealed that activation of both epidermal growth factor and IL-6 signaling is stronger after APAP hepatotoxicity (Bhushan et al. 2020), while other data may indicate that HGF signaling is less important (Clemens, McGill, et al. 2019). Consistent with a major role for IL-6, James et al. (James, Lamps, et al. 2003) demonstrated that IL-6 KO mice have reduced PCNA expression in the liver at 48-72 h after APAP overdose, indicating the IL-6/STAT3 signaling is important in repair after APAP overdose similar to partial hepatectomy. The same group demonstrated that treatment with recombinant vascular endothelial growth factor (VEGF) stimulates regeneration (Donahower et al. 2010). Around the same time, Apte et al. (Apte et al. 2009) demonstrated that ablation of β-catenin decreases markers of liver regeneration at 24 h after APAP overdose in mice. The role of Wnt/β-catenin signaling is further supported by the observation that β-catenin signaling is only increased after APAP overdoses from which mice recover, not after lethal doses (Bhushan et al. 2014). In addition, overexpression of stable β-catenin enhances liver regeneration after APAP (Bhushan et al. 2014), and treatment with an inhibitor of glycogen synthase kinase 3 β (GSK3β), which itself inhibits β-catenin signaling, has the same effect (Bhushan et al. 2017). More recently, a role for the phospholipid phosphatidic acid (PA) in Wnt/β-catenin signaling after APAP overdose has been demonstrated. Tissue and circulating levels of PA dramatically and persistently increase during APAP hepatotoxicity and recovery in mice (Lutkewitte et al. 2018). Importantly, inhibition of PA synthesis decreases GSK3β phosphorylation, β-catenin signaling and PCNA expression (Lutkewitte et al. 2018; Clemens, Kennon-McGill, et al. 2019), which is consistent with a role of PA in regulation of GSK3β, while addition of a GSK3 inhibitor blocks those effects (Clemens, Kennon-McGill, et al. 2019). Overall, it is clear the IL-6, EGFR and Wnt/β-catenin signaling are critical for proper repair and regeneration after APAP overdose.

Finally, there is also some evidence that mitochondrial biogenesis is important for liver repair after APAP overdose. It has been observed that proliferating hepatocytes surrounding areas of tissue necrosis have more mitochondria, indicating increased mitochondrial biogenesis in those cells (Ni et al. 2013). Increased expression of PPARγ co-activator 1α (Pgc-1α), nuclear respiratory factor 1 (Nrf1), and dynamin-related protein 1 (Drp1) has also been observed with time courses that resemble liver regeneration (Du et al. 2017). Most importantly, treatment with SRT1720 increased markers of mitochondrial biogenesis and accelerated regeneration after APAP overdose in mice (Du et al. 2017). Together, these data indicate that mitochondrial biogenesis is likely important to provide energy for liver repair after APAP hepatotoxicity.

Role of Inflammation

The significance of sterile inflammation in APAP hepatotoxicity is controversial. Numerous reviews on this topic have been published in the last several years (Woolbright and Jaeschke 2017, 2018), so we will describe these issues only briefly. Laskin et al. observed infiltration and activation of monocytes in the liver in rats treated with large doses of APAP (Laskin and Pilaro 1986; Laskin et al. 1986). Based on that, they hypothesized that macrophages in the liver contribute to APAP hepatotoxicity. Consistent with their early results, they later discovered that treatment with the Kupffer cell inactivators gadolinium chloride (GdCl) and dextran sulfate significantly reduced APAP hepatotoxicity (Laskin et al. 1995) and that was reproduced in mice (Michael et al. 1999). However, it was later discovered that complete ablation of Kupffer cells using liposome-entrapped clodronate actually aggravated APAP-induced liver injury (Ju et al. 2002), indicating that there may be another mechanism to explain the earlier results. Furthermore, NADPH oxidase KO mice were not protected against APAP hepatotoxicity, and displayed similar evidence of oxidative stress compared to wildtype controls, demonstrating that macrophages do not contribute to the oxidative stress or injury (James LP, McCullough, et al. 2003). Finally, CCR2 knockout mice were not protected against APAP, and in fact had delayed resolution of injury (Holt et al. 2008). Taken together, these and other data strongly indicate that macrophages do not contribute to APAP toxicity and may even be beneficial for repair. For example, it was recently demonstrated that netrin-1 promotes regeneration and recovery after APAP hepatotoxicity in mice through a mechanism involving Kupffer cells (Duan et al. 2020).

Another inflammatory cell type often implicated in APAP hepatotoxicity is the neutrophil. Mitchell, Jollow, Potter, Gillette and Brodie, described neutrophil accumulation in the liver after APAP overdose in mice in their original 1973 reports and, we now know that they are an important driver of injury in rodent ischemia-reperfusion models of acute liver injury. One of the first studies to directly test the role of neutrophils in APAP toxicity revealed that neutrophil antiserum reduced APAP-induced injury in rats (Smith et al. 1998). However, the use of polyclonal serum may have confounded the results. Indeed, later experiments revealed that treatment with a specific CD18-neutrophil antibody had no effect on APAP hepatotoxicity in mice (Lawson et al. 2000). Since then, numerous studies have been published presenting wildly conflicting data. For example, some research groups have found that knockout of inflammasome components reduces APAP-induced liver injury (Imaeda

et al. 2009), while others have found no effect (Williams et al. 2011). Overall, the role of neutrophils in APAP hepatotoxicity remains controversial.

Several groups have reported important roles for cytokines in APAP-induced liver injury. Cytokines clearly increase after APAP overdose, but again their effects are controversial. For example, interleukin-22 (IL-22) binding protein (IL22BP) knockout mice have increased IL-22 signaling and more severe injury than controls (Kleinschmidt et al. 2017), but treatment with exogenous IL-22 is protective against APAP (Feng et al. 2014). Similarly, there is some evidence that IL-6 can exacerbate the liver injury in certain conditions (Woolbright and Jaeschke 2018), but treatment with exogenous IL-6 is protective (Gao et al. 2019) and IL-6 KO mice have worse outcomes and delayed injury resolution {James et al. 2003}. Additional research is needed to fully understand the importance of cytokines in APAP hepatotoxicity.

Translation of APAP Hepatotoxicity Mechanisms to Humans

Despite decades of research using animal models and cell culture models, the mechanisms of APAP hepatotoxicity have only recently been fully translated to humans. Davis et al. (Davis et al. 1975) demonstrated that rats had impaired bromosulphthalein glutathionylation after APAP overdose due to hepatic glutathione depletion, and they observed a similar reduction of bromosulphthalein metabolism in APAP overdose patients, indicating that APAP depletes glutathione in humans similar to rodents. Lauterburg and Mitchell later confirmed that by demonstrating that increasing therapeutic doses of APAP result in greater incorporation of cysteine derived from radiolabeled cystine into the NAC-APAP metabolite in humans (Lauterburg and Mitchell 1987). A few years later, the first evidence of APAP-protein binding in APAP overdose patients was published (Hinson et al. 1990), later confirmed in larger studies (Webster et al. 1996; James et al. 2001; Davern et al. 2006; James et al. 2007). Finally, it was recently shown that serum values for APAP-protein adducts in early-presenting overdose patients can predict development of severe liver injury (Chiew et al. 2020), which is consistent with the idea that protein binding is a driver of injury in humans like mice. Taken together, those data demonstrate that glutathione depletion and protein binding are also critical in the mechanism of toxicity in humans.

More recently, using furosemide as a negative control for mitochondrial dysfunction in hepatotoxicity, it was reported that glutamate dehydrogenase (GLDH), mitochondrial DNA (mtDNA) and long-chain acylcarnitines in plasma are specific biomarkers for mitochondrial damage in the context of liver injury (McGill, Sharpe, et al. 2012; McGill et al. 2014). Importantly, both GLDH and mtDNA were found to be elevated in APAP overdose patients with liver injury (peak ALT≥1,000 U/L) (McGill, Sharpe, et al. 2012). In addition, long-chain acylcarnitine values were greater in blood from patients with delayed NAC treatment than from those who received NAC early (Bhattacharyya et al. 2013; Bhattacharyya et al. 2014). Furthermore, nuclear DNA fragments have also been reported in plasma after APAP overdose in humans (Craig et al. 2011; McGill, Sharpe, et al. 2012; McGill et al. 2014), and nuclear DNA fragmentation is mediated by endonucleases released from damaged mitochondria (discussed above). Altogether, these data clearly demonstrate that APAP hepatotoxicity causes mitochondrial damage and DNA fragmentation in humans in addition

to mice. Importantly, these appear to be critical events, as serum values for GLDH, mtDNA, and nuclear DNA fragments are at least modestly predictive of death in APAP-induced acute liver failure patients (McGill et al. 2014). Finally, caspase 3 activity as a biomarker of apoptosis was not detectable in plasma from APAP overdose patients, despite being elevated in plasma from mice with liver apoptosis due to LPS/Galactosamine treatment, indicating that apoptosis is not a major mechanism of cell death in humans (McGill, Sharpe, et al. 2012). Overall, these data indicate that the mechanisms of APAP-induced liver injury are similar in humans and mice.

Additional translational data have come from recent studies using cultured human hepatocytes. HepaRG is a unique bipotential human liver cell line that can differentiate into hepatocytes and bile duct epithelium-like cells. Importantly, HepaRG cells express most major drug metabolizing enzymes and transporters at levels similar to primary human hepatocytes. It has been demonstrated that APAP is toxic specifically to hepatocytes within HepaRG cultures, with a time course of injury that resembles APAP overdose patients (McGill et al. 2011). APAP also causes glutathione depletion, protein binding, mitochondrial depolarization and oxidative stress in HepaRG cells, and each of those precedes the cell death, indicating that they may be important for the mechanism (McGill et al. 2011). More recently, the mechanisms of APAP toxicity were explored in primary human hepatocytes (PHH) freshly isolated from resected liver tissue. Like HepaRG cells, fresh PHH develop toxicity with a time course that resembles humans (Xie et al. 2014). And, again, mitochondrial depolarization and oxidative stress precede the cell death. Importantly, JNK activation was also observed in PHH after APAP treatment, and the JNK inhibitor SP600125 reduced APAP toxicity in that model (Xie et al. 2014). Finally, there is evidence that 4-methylpyrazole protects PHH against APAP toxicity by inhibiting cytochromes P450 and reducing protein alkylation (Akakpo et al. 2018). Collectively, the data from APAP overdose patients and cultured human hepatocytes strongly indicate that the basic mechanisms of APAP hepatotoxicity in humans and mice are at least similar, if not identical.

Novel Treatments for APAP Overdose

New treatments for APAP overdose could be beneficial for some patients. Although NAC is highly protective when administered early, it loses efficacy in patients ≥10 h after APAP overdose (Rumack et al. 1981) and recent data indicate that most APAP overdose patients present later than that (Craig et al. 2012). However, life-threatening acute liver injury is relatively rare compared to other conditions, and there is a general perception that NAC is good enough. As a result, few pharmaceutical companies have demonstrated interest in development of new drugs to treat APAP hepatotoxicity. To address that, some research groups have focused on re-purposing existing drugs.

Currently, two drugs are the focus of clinical investigation. Mangifodopir is a manganese-containing compound that approved in many countries as an imaging contrast agent. The manganese ions dissociate and enter normal tissues, which makes them appear brighter by MRI. It has also been tested as a MnSOD mimetic, due to the presence of manganese. In the latter case, dissociation is undesirable. Calmangifodopir (CMF) was developed to address that. CMF is a mangifodopir derivative in which all but one manganese is replaced

by calcium, which prevents release of the single remaining manganese and facilitates its function as an antioxidant. Mangifodopir itself has been shown to be hepatoprotective after APAP overdose and ischemia-reperfusion injury in mice (Bedda et al. 2003; Coriat et al. 2011). Importantly, a recent phase I clinical trial in which CMF treatment was combined with a truncated NAC regimen revealed that addition of CMF modestly decreased the serum liver injury biomarkers keratin 18 and caspase-cleaved keratin 18 in overdose patients with no effect on serious adverse event reporting (Morrison et al. 2019). These data demonstrate that CMF is a safe and potentially efficacious adjunct to NAC.

Some have promoted the drug 4-methylpyrazole (4MP; a.k.a. fomepizole) as another novel treatment for APAP overdose patients. 4MP is widely used to treat ethylene glycol and alcohol poisoning because it is an alcohol dehydrogenase inhibitor. It has been known for some time that 4MP can also reduce bioactivation of APAP (Thummel et al. 1989) and protect against APAP hepatotoxicity in rats (Burk et al. 1990; Brennan et al. 1994). In 2013, a case report appeared describing a patient who had apparently consumed an extremely large dose of APAP, with a peak serum APAP concentration in a range which few people have been reported to survive (Zell-Kanter et al. 2013). The authors treated the patient with NAC, along with a single dose of 4MP due to suspected alcohol co-ingestion, and the patient recovered. This led to speculation that 4MP could be a useful adjunct to NAC (Yip and Heard 2016). Akakpo et al. subsequently demonstrated that 4MP reduces APAP-induced liver injury in mice by blocking reactive metabolite formation as a pre-treatment and reducing JNK activation as a post-treatment (Akakpo et al. 2018; Akakpo et al. 2019). Most recently, a small crossover trial in volunteer subjects revealed that 4MP also reduces APAP reactive metabolite formation in humans (Kang et al. 2020). These data indicate that 4MP is another promising adjunct to NAC therapy, though we need additional clinical trials to confirm that.

Summary

In the past 65 years, we have seen the introduction of APAP as a commercially viable analgesic and antipyretic, the rise of APAP overdose to both a popular drug and the primary cause of acute liver failure in several countries, the introduction of NAC as an antidote, tremendous advances in understanding of the fundamental toxicity mechanisms, and now the emergence of a new generation of therapeutics for overdose patients. Along the way, we have learned an incredible amount about the basic mechanisms of xenobiotic hepatotoxicity, as well as liver injury more generally, by studying the pathophysiology of APAP hepatotoxicity in rodents and humans. In the future, we hope to see the widespread adoption of new treatments for APAP overdose patients, and we will continue to explore the mechanisms of APAP-induced liver injury and subsequent repair in mice as a clinically relevant model of liver disease.

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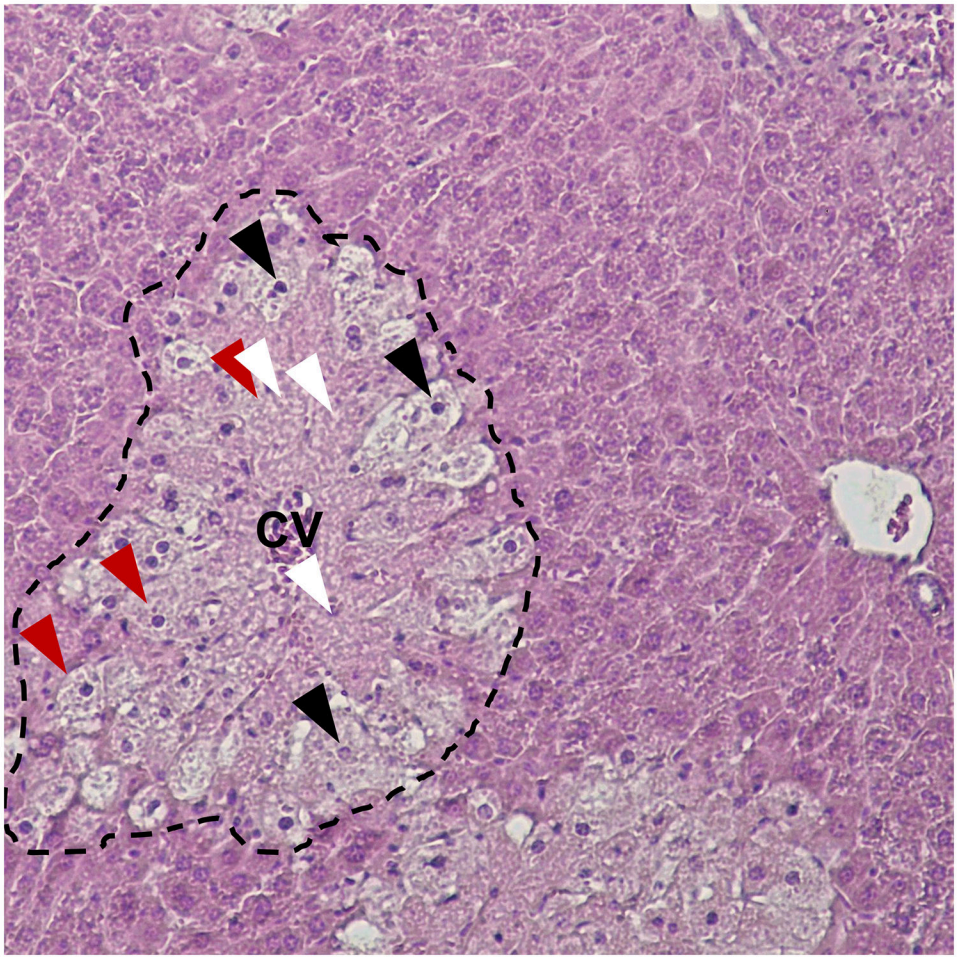

Figure 1. Histopathology of APAP-induced liver injury.

A mouse was treated with 300 mg/kg APAP. The liver was collected 24 h later. Liver tissue was fixed in 10% phosphate-buffered formalin and embedded in paraffin wax. 5 μm sections were cut, mounted on glass slides, and stained with hematoxylin and eoson. The dotted line indicates an area of hepatocellular necrosis displaying eosinophilia and loss of basophilic nuclei. Black arrowheads: Pyknosis and karyorrhexis. White arrowheads: Karyolysis. Red arrowheads: Cell swelling.

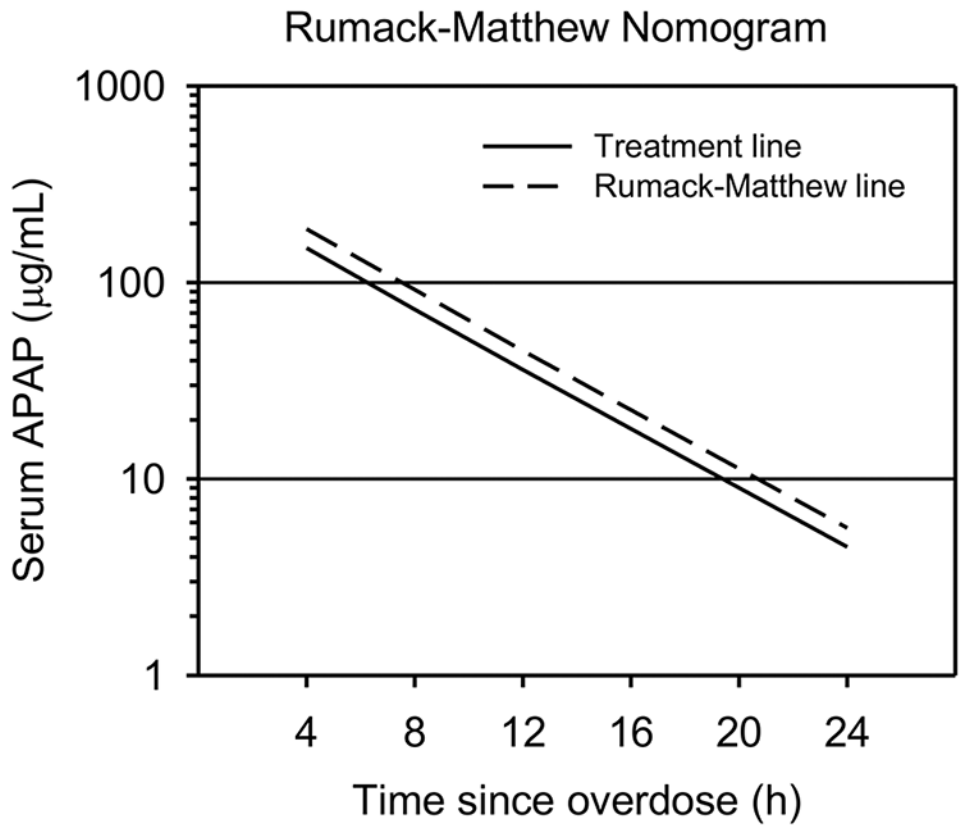

Figure 2. The Rumack-Matthew nomogram.

When measured between 4 and 24 hours after APAP overdose, values above the dashed line (Rumack-Matthew line) are likely to result in hepatotoxicity, values between the dashed line and the solid line (Treatment line) may or may not lead to hepatotoxicity, and values below the solid line are unlikely to cause hepatotoxicity. All patients with values above the Treatment line should receive NAC.

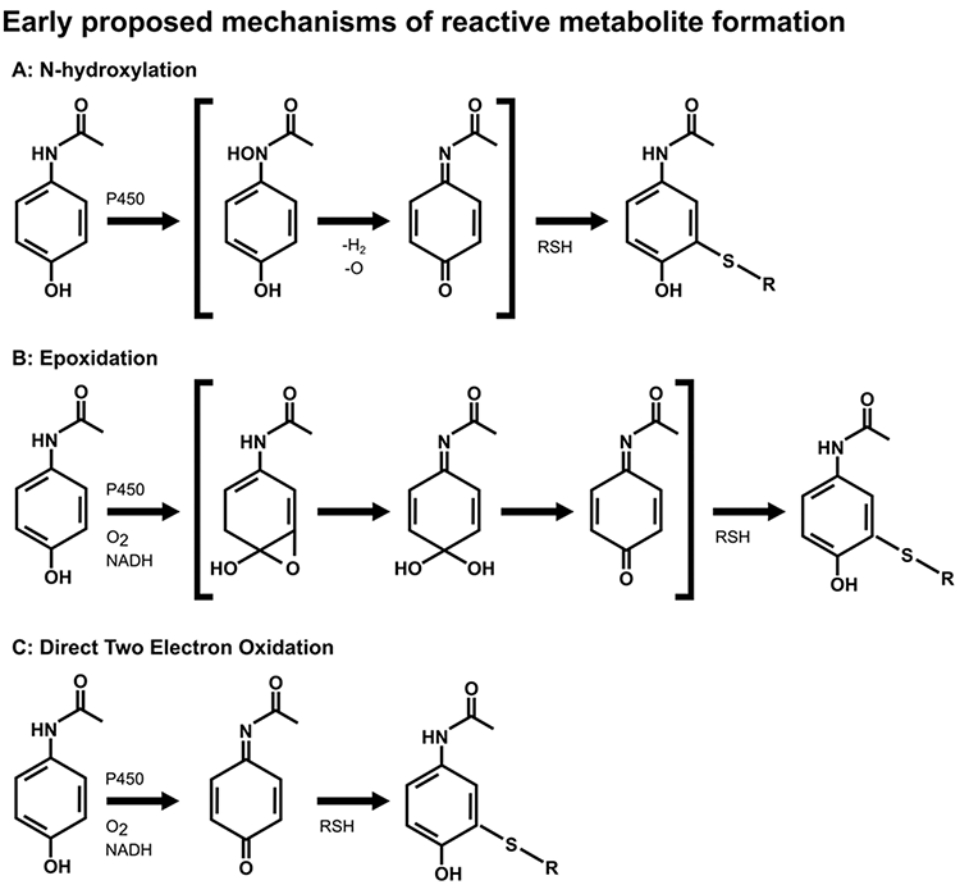

Figure 3. Proposed mechanisms of NAPQI formation.

Initially, it was thought that formation of the reactive metabolite of APAP may precede through N-hydroxylation (A) or epoxidation (B), as shown. These mechanisms were proven to be incorrect. (C) It is now thought that APAP is directly oxidized by a two-electron oxidation, a previously unrecognized cytochrome P-450 mechanism. Once formed, NAPQI readily reacts with sulfhydryl groups, such as on GSH or cysteine residues in proteins.

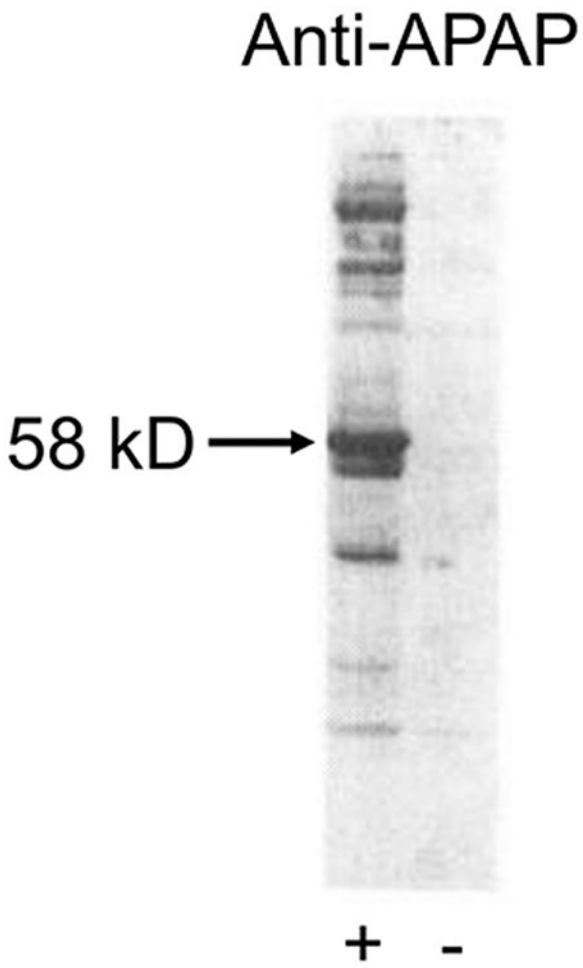

Figure 4. Anti-APAP immunoblot.

Cytosolic fractions from the livers of control (−) and 600 mg/kg APAP-treated (+) mice, isolated 2 hours after treatment. Western blotting was performed using an anti-APAP antibody. A dominant band is visible around 58 kD. Adapted from(Cohen and Khairallah 1997), with permission from the publisher.

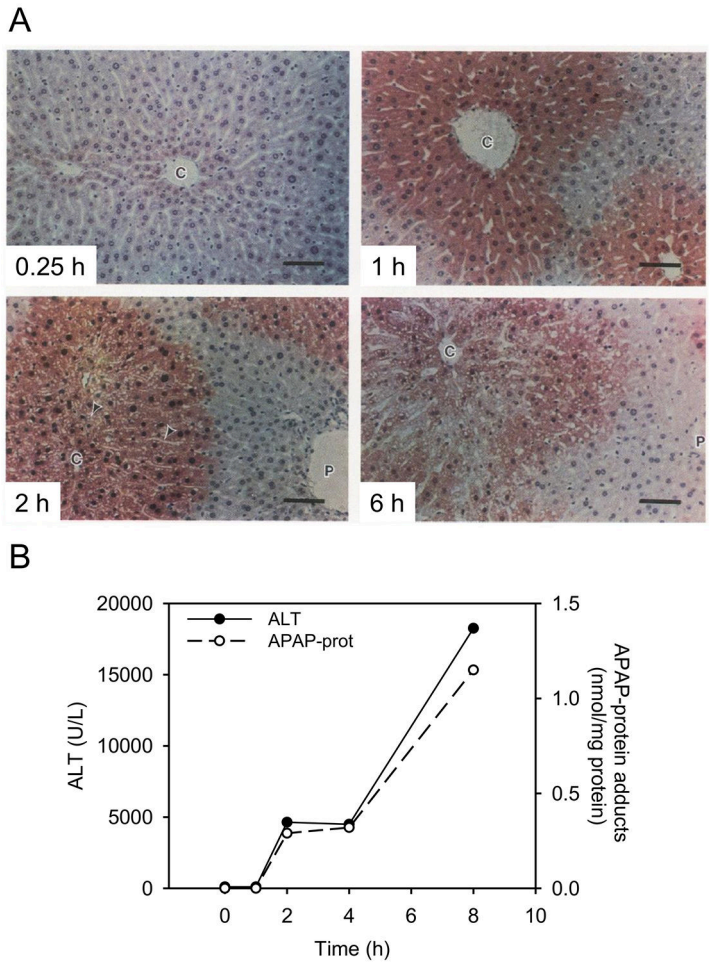

Figure 5. Measurement of covalent APAP-protein adducts in the liver.

Mice were treated with a hepatotoxic dose of acetaminophen (APAP). Livers and serum were collected at multiple time points after APAP overdose. (A) Immunohistochemistry for APAP. (B) Serum alanine aminotransferase (ALT) activity (left y-axis) and serum APAP-protein adducts (right y-axis) over time. Adapted from (Roberts D. W. et al. 1991) and (McGill et al. 2013) with permission.

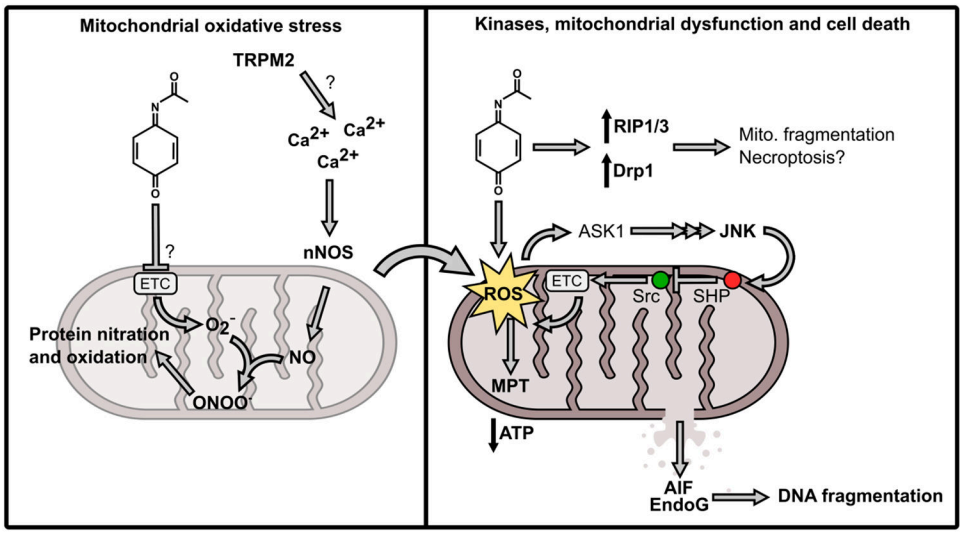

Figure 6. Mechanisms of APAP hepatotoxicity.

Acetaminophen (APAP) hepatotoxicity begins with formation of a reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI). Left side: NAPQI depletes hepatic glutathione (GSH) and binds to proteins. The initial protein binding leads to oxidative stress through an unknown mechanism that may involve covalent modification and inhibition of electron transport chain (ETC) complex proteins leading to increased superoxide [O_2^-]. At the same time, activation of neuronal nitric oxide synthase (nNOS)/NOS1 occurs, possibly through redox modification or covalent modification of the transient receptor potential melastatin 2 (TRPM2) which increases intracellular calcium (Ca^{2+}). Nitric oxide (NO) from nNOS then reacts with O_2^- to form peroxynitrite ($ONOO^-$), which then reacts with tyrosine and other residues on intracellular proteins. Right side: The initial reactive oxygen species (ROS) also activate apoptosis-signaling kinase 1 (ASK1), ultimately leading to activation of the c-Jun N-terminal kinases 1 and 2 (JNK). JNK then translocates to mitochondria, binding to the protein Sab in the outer mitochondrial membrane (OMM). This interaction causes release of the protein tyrosine phosphatase, nonreceptor type 6 (SHP) from Sab on intermembrane side of the OMM. SHP then translocates to the inner mitochondrial membrane and inhibits the ETC enhancer Src, resulting in reduced ETC activity and therefore increased ROS production. This forms a feed-forward loop in which ROS activate JNK, and JNK increases ROS. Eventually, the mitochondrial membrane permeability transition (MPT) occurs with loss of ATP production. The mitochondria swell and the membranes rupture, liberating the endonucleases apoptosis-inducing factor (AIF) and endonuclease G. The free nucleases can then fragment nuclear DNA. In parallel, expression and activity of the receptor-interacting protein (RIP) kinases 1 and 3 increase, along with Drp1-mediated mitochondrial fragmentation, through underexplored mechanisms. Ultimately, the hepatocyte dies by oncotic necrosis.