CHAPTER

19

Mechanisms of Acetaminophen-Induced Liver Disease

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OUTLINE

INTRODUCTION

Acetaminophen (paracetamol, *N*-acetyl-*p*-aminophenol) is a widely used analgesic and antipyretic drug [1–3]. It is sold under a variety of trade names and over the counter in various combination products for cold and flu symptoms. Acetaminophen was initially synthesized in the late 1800s and was originally reported to have analgesic activity by von Mering in 1893. However, it was not until the latter half of the twentieth century that it became widely used. Its increased use was initially associated with the recognition that phenacetin, a structurally related drug that is metabolized to acetaminophen, was nephrotoxic in abusers [4]. In the 1960s, it was recognized that in overdose acetaminophen produced a centrilobular hepatic necrosis [5]. The clinical toxicology of acetaminophen is reviewed in Chapter 20.

Subsequent to the report that acetaminophen was hepatotoxic in overdose, Boyd and Bereczky reported that acetaminophen also produced a centrilobular hepatic necrosis in rodents [6]. Since that time, the pathology has been evaluated in multiple laboratories and the consensus is that the primary toxicity is a hepatic necrosis [7–10]. Recent research has evaluated the role of programmed cell death, in particular apoptosis, in acetaminophen hepatotoxicity. Gujral et al. [11] used histological analysis of liver sections to determine necrosis versus apoptosis in acetaminophen-treated mice. At 24 h following a toxic dose of acetaminophen, they reported that 67% ± 12% of hepatocytes were necrotic and 0.35% were apoptotic. These data have led Jaeschke and coworkers to conclude that apoptosis is not a significant pathway of toxicity for acetaminophen [11–16].

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REACTIVE METABOLITE FORMATION

In 1973, Mitchell, Jollow, Potter, Gillette, and Brodie reported that a reactive metabolite that covalently bound to protein was causative in the hepatotoxicity of acetaminophen in mice [8,17–19]. This was the first study reporting a role for a reactive metabolite in the toxicity of a clinically used drug. At nontoxic doses, the reactive metabolite was efficiently detoxified by glutathione (GSH), forming an acetaminophen-GSH conjugate. However, following toxic doses of acetaminophen, hepatic GSH was depleted by as much as 80–90% and the reactive metabolite covalently bound to protein. Depletion of hepatic GSH by administration of diethyl maleate increased hepatotoxicity and covalent binding, whereas administration of cysteine to increase GSH decreased toxicity and covalent binding. The amount of covalent binding correlated with the relative hepatotoxicity [19,20]. Covalent binding was determined to be mediated by metabolism by cytochrome P450 (CYP) [18].

The identification of the reactive metabolite of acetaminophen was difficult because of its instability. Initially, it was postulated that the metabolite was *N*-hydroxyacetaminophen [18]. However, the synthetic *N*-hydroxyacetaminophen was too stable to be the reactive metabolite, and it was clearly shown that it was not an acetaminophen metabolite [21]. In addition, the reactive metabolite was shown not to be an epoxide [22–24]. The reactive metabolite was then proposed to be *N*-acetyl-*p*-benzoquinone imine (NAPQI) [25], but this was difficult to prove because of its instability. The problem was that it was easily reduced by NADPH, which was used in microsomal incubation mixtures for CYP activity.

In 1982, Dahlin and Nelson synthesized NAPQI [26]. They showed that it reacted with GSH to form 3-(glutathione-*S*-yl)acetaminophen, a known biliary metabolite of acetaminophen [27]. Utilizing purified CYP and cumene hydroperoxide, a system that did not utilize reducing conditions, they showed that NAPQI was a metabolite of acetaminophen [28]. Subsequently, evidence was presented that NAPQI, and not the free radical *N*-acetyl-*p*-benzosemiquinone imine, was the microsomal metabolite that reacted with GSH to form a conjugate [29]. NAPQI was postulated to be formed by a direct two-electron oxidation that was a new mechanism for CYP [29–31].

The reaction of NAPQI with GSH occurs by conjugation to form 3-(glutathione-*S*-yl)acetaminophen and by reduction to acetaminophen [26,27]. The second-order rate constant for the reaction of NAPQI with GSH was reported to be (3.2×10^4 $M^{-1}s^{-1}$). The reaction was very rapidly catalyzed by glutathione *S*-transferase P (GSTP1–1) [32]. Thus, detoxification of NAPQI is extremely rapid, which may explain why covalent binding to proteins was not observed in hepatocytes until GSH was almost completely depleted [19].

In initial work describing the importance of hepatic GSH in acetaminophen-induced hepatotoxicity in mice, Mitchell et al. [19] showed that administration of cysteine prevented hepatotoxicity. This finding led to the development of *N*-acetylcysteine (NAC) as the antidote [33–35]. NAC was used in preference to cysteine because it was already being used as a mucolytic agent (Mucomyst). The mechanism by which NAC inhibits acetaminophen toxicity has been reported to be increased detoxification of NAPQI by a direct conjugation [36]; however, conversion of NAC to cysteine will increase GSH synthesis and repletion of GSH levels may also be important. The metabolism of acetaminophen is presented in Figure 19-1.

Cytochrome P450s in Reactive Metabolite Formation

In the late 1980s and early 1990s, the various isoforms of CYP were purified. Upon their purification, there was a significant effort to determine the isoforms that metabolize acetaminophen to NAPQI. Using in vitro metabolism studies in which NAPQI was trapped as the acetaminophen-GSH conjugate and chromatographically quantified, it was determined that the major CYP isoforms important in acetaminophen metabolism are CYP2E1, CYP1A2, and CYP3A4 [37–40]. CYP2E1, the alcohol-inducible form, was determined to be the most important. Snawder and coworkers reported the K_m for CYP2E1 metabolism of acetaminophen to be 0.18 mM, whereas the K_m for CYP1A2 was roughly an order of magnitude higher [40]. Later the importance of CYP2E1 and CYP1A2 in acetaminophen toxicity was clearly demonstrated in genetically altered (knockout) mice that did not express these enzymes [41,42]. In wild-type mice, the lethal dose, 50% (LD_{50}), for acetaminophen was approximately 400 mg/kg, whereas the LD_{50} in CYP2E1 knockout mice was approximately 1,000 mg/kg. In CYP1A2 knockout mice, the LD_{50} was roughly 500 mg/kg. In double CYP2E1/CYP1A2 knockout mice, the LD_{50} was >1,200 mg/kg, the highest dose tested. These LD_{50} data correlated with the relative increases in serum ALT (alanine aminotransferase) levels and histopathological evidence of liver necrosis. Serum ALT levels increased in wild-type animals at doses of 400 mg/kg and greater; however, ALT increases were not observed in the double knockout mice at an acetaminophen dose of 1,200 mg/kg. Thus,

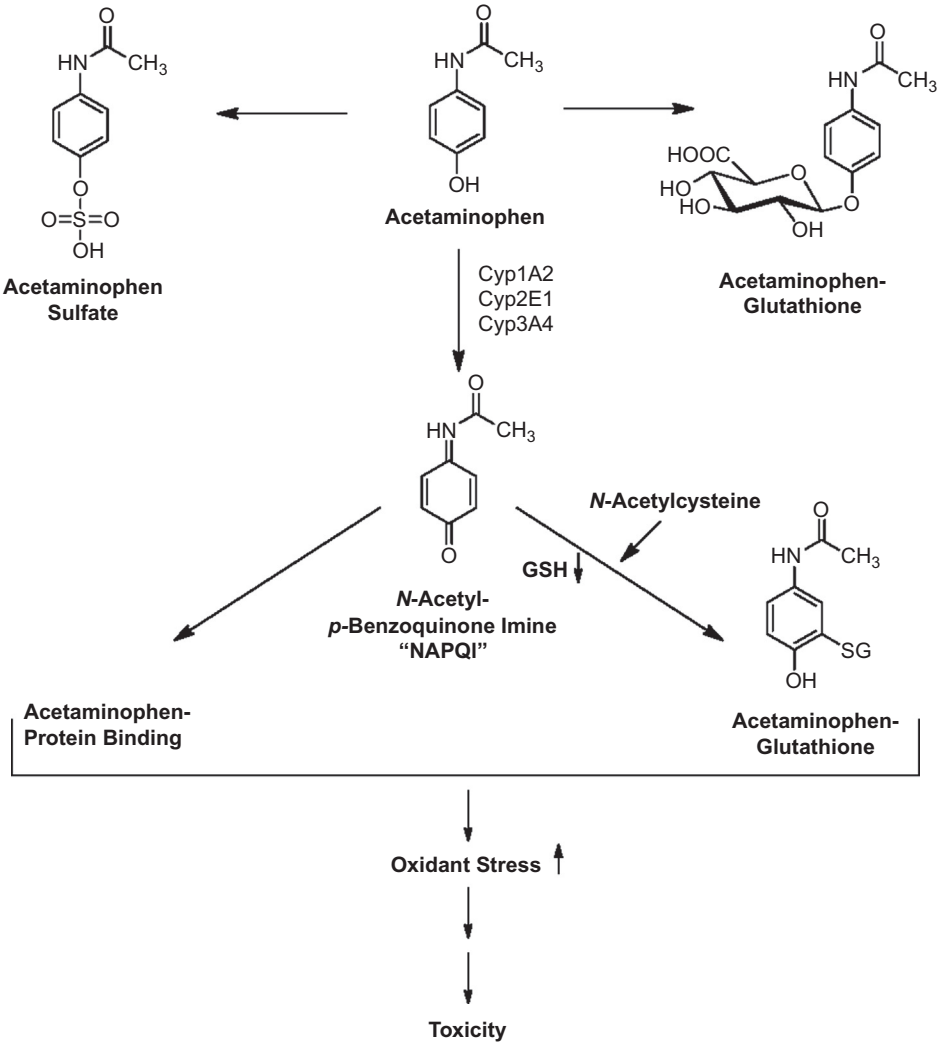

FIGURE 19-1 **Mechanism of acetaminophen metabolism.** GSH, glutathione.

the major CYP important for the acetaminophen hepatotoxicity is CYP2E1, followed by CYP1A2. Additional evidence that CYP3A may contribute to production of the reactive metabolite and development of toxicity has been presented using inducers such as phenobarbital [43].

PROTEIN COVALENT BINDING

Covalent binding of acetaminophen to proteins was found to correlate with acetaminophen-induced hepatotoxicity [17]. Covalent binding was initially determined using radiolabeled acetaminophen. The radiolabeled drug was administered to the animal and the liver was then removed and homogenized. A sample of protein was precipitated by acid or solvent and washed multiple times with solvent. Subsequently, the amounts of radioactivity and protein in the sample were determined and the nanomoles of acetaminophen per mg protein calculated. The covalent binding of the adduct was ascertained by protease digestion of the tissue sample and showed that the acetaminophen was attached to an individual amino acid. The assay was reliable and furnished a significant amount of important data. Subsequently, immunochemical approaches were developed for analysis of acetaminophen covalently bound to cysteine groups in proteins [44,45]. These assays provided more specific data on covalent binding. Western blot analyses of liver proteins from mice treated with toxic doses of acetaminophen indicated that a limited number of proteins were adducted [46,47]. Competitive enzyme-linked immunosorbent assay (ELISA) and Western blot data indicated that maximum levels of adducts occurred in the liver after 1–2 h. With the subsequent lysis of hepatocytes, acetaminophen-protein adducts were observed in the serum and Western blot assays indicated that these adducts were of hepatic origin [47]. The appearance of acetaminophen-protein adducts in serum correlated

with increases of serum ALT and AST (aspartate aminotransferase) [48]. Thus, acetaminophen-protein adducts in serum were a biomarker for the formation of hepatic acetaminophen-protein adducts and a correlate of acetaminophen toxicity in experimental animals. This finding was also utilized to show the presence of acetaminophen-protein adducts in the serum of acetaminophen overdose patients. The highest levels of adducts were found in the patients with the most severe toxicity [49].

Immunohistochemical analysis of liver sections from treated mice revealed a high correlation between the presence of acetaminophen-protein adducts in hepatocytes and the development of necrosis in the cells [10]. Adducts were detectable in the hepatocytes of liver sections within 15 min of dosing. By 1 h, staining intensity was maximal and adducts were confined to the centrilobular hepatocytes. Adducts were not present in periportal hepatocytes. At 2–6 h, hepatocytes containing adducts were developing necrotic changes, as evidenced by vacuolization and pyknotic nuclei. The only hepatocytes observed to develop necrosis were those containing acetaminophen-protein adducts. By 24 h, all of the observed necrotic cells contained adducts and the majority of hepatocytes containing adducts developed necrosis [10]. These data indicate an excellent correlation between the presence of acetaminophen-protein adducts and necrosis.

Matthews et al. [50] presented evidence that the acetaminophen in the protein adducts was covalently adducted to cysteine groups. Comparison of Western blots of liver homogenates from acetaminophen-treated mice using an antisera specific for acetaminophen and an antisera specific for 3-(cysteine-*S*-yl)acetaminophen indicated an identical adduct profile. Thus, no evidence was obtained that acetaminophen was attached to amino acids other than cysteine in proteins.

DeLeve and coworkers [51] examined acetaminophen toxicity in purified liver sinusoidal endothelial cells (LSECs). Previous data indicated that hepatic endothelial cells contain CYP enzymes [52,53]. Therefore, activation of acetaminophen by CYP enzymes in endothelial cells may produce toxicity. LSECs were isolated from two strains of mice: acetaminophen was not toxic to cultured LSECs from Swiss Webster mice but was toxic to cultured LSECs from C3H/HEN mice. GSH was depleted before the development of toxicity in LSECs of the C3H/HEN mice; however, it was not depleted in LSECs from the Swiss Webster mice. Addition of the suicide CYP inhibitor, aminobenzotriazole, inhibited development of toxicity in the C3H/HEN cells. These data suggested involvement of NAPQI as a toxic metabolite to LSECs. However, the two strains of mice appeared to be equally sensitive to the hepatic necrosis produced by toxic doses of acetaminophen in vivo. Interestingly, coculture of endothelial cells with the corresponding hepatocytes resulted in toxicity to LSECs of both strains, but the relationship to covalent binding was not defined [51].

In an attempt to understand the role of covalent binding in the hepatotoxicity of acetaminophen, specific proteins adducted with acetaminophen were isolated and sequenced [54]. The working hypothesis was that covalent binding to a specific protein in the liver caused the toxicity and that the toxic mechanism could be determined by identifying the protein adducts. A number of proteins were identified by this traditional approach [55–59]. Subsequently, Burlingame's laboratory, utilizing the then newly developed mass spectral methods, identified a number of additional proteins containing covalently bound acetaminophen [60]. The proteins reported to be adducted by acetaminophen are shown in Table 19-1.

TABLE 19-1 Acetaminophen-Protein Adducts Formed in Acetaminophen Toxicity in Mice

Adduct	Reference
Glutamine synthetase	[56]
Glutamate dehydrogenase	[57]
Aldehyde dehydrogenase	[58]
Selenium (acetaminophen) binding protein	[55]
N-10 formyltetrahydrofolate dehydrogenase	[59]
Glutathione peroxidase	[60]
Thioether *S*-methyltransferase	[60]
Aryl sulfotransferase	[60]
Pyrophosphatase	[60]
Tropomyosin 5	[60]
Proteasome subunit C8	[60]
Methionine adenosyltransferase	[60]
Protein synthesis initiation factor 4A,	[60]
ATP synthase α subunit	[60]
Carbonic anhydrase III	[60]
Urate oxidase	[60]
2,4-Dienyl CoA reductase	[60]
Osteoblast specific factor 3	[60]
Glutathione transferase π	[60]
Sorbitol dehydrogenase	[60]
Glycine *N*-methyltransferase	[60]
3-Hydroxyanthranilate 3,4-dioxygenase	[60]

Even though there is an excellent correlation between protein covalent binding of acetaminophen and development of hepatotoxicity, it may be that severe GSH depletion in individual hepatocytes is the critical factor necessary to produce toxicity and that covalent binding is just a biomarker of NAPQI formation. Nagai and coauthors showed that GSH depletion in cultured hepatocytes by both diethyl maleate and phorone caused necrosis [61]. However, it is unknown whether these compounds covalently bind to protein, and it is possible that covalent binding was important for the observed necrosis. Henderson and coworkers [62] examined acetaminophen-induced GSH depletion and hepatotoxicity in GSTP1−1 knockout mice. This is the glutathione transferase that catalyzes the conjugation of NAPQI with GSH [32]. Following a toxic dose of acetaminophen, hepatic GSH was depleted by >90% in wild-type mice, but by only 70% in the knockout mice. Unexpectedly the knockout mice did not develop as much hepatic necrosis as did the wild-type mice, but both groups of mice had similar levels of acetaminophen covalent binding. These data appear to separate the covalent binding of acetaminophen to proteins from the development of the toxicity. However, these data may simply indicate that both covalent binding and severe GSH depletion are necessary for the development of hepatotoxicity. So far the role of protein binding in acetaminophen toxicity is unclear, and a better understanding of the role of protein binding in toxicity, if there is any role at all, will require additional studies. However, it is now well established that covalent binding is an excellent biomarker of the formation of the toxic metabolite, NAPQI.

Acetaminophen-protein adducts have proven to be a reliable biomarker of metabolic activation and toxicity. Analysis of adducts has been used extensively to determine if experimental conditions affect acetaminophen metabolism. For example, in studies utilizing genetically altered mice, analysis of acetaminophen-protein adducts has shown that activation of acetaminophen metabolism is unaffected by the genetic change [63,64]. However, in other studies, misinterpretations have occurred as a result of not knowing the effect of the treatment on acetaminophen metabolism to a covalent adduct. For example, Masubuchi et al. reported that the mitochondrial permeability transition (MPT) inhibitor cyclosporin A (at 50 mg/kg) inhibited acetaminophen hepatotoxicity in mice [65], an effect previously reported to occur in isolated hepatocytes [13,66]. The finding that liver GSH was maximally depleted with or without cyclosporin A was taken as evidence that CYP metabolism of acetaminophen to NAPQI was not inhibited. However, GSH depletion per se is inadequate to show that CYP metabolism of acetaminophen to NAPQI was not inhibited. Chaudhuri et al. [67] recently reported that cyclosporin A at doses as low as 20 mg/kg inhibit acetaminophen-protein binding. Thus, even though MPT is inhibited by cyclosporin A, the data of Chaudhuri et al. [67] suggest that the mechanism by which Masubuchi et al. found that cyclosporin A inhibited toxicity was through decreased metabolic activation [65]. In a second example, Latchoumycandane et al. [68,69] reported that the JNK/MAPK8 (mitogen-activated protein kinase 8) inhibitor leflunomide protected mice from acetaminophen-induced liver toxicity and concluded that the mechanism of protection was via JNK inhibition [68]. GSH depletion was not significantly different in the livers of the acetaminophen-treated mice with and without leflunomide. However, in subsequent research Tan et al. found that leflunomide inhibited the metabolic activation of acetaminophen and was an inhibitor of CYP1A2 [70]. Thus, leflunomide may have inhibited acetaminophen toxicity by inhibiting NAPQI formation. A third example is the finding that *S*-adenosylmethionine inhibits acetaminophen toxicity in mice [71]. It was concluded that the mechanism for this involved maintenance of GSH levels. However, Caro and Cederbaum have shown that this compound inhibits CYP2E1, the critical CYP in acetaminophen metabolic activation [72]. Therefore, not only may treatment with drugs alter acetaminophen toxicity but solvents in which the treatment drugs are frequently administered may also inhibit the metabolism of acetaminophen to NAPQI and thus confound the interpretation of the results. For example, dimethyl sulfoxide [73−75] has been reported to inhibit CYP metabolism of acetaminophen to NAPQI and to decrease acetaminophen hepatotoxicity. This is an effect that occurs with many solvents, including ethanol [75]. Ethanol will induce CYP2E1 and increase acetaminophen toxicity by increasing NAPQI formation, but will also inhibit the metabolic activation of acetaminophen [75]. Thus, in the evaluation of mechanisms of acetaminophen hepatotoxicity, it is necessary to show that acetaminophen-protein binding is not inhibited by the drug treatment.

In 2002, Muldrew et al. developed a highly sensitive and specific assay for the detection of acetaminophen-protein adducts (3-(cysteine-*S*-yl)acetaminophen) in proteins In this assay, the protein sample is first digested with protease and the resulting 3-(cysteine-*S*-yl)acetaminophen is quantified by a high performance liquid chromatography-electrochemical detection (i.e., HPLC-EC) assay. This assay has allowed the determination of acetaminophen-protein adducts in samples without using radiolabeled acetaminophen in animal experiments or quantification of protein samples by immunoassays. The assay has been

used to quantify acetaminophen-protein adducts in humans, as described in Chapter 20.

TOXICITY OCCURS IN TWO PHASES

Using freshly isolated hepatocytes, Boobis and coworkers have shown that acetaminophen toxicity occurs in two phases [76,77]. Incubation of acetaminophen with freshly isolated hamster hepatocytes for 90 min resulted in depletion of GSH and covalent binding of radiolabeled acetaminophen to protein. Subsequent washing of the hepatocytes to completely remove the acetaminophen, followed by reincubation of the hepatocytes in buffer, resulted in toxicity in the reincubation phase. By 4.5 h, toxicity had occurred in 60% of the hepatocytes. Further, acetaminophen toxicity in freshly isolated mouse hepatocytes also occurs in two phases [66,78,79]. Thus, acetaminophen toxicity occurs in two phases in both systems. As discussed above, the metabolic phase has been thoroughly investigated and is characterized by CYP metabolism to NAPQI, resulting in GSH depletion and covalent binding. The toxicity phase is poorly understood and has been the focus of research in many different laboratories in recent years. A number of different events appear to occur in the toxicity phase, including altered ion imbalance, altered mitochondrial function, increased oxidative stress, and increased signal transduction processes.

ALTERATIONS IN CALCIUM METABOLISM

The role of altered calcium metabolism has been postulated to be important in various drug- and chemical-induced toxicities [80,81], including acetaminophen-induced hepatic necrosis [82–85]. Burcham and Harmon [83] reported that toxic doses of acetaminophen to mice resulted in an increase in mitochondrial calcium levels. At 3 h following acetaminophen administration, there was a twofold increase in mitochondrial calcium, and by 24 h there was a 3.5-fold increase. They found no significant increases in cytosolic or microsomal calcium levels. Similarly, Timerstein and Nelson reported that following a toxic dose of acetaminophen to mice, there were increases in hepatic mitochondrial calcium levels at 1 h and 6 h (177% and 396%, respectively) [85]. However, with the nontoxic acetaminophen analog, 3-hydroxyacetaminophen, there was only a minor increase in mitochondrial calcium. Importantly, following toxic doses of acetaminophen to mice, there is a large decrease in plasma membrane calcium ATPase activity [85,86]. This enzyme is important in the removal of calcium from the cell because extracellular calcium enters the cell due to a large electrochemical gradient driving this ion into the cell [87]. Inhibition of the enzyme results in calcium accumulation in the cell. In both reports, the authors suggested that covalent binding of the reactive metabolite of acetaminophen to the calcium ATPase was responsible for the loss of its activity [85,86]; however, this enzyme has not been reported to be adducted by NAPQI. Oxidative stress is another mechanism that may explain the inhibition of this enzyme in acetaminophen hepatotoxicity. Squier and coworkers [88,89] have reported that increased oxidative stress causes oxidation of critical methionine residues in calmodulin, which is important in the control of calcium ATPase activity, thus leading to decreased enzyme activity.

The effect of the calcium-specific chelators on acetaminophen-induced toxicity in freshly isolated hamster hepatocytes was examined by Boobis et al. [90]. Addition of the calcium chelator, Quin 2-AM, to freshly isolated hamster hepatocytes in the reincubation phase prevented the loss of viability; however, membrane blebbing was not substantially altered. It was concluded that alterations in cytosolic calcium are linked less directly to plasma membrane blebbing than to loss of cell viability [90]. Also, they reported that the increase in cytosolic calcium correlated with the development of toxicity [91].

Corcoran and coworkers reported nuclear damage in acetaminophen liver toxicity and investigated its role as a mechanism of toxicity. They reported that following a toxic dose of acetaminophen to mice there was an increase in nuclear calcium levels that appeared to approximately coincide in time with the development of the toxicity. Electrophoretic analyses revealed a marked decline in slowly migrating large DNA fragments accompanied by an accumulation of small DNA fragments. It was concluded that this was diagnostic of calcium-activated endonuclease digestion of the DNA and postulated to be important in development of the toxicity [92,93]. Similar results were obtained using cultured hepatocytes [94]. Importantly, it was found that the calcium ion chelator, ethylene glycol tetraacetic acid (EGTA), blocked the toxic cell death. Since EGTA is ionized and does not enter the cells, these data suggest a role for extracellular calcium in acetaminophen toxicity.

The effect of calcium antagonists on acetaminophen-induced hepatotoxicity have been examined in experimental animals by a number of investigators [93,95,96]. Generally, these drugs inhibited acetaminophen toxicity; however, acetaminophen metabolism leading to covalent binding was not examined.

OXIDATIVE STRESS AND TOXICITY

Oxidative stress is believed to be a general mechanism by which many toxicities occur, and it may be important in acetaminophen toxicity. Oxidative stress, or oxidant stress, is believed to be caused an increase in reactive oxygen species (ROS) and/or reactive nitrogen species (RNS). The major molecular pathways of oxidative stress are presented in Figure 19-2. Each of these pathways has been examined for its relative importance in acetaminophen-induced hepatotoxicity. As shown in Figure 19-2, each mechanism may be initiated by the superoxide anion ($O_2^{\bullet -}$). This ROS may be formed by a number of mechanisms, including metabolism of substrates by CYP2E1 or other enzymes [97], from mitochondria [98,99], or by activation of NADPH oxidase (NOX) [100]. Superoxide is detoxified by superoxide dismutase (SOD), an enzyme that converts two molecules of superoxide to one molecule of hydrogen peroxide and one molecule of oxygen. Hepatocytes contain two SOD enzymes. SOD1 [superoxide dismutase (Cu-Zn)] is in the cytosol and SOD2 [superoxide dismutase (Mn)] is in mitochondria. Hydrogen peroxide may also be produced by various oxidases.

Oxidative stress occurring by pathway A (Fig. 19-2) occurs with formation of RNS. In this pathway, nitric oxide (NO) reacts very rapidly with superoxide (approximately 9×10^9 $M^{-1}sec^{-1}$) to form peroxynitrite [101]. Peroxynitrite catalyzes both the one-electron oxidation of a variety of biological substances and formation of the nitrogen dioxide radical. Peroxynitrite oxidizes tyrosine to the phenoxy free radical, which rearranges to form a carbon-centered radical and reacts with the nitrogen dioxide radical to form 3-nitrotyrosine [102]. 3-Nitrotyrosine is a biomarker of peroxynitrite formation and nitrogen stress [101]. Importantly, in this pathway the presence of excess levels of nitric oxide may lead to dinitrogen trioxide by reaction of the nitrogen dioxide radical with nitric oxide (also a free radical). Dinitrogen trioxide is a nitrosylating agent (i.e., it will add NO to nucleophiles). GSH may be *S*-nitrosylated. *S*-Nitrosylation of cysteine groups on proteins is a posttranslational modification that is believed to be an important signaling mechanism [103,104]. *S*-Nitrosylation may also occur by reaction of nitric oxide with the thiyl radical of cysteine.

Toxicity by pathway C (Fig. 19-2) is believed to be primarily caused by reduction of hydrogen peroxide by transitional metal ions, such as ferrous ions, to produce the hydroxyl radical (the Fenton or Haber-Weiss Reaction). This hydroxyl radical is an extremely reactive, diffusion-limited species. It can oxidize a variety of biological molecules, including lipids, DNA, and proteins. Oxidation of lipids will initiate lipid peroxidation. The resulting ferric ion formed by this reaction can be reduced back to the ferrous ion by superoxide. In addition, the hydroxyl radical will oxidize guanine residues in DNA to 8-hydroxyguanine and protein lysine groups to the corresponding aldehyde. In addition to reduction, hydrogen peroxide can be metabolized by myeloperoxidase (MPO) in neutrophils in the presence of chloride ions to produce hypochlorous acid (HOCl) (pathway B, Fig. 19-2). At neutral pH, sodium hypochlorite (hypochlorous acid; HOCl) chlorinates tyrosine, forming the biomarker 3-chlorotyrosine. Early research aimed at understanding oxidative stress in acetaminophen toxicity assayed for lipid peroxidation (pathway C, Fig. 19-2). By this mechanism, the lipids are oxidized to free radical intermediates, which lead to peroxidation of lipids

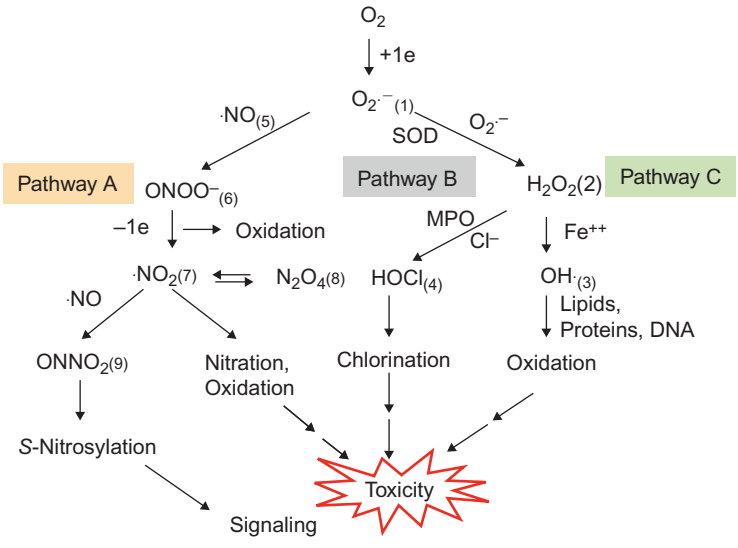

FIGURE 19-2 **Mechanisms of oxidative stress.** The intermediates are superoxide (1), hydrogen peroxide (2), hydroxyl radical (3), hypochlorous acid (4), nitric oxide (5), peroxynitrite (6), nitrogen dioxide radical (7), dinitrogen tetroxide (8), and dinitrogen trioxide (9).

with disruption of membrane integrity and lysis of the plasma membranes [105]. Since GSH, which is depleted by the metabolite NAPQI, is the cofactor for glutathione peroxidase (GPx), detoxification of peroxides is inhibited in acetaminophen-induced toxicity. Wendel and coworkers [106] reported that toxic doses of acetaminophen to mice caused increased exhalation of ethane, a product of lipid peroxidation. Younes et al. [107] did not find that toxic doses of acetaminophen in mice caused lipid peroxidation (ethane exhalation), but coadministration of ferrous sulfate increased ethane exhalation without an increase in toxicity. Subsequently, Gibson et al. [108] examined hepatic protein oxidation (carbonyl formation) following toxic doses of acetaminophen to mice. As with lipid peroxidation, protein aldehyde formation (protein oxidation) is also mediated by a Fenton-type mechanism (pathway C, Fig. 19-2) [109,110]. Increased protein aldehyde formation was not observed following toxic doses of acetaminophen. Thus, the lack of evidence for lipid peroxidation/protein oxidation under conditions of acetaminophen toxicity [107] suggests that the toxicity was not mediated by lipid or protein oxidation per se.

Even though the mechanism of toxicity does not appear to be mediated by lipid or protein oxidation per se, research using iron chelators has suggested that oxidative stress may be a key step leading to acetaminophen toxicity. Iron chelators such as deferoxamine have been reported to decrease the toxicity of acetaminophen in both rat and mouse hepatocytes [111,112]. Moreover, administration of deferoxamine to either rats [113] or mice significantly delayed development of toxicity without altering covalent binding [114]. Administration of ferrous sulfate restored the sensitivity of mice to acetaminophen toxicity [114]. These data are consistent with a Fenton-type mechanism of oxidative stress playing a critical role in the development of hepatotoxicity.

The source of the iron that plays a role in acetaminophen toxicity has recently been investigated by Kon et al. [115]. They reported that in acetaminophen-treated mouse hepatocytes there was an increase in free ferrous ions, which occurred as a result of disruption of lysosomes. Ferrous ion chelators decreased this toxicity. These data suggest that uptake of ferrous ions into the mitochondria and the resulting oxidative stress was a trigger for MPT and cell death [115,116].

In 1998, it was reported that livers from mice treated with a toxic dose of acetaminophen developed 3-nitrotyrosine in the hepatic proteins of the centrilobular regions [117]. Immunohistochemical analysis of 3-nitrotyrosine in proteins and acetaminophen-protein adducts in the livers of acetaminophen-treated mice revealed that the two types of adducts colocalized in the same hepatocytes and that these cells were necrotic. Neither nitrated tyrosine residues in proteins nor acetaminophen-adducted proteins were observed in the periportal regions and were not present in the saline-treated control. In addition, neither adduct was observed in cells that were not necrotic. The presence of nitrated protein in the livers of the treated mice indicates that oxidative stress is occurring, and oxidative stress takes place only in the hepatocytes containing protein adducts that are developing necrotic changes. Importantly, peroxynitrite is detoxified by GSH/GPx [118] and GSH is depleted by NAPQI in acetaminophen toxicity. As discussed above, there is an excellent correlation between the presence of acetaminophen-protein adducts and the development of necrosis [10]. Also, it has been shown that the only hepatocytes undergoing necrosis and containing acetaminophen-protein adducts are those that contain CYP2E1 [119]. Collectively, these data are consistent with a hypothesis that metabolism of acetaminophen, primarily by CYP2E1, results in NAPQI formation, which depletes GSH and covalently binds to proteins. The depletion of GSH is believed to be a critical step, since it results in loss of the ability of the hepatocyte to detoxify peroxide and peroxynitrite, the intermediates that produce oxidative stress.

Mechanisms of producing oxidant stress have been investigated by determining the effect of inhibitors on acetaminophen toxicity and by determining toxicity in genetically altered mice. In 1998, Gardner and coworkers [120] reported that toxic doses of acetaminophen to rats induced iNOS (inducible nitric oxide synthase) in the centrilobular hepatocytes, and that toxicity correlated with the level of iNOS expression. Moreover, treatment of rats with the iNOS inhibitor, aminoguanidine, decreased acetaminophen hepatotoxicity. These data suggested that nitric oxide formed by iNOS may be important in toxicity in rats. Subsequently, acetaminophen hepatotoxicity was examined in iNOS knockout mice. Even though iNOS knockout mice had significantly lower serum ALT levels than wild-type mice, histological evaluation of the acetaminophen-induced hepatic necrosis indicated that there was no significant difference in the amount of hepatic necrosis between wild-type and iNOS knockout mice [63]. Similarly, Bourdi et al. [121] reported that wild-type mice and iNOS knockout mice were equally sensitive to the hepatotoxic effects of acetaminophen. Further, the iNOS inhibitor aminoguanidine did not alter acetaminophen toxicity in mice [122]. These data suggested that iNOS-mediated protein nitration was not an important factor leading to acetaminophen toxicity in mice. However, Gardner reported that the acetaminophen-induced increase in serum AST was higher in wild-type mice than in the iNOS knockout mice [123] and that aminoguanidine decreased serum AST levels in treated mice.

Another possible mechanism that was investigated as a potential source of increased oxidative stress in acetaminophen hepatotoxicity is NADPH oxidase (NOX). NOX is a major enzyme in the respiratory burst that generates superoxide. Activation of Kupffer cells (KCs) or recruited macrophages following covalent binding was envisioned to cause their activation and cell death by the resulting oxidant stress [124–126]. However, in mice deficient in gp91phox (cytochrome *b*-245 heavy chain), the primary subunit of NOX, acetaminophen hepatotoxicity was not significantly different from the hepatotoxicity observed in wild-type mice [127]. Further, the mice had similar levels of nitrated protein. In addition, cotreatment of acetaminophen and the NOX inhibitors diphenylene iodonium chloride or apocynin did not reduce toxicity [128].

The potential role of hypochlorite in acetaminophen has also been investigated. Hypochlorite is produced by neutrophils by a mechanism involving MPO utilization of hydrogen peroxide and chloride ions (pathway B, Fig. 19-2) and reacts with tyrosine residues to form 3-chlorotyrosine. However, chlorotyrosine adducts were not detected in the livers of acetaminophen-treated mice [129], but were readily detected in endotoxemia, a known neutrophil-mediated hepatotoxic injury [130].

CYP2E1 catalytic activity occurs with uncoupling (superoxide formation) [97,131]. This may also be a source of increased oxidative stress in the hepatocyte and important in acetaminophen toxicity. As discussed above, CYP2E1 and CYP1A2 are the major enzymes that metabolize acetaminophen to the reactive metabolite NAPQI [41,132]. Metabolism of acetaminophen to NAPQI may result in increased superoxide, leading to increased levels of hydrogen peroxide and/or peroxynitrite [41,133,134]. In metabolomic studies, acetaminophen-treated wild-type and CYP2E1 knockout mice were examined for urinary metabolites derived from NAPQI (acetaminophen-cysteine conjugate, acetaminophen-NAC conjugate, and acetaminophen-GSH conjugate). In these studies, the levels of urinary NAPQI-derived metabolites at toxic doses of 200 mg/kg and 400 mg/kg to CYP2E1 knockout mice were not significantly different from those in wild-type mice, and maximal hepatic GSH depletion was similar. These data suggest that metabolism of acetaminophen to the reactive metabolite GSH conjugate was the same in wild-type and CYP2E1 null mice; thus, the initial GSH concentrations in both wild-type and knockout mice were the same. However, administration of toxic doses of acetaminophen to wild-type mice resulted in a significant increase in hepatic hydrogen peroxide concentrations compared to the knockout mice. These data suggest that excess levels of superoxide were formed in acetaminophen metabolism by CYP2E1, and these were dismutated to hydrogen peroxide. Thus, it appears that there was increased oxidant stress in the wild-type mice [132].

MITOCHONDRIAL PERMEABILITY TRANSITION

A number of early studies examined the role of mitochondrial dysfunction in acetaminophen toxicity. Jollow et al. reported that mitochondria were a target for the acetaminophen reactive metabolite, NAPQI [17], and a number of arylated proteins were subsequently identified in mitochondria [47,135]. Electron microscopic examination of livers from acetaminophen-treated mice by Racz's laboratory indicated alterations in mitochondrial morphology consisting of enlargement and pallor [136]. Functional alterations in the ability to sequester calcium have also been reported [85]. Inhibition of mitochondrial respiration at complexes I and II, but not at complex III, was reported in the isolated mouse hepatocyte suspension assay [137] and in vivo [138]. In addition, ATP levels in vivo and in treated hepatocytes were decreased. Similar changes have been shown by addition of NAPQI to hepatocytes [137,139,140]. Moldeus, Orrenius, and coworkers reported that the addition of NAPQI to isolated rat liver mitochondria caused release of sequestered calcium [141,142]. Amimoto et al. [143] showed that following toxic doses of acetaminophen to mice there was a dramatic loss of the reduced forms of coenzyme Q (ubihydroquinone or ubiquinol; CoQ_9H_2 and $CoQ_{10}H_2$), intermediates important in the transfer of reducing equivalents between complexes I and II and complex III.

MPT has been reported to be an important mechanism in the development of acetaminophen-induced hepatotoxicity [13,66]. Briefly, MPT is an abrupt increase in the permeability of the inner mitochondrial membrane to ions and both anionic and cationic solutes of masses less than 1.5 kDa. This occurs with inner mitochondrial membrane depolarization, uncoupling of oxidative phosphorylation, release of intramitochondrial ions and metabolic intermediates, mitochondrial swelling, and decreased ATP synthesis [144]. MPT may be induced by increased cytosolic oxidant stress and calcium. A number of oxidants have been reported to lead to MPT including t-butyl hydroperoxide (t-BuOOH) [145] and peroxynitrite [146]. In hepatocytes, t-BuOOH causes oxidation of pyridine nucleotides, mitochondrial depolarization, decreased ATP levels, and loss of hepatocyte viability. t-BuOOH treatment causes a 15-fold increase in mitochondrial ROS formation (oxidation of dichlorodihydrofluorescein) [145,147]. Thus, MPT occurs as a result of

oxidative stress and leads to a large increase in oxidative stress.

MPT occurs as a result of the opening of a voltage- and calcium-dependent high conductance channel or pore called the *permeability transition pore* [148,149]. A major protein in the MPT pore is cyclophilin D, which binds avidly to cyclosporin A; thus, cyclosporin A is an excellent inhibitor of MPT [148,150]. Baines et al. [151] reported that mitochondria from cyclophilin D knockout mice were dramatically less sensitive to oxidant stress and calcium-induced MPT than were those from wild-type mice. Also, primary hepatocytes from these mice were less sensitive to calcium overload and oxidative stress-induced cell death than were hepatocytes from wild-type mice [151].

It has been shown that MPT inhibitors decrease the toxicity of acetaminophen in hepatocytes. Using cultured hepatocytes, Kon and coworkers [13,152] reported that acetaminophen toxicity was inhibited by cyclosporin A and by the nonimmunosuppressive cyclosporin A analog, NM811. Cyclosporin A did not alter the rate of acetaminophen-induced GSH depletion, which suggested that the prevention of toxicity did not occur by inhibition of acetaminophen metabolism to NAPQI. Rather, toxicity occurred with a loss of mitochondrial membrane potential. Reid et al. [66,79] examined the effect of MPT inhibitors in freshly isolated mouse hepatocytes using the approach of Boobis et al. [76,77]. Thus, acetaminophen was incubated with mouse hepatocytes for 2 h to allow GSH depletion and covalent binding to occur. This was followed by washing to remove acetaminophen and subsequent reincubation with medium alone (for 3–5 h). Inclusion of the MPT inhibitors cyclosporin A, trifluoperazine, or dithiothreitol in the reincubation phase completely inhibited toxicity. Also, addition of the acetaminophen antidote, NAC, in the reincubation phase completely inhibited toxicity. Toxicity was shown to occur with MPT and loss of mitochondrial membrane potential; these were prevented by the addition of cyclosporin A and NAC in the reincubation phase. Utilization of a redox-sensitive dye indicated that toxicity occurred along with a large increase in oxidant stress in the reincubation phase. The large increase in oxidant stress was eliminated by the addition of cyclosporin A or NAC to the reincubation phase [66,79].

Similarly, McLean and coworkers examined acetaminophen toxicity in rats pretreated with the CYP inducer, phenobarbital, and in liver slices from these animals. They found that a cocktail consisting of the MPT inhibitors cyclosporin A and trifluoperazine with fructose (a glycolytic substrate) inhibited acetaminophen toxicity [153]. Dimova also reported that the MPT inhibitor trifluoperazine decreased acetaminophen-induced hepatotoxicity in mice [95].

Two recent studies examined acetaminophen toxicity in mice genetically deficient in cyclophilin D. Baines et al. [151] had previously reported that mitochondria from cyclophilin D knockout mice were dramatically less sensitive to MPT compared to those from wild-type mice. Ramachandran et al. [154] reported that the cyclophilin D-deficient mice treated with a low dose of acetaminophen (200 mg/kg) were much less susceptible to acetaminophen hepatotoxicity than were wild-type mice. Oxidant stress was blunted in the genetically altered mice, but not eliminated. Subsequently, LoGuidice and Boelsterli [155] used a large acetaminophen dose (600 mg/kg) and found that mice genetically deficient in cyclophilin D were equally sensitive to the hepatotoxic effects of acetaminophen as were wild-type mice. Protein nitration was also observed in both genetically altered mice and the wild-type mice.

To determine the mechanism of acetaminophen-induced MPT, Burke et al. [79] performed additional experiments using freshly isolated mouse hepatocytes. Immunoassays (e.g., ELISA) indicated that the amounts of protein nitration correlated with the development of toxicity. These data suggested a role for peroxynitrite (pathway A, Fig. 19-2). Importantly, the MPT inhibitor cyclosporin A inhibited nitration and toxicity, as did NAC. Peroxynitrite is known to rapidly react with thiols such as NAC [156]. Moreover, the nNOS (neuronal NOS) inhibitor, 7-nitroindazole, inhibited toxicity and nitration; however, the iNOS inhibitor SAIT [*S*-(2-aminoethyl)isothiourea] had no effect on either toxicity or nitration. In addition, 7-nitroindazole blocked the acetaminophen-induced loss of mitochondrial membrane potential, consistent with blockade of MPT [79]. Importantly, nNOS is believed to be present in mitochondria as mtNOS (mitochondrial NOS) [157,158].

Examination of acetaminophen hepatotoxicity in nNOS knockout mice indicated that increases in serum ALT and AST were significantly delayed compared to in wild-type mice. Thus, increases in serum ALT and AST were significantly higher in wild-type mice at 4 and 6 h after acetaminophen than in the nNOS knockout mice, but by 8 h there was no significant differences. In the wild-type mice, serum ALT and AST correlated with levels of protein nitration. Only low levels of nitrated proteins were observed in acetaminophen-treated nNOS knockout mice. These in vitro and in vivo data suggest that MPT in wild-type mice occurs by a mechanism involving activation of nNOS, formation of peroxynitrite with protein nitration, loss of mitochondrial membrane potential, and toxicity. However, the finding of delayed hepatotoxicity in nNOS knockout mice associated with only low levels of nitrated proteins suggests that mechanisms other than RNS may be involved in the toxicity [159].

It has recently been shown that acetaminophen-induced toxicity occurs with a loss of SOD2 activity, which occurs by nitration of a tyrosine residue on the enzyme [160]. As shown in Figure 19-2, SOD2 shunts superoxide produced in mitochondria toward hydrogen peroxide, which can subsequently diffuse out of the mitochondria and be detoxified by GPx or catalase. Such shunting results in a decreased reaction with nitric oxide, leading to less peroxynitrite formation. Loss of SOD2 activity would be expected to result in an increased steady-state concentration of superoxide in the mitochondria, with increased formation of peroxynitrite. The importance of this enzyme in acetaminophen toxicity has been shown in rats and mice. Yoshikawa et al. reported that knockdown of SOD2 in the rat enhanced hepatotoxicity [161]. Fuhimoto et al. examined acetaminophen in SOD2 heterozygous knockout mice (homozygous knockout is embryonic lethal). They found that acetaminophen-induced hepatic necrosis was significantly greater in the heterozygote than in wild-type mice [162]. Similar toxicity data have been recently reported by Ramachandran et al., but these investigators also found a significant increase in 3-nitrotyrosine in proteins [163].

The only major nitrated protein observed in the hepatic mitochondria of acetaminophen-treated mice was SOD2 [159,160]. The paucity of other nitrated proteins suggests that peroxynitrite may be interacting at other sites in the mitochondria. Schopfer et al. [164] have reported that peroxynitrite will oxidize reduced coenzyme Q. The mechanism is by a one-electron oxidation to form the ubisemiquinone, an intermediate believed to react with molecular oxygen to form superoxide. The resulting superoxide could react with nitric oxide, forming additional peroxynitrite and oxidation of additional coenzyme Q. This mechanism (shown in Fig. 19-3) would explain the finding by Amimoto et al. [143] that following toxic doses of acetaminophen to mice there was a dramatic loss of reduced forms of coenzyme Q (CoQ_9H_2 and $CoQ_{10}H_2$). Oxidation of coenzyme Q by peroxynitrite would disrupt electron flow in the mitochondria, decrease mitochondria membrane potential, and decrease ATP synthesis. All of these events are observed in acetaminophen-induced hepatotoxicity.

ANTIOXIDANT DEFENSE MECHANISMS

Antioxidant defense mechanisms have evolved to protect cells. In the normal cells, these defense mechanisms comprise many enzymes and small molecular weight compounds to detoxify and metabolize oxidants. Some important enzymes include catalase, glutathione synthesis enzymes [e.g., GCLC (glutamate-cysteine ligase catalytic subunit)], GPxs, epoxide hydrolase, GSTs, heme oxygenases (HOs), quinone oxidoreductase, SODs, and transporters. Maintaining the cell in a reducing environment is critical to its survival and defense mechanisms are augmented in surviving cells exposed to increased oxidant stress. NFE2-related factor 2 (NRF2) is a major antioxidant defense mechanism in hepatocytes [165]. Under normal nonstressed, conditions, Nrf2 normally associates with Kelch-like ECH-associated protein 1 (Keap1), resulting in its ubiquitylation and proteasomal degradation. However, under conditions of increased oxidation stress, cysteine groups on Keap1 are oxidized, which disrupts the association with Nrf2 leading to Nrf2 accumulation. Nrf2 then translocates to the nucleus, where it activates the antioxidant response element, resulting in increased synthesis of a large number of antioxidant defense enzymes.

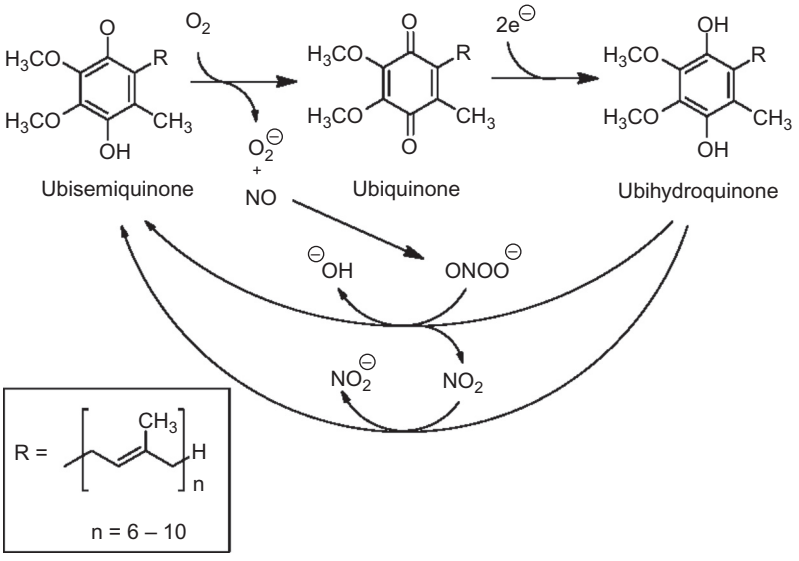

FIGURE 19-3 Peroxynitrite oxidation of ubihydroquinone (coenzyme Q). *Adapted from Schopfer et al. [164].*

The importance of Nrf2 in acetaminophen toxicity was initially reported by two different laboratories [166,167]. Both groups showed that Nrf2 knockout mice had increased susceptibility to acetaminophen hepatotoxicity and lethality compared to wild-type mice. Both groups reported an impaired ability of the Nrf2 knockout mice to replenish their GSH stores and a decreased ability to glucuronidate the parent drug. Subsequently, Park's laboratory reported the appearance of Nrf2 in the nucleus of mice as early as 1 h following acetaminophen treatment. This occurred in a dose-dependent manner, and induction occurred at both toxic and nontoxic doses. Also, they reported significant induction at 1 h of *Ephx1* (encoding epoxide hydrolase), *Hmox1* (encoding H01), and *Gclc* mRNAs [168]. In a subsequent study, they found that addition of NAPQI to mouse liver cells caused a concentration-dependent accumulation of Nrf2 in the nucleus. Data were presented that NAPQI reacted with the cysteine groups of Keap1 to form adducts, which suggested that, in addition to the oxidative stress, acetaminophen-cysteine adducts may be important in Nrf2 induction in acetaminophen toxicity [169]. Further evidence for the importance of the Nrf2 pathway in acetaminophen toxicity was obtained by studying acetaminophen hepatotoxicity in mice pretreated with the triterpenoid, CDDO-Im, and with oleanolic acid to activate the Nrf2 pathway. Acetaminophen toxicity was significantly decreased in mice pretreated with either CDDO-Im [170] or oleanolic acid [171]. Moreover, both pretreatments resulted in nuclear accumulation of Nrf2 and induction of *Hmox1*, *Cryz* (encoding quinone oxidoreductase), and *Gclc* mRNA [170]. In addition to induction of antioxidant enzymes, activation of the Nrf2 pathway results in induction of enzymes such as transporters that also play a role in metabolism [172]. Figure 19-4 shows the mechanism of induction of antioxidant defense enzymes in acetaminophen hepatotoxicity.

Hypoxia-inducible factor 1-alpha (HIF1-α) is another nuclear factor that is elevated during in acetaminophen toxicity [173]. Its importance as a master regulator of many genes, including those involved in angiogenesis, apoptosis, cell proliferation/survival, energy metabolism, and regeneration, is well described [174,175]. In addition to being induced by hypoxia, this gene can be induced by oxidative stress [176–178]. Incubation of acetaminophen with freshly isolated hepatocytes under a stream of 95% oxygen resulted in a time-dependent induction of HIF1-α subsequent to GSH depletion. HIF1-α induction was blocked by the addition of cyclosporin A, a treatment that inhibits MPT and the associated increase in oxidative stress [173]. Subsequently, it was shown in mice that HIF1-α induction occurred 1 h following acetaminophen treatment at doses that did not produce toxicity (15 mg/kg, 30 mg/kg, and 100 mg/kg). Examination of pimonidazole binding to proteins as an assay for hypoxia indicated that hypoxia did not occur until 4 h, a time coincident with the developing necrosis and well after the initial induction of HIF1-α [67]. These data support the hypothesis that early HIF1-α induction was mediated by oxidative stress but indicate that hypoxia may be a late mechanism contributing to HIF1-α induction in acetaminophen hepatotoxicity. The late hypoxia may be a result of the necrosis and disrupted cells, which may physically occlude blood flow. Thus, activation of Nrf2 and induction of HIF1-α are mechanisms that prepare surviving cells for a subsequent defense against oxidative stress, improve their chance of survival, and prepare the liver for regeneration. This mechanism is presented in Figure 19-4.

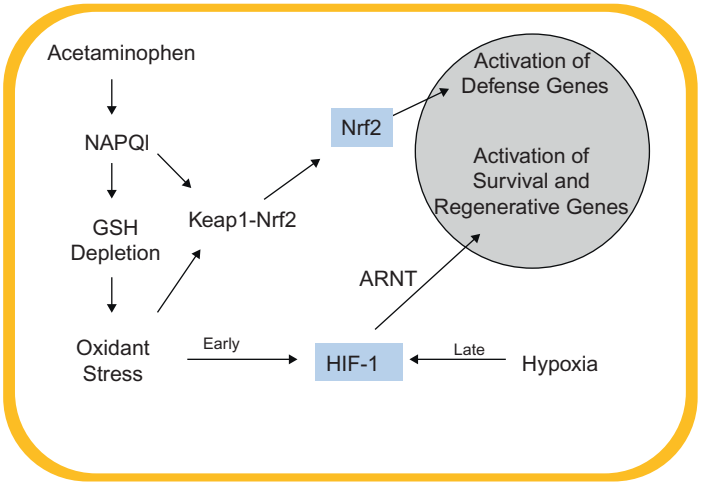

FIGURE 19-4 **Acetaminophen-induced activation of defense and survival genes.** ARNT, aryl hydrocarbon receptor nuclear translocator (HIF1-β); GSH, glutathione; HIF1-α, hypoxia-inducible factor 1-alpha; Keap1, Kelch-like ECH-associated protein 1; NAPQI, *N*-acetyl-*p*-benzoquinone imine; Nrf2, nuclear factor erythroid 2-related factor 2.

CELL SIGNALING AND MITOCHONDRIAL PERMEABILITY TRANSITION

JNK, a member of the mitogen-activated kinases superfamily, may be an important signaling mechanism in acetaminophen-induced liver toxicity. JNK is believed to be important in regulating various cellular functions such as proliferation, survival, and death. It may be activated by a variety of stimuli including cytokines [e.g., tumor necrosis factor (TNF-α) and interleukin-1 (IL-1)] and by exposure of cells to various environmental stresses, such as osmotic stress, oxidative stress, or radiation. There are two primary JNKs in liver, JNK1 and JNK2, and these are activated by phosphorylation [179]. Activation of the JNKs has been implicated in acetaminophen toxicity [180–184] and was recently reviewed by Kaplowitz and coworkers [185].

Following a toxic dose of acetaminophen to mice, there is an early transitory phase of JNK activation and a later sustained phase. The transitory phase may be mediated by TNF-α but does not cause toxicity. Subsequently, there is a sustained or prolonged activation that is believed to be important in toxicity. JNK1 and JNK2 are activated in acetaminophen toxicity in both mice and isolated hepatocytes [68,69,180,181,184,186].

Kaplowitz's laboratory examined the effects of JNK inhibitors, JNK knockout mice, and JNK antisense on acetaminophen toxicity [181,182]. They found that pretreatment of mice with SP600125, which inhibits activation of both JNK1 and JNK2, protected the mice from developing acetaminophen-induced hepatic necrosis in vivo and inhibited acetaminophen toxicity in vitro. This drug had no effect on acetaminophen-induced GSH depletion or protein covalent binding, which indicated that the drug did not inhibit metabolism. Moreover, SP600125 inhibited phosphorylation of the AP1 transcription factor, an indicator of JNK activity. The authors found that acetaminophen hepatotoxicity was unaltered in JNK1 knockout mice but was decreased by approximately 50% in JNK2 knockout mice (double knockout mice are embryo lethal). Knockdown of JNK1 by an antisense oligonucleotide did not alter acetaminophen hepatotoxicity, but antisense-mediated knockdown of JNK2 decreased toxicity by approximately 80%. JNK1 and JNK2 antisense oligonucleotides together protected mice from acetaminophen hepatotoxicity. Cumulatively, these data, plus data obtained from other laboratories, suggest that activation of both JNK1 and JNK2 are necessary for the development of acetaminophen hepatotoxicity, but that the major activity is conferred by JNK2 [181,182,187].

Nakagawa et al. investigated the importance of ASK1 (apoptosis signal-regulating kinase 1) as an upstream activator of JNK in acetaminophen toxicity [188]. ASK1 is a ubiquitously expressed MAP3K (mitogen-activated protein kinase kinase kinase) that is activated by various types of stresses, including calcium influx, endoplasmic reticulum stress, lipopolysaccharide, oxidative stress, and TNF-α. ASK1 is believed to be associated with thioredoxin. Under conditions of oxidative stress, thioredoxin is converted to its oxidized form and ASK1 is released to be self-activated [188]. Although the physiological relevance of ASK1 in the control of JNK activation is unclear, genetic deletion studies have suggested that different stimuli may act through ASK1 to activate JNK signaling [188]. ASK1 knockout mice were found to be significantly less sensitive to acetaminophen hepatotoxicity than were wild-type mice. Moreover, phosphorylation of JNK1 and JNK2 was dramatically decreased in ASK1 knockout mice. In agreement with a previous report by Gunawan et al. [181], this study found that at 24 h acetaminophen toxicity was decreased in JNK2 knockout mice, but not in JNK1 knockout mice, and that toxicity was almost completely abrogated by the JNK inhibitor SP600125.

In addition to a role for ASK1 in JNK activation, Shinohara et al. reported that glycogen synthase kinase-3 beta (GSK-3β) may play a role in JNK activation in acetaminophen toxicity [189]. Silencing GSK-3β protected mice from acetaminophen-induced increases in serum ALT levels and hepatic necrosis, and JNK activation was delayed. However, silencing GSK-3α or AKT2 had no effect on acetaminophen toxicity.

Translocation of activated JNK and GSK-3β to hepatocyte mitochondria may be a critical event in acetaminophen hepatotoxicity, as well as in many other hepatotoxicities [116]. JNK translocation promotes the release of a variety of mitochondrial proteins. A time course for JNK translocation to mitochondria indicated it corresponded to a decrease in mitochondrial respiration [182]: mitochondrial GSH depletion occurred by 1–2 h, JNK activation in liver homogenate by 2–4 h, JNK translocation to mitochondria by 4 h, and increased toxicity (serum ALT by 6 h). SP600125 did not alter GSH depletion (NAPQI formation) but blocked JNK activation and translocation to mitochondria, as well as subsequent toxicity. Mitochondria from livers of acetaminophen-treated mice showed decreased State 3 respiration and decreased respiratory control ratios, whereas mice treated with acetaminophen plus JNK inhibitor were partially protected from these losses. Moreover, addition of activated JNK to isolated mitochondria from acetaminophen-treated mice (after GSH depletion and covalent binding but before

JNK translocation) plus JNK inhibitor led to a loss of mitochondrial respiration. Cyclosporin A blocked this decrease, which suggests that JNK translocation is a critical mediator of acetaminophen-induced MPT [182].

Win et al. [190] examined the role of Sh3bp-5 (SH3 domain-binding protein 5) as a target of activated JNK in the mitochondria. Sh3bp-5 is a scaffold protein containing a kinase interaction motif that is important in the binding of JNK. These investigators found Sh3bp-5 was expressed in the mitochondrial outer membrane of mouse liver. In acetaminophen-treated mice, Sh3bp-5 colocalized with activated JNK in hepatic mitochondria. Immunoprecipitation of activated JNK from acetaminophen-treated hepatocytes resulted in coprecipitation of Sh3bp-5, and immunoprecipitation of Sh3bp-5 resulted in coprecipitation of activated JNK. Sh3bp-5 silencing in mouse livers did not alter acetaminophen-induced GSH depletion or covalent binding (NAPQI formation), but it dramatically decreased hepatotoxicity (decreased serum ALT levels and histopathological evidence of liver necrosis). Not only was there decreased necrosis in the livers of the acetaminophen-treated mice with silenced Sh3bp-5 but there was also less nitration of hepatocytes in the centrilobular areas. Moreover, mitochondria from acetaminophen-treated Sh3bp-5-silenced mouse livers had no loss of the respiratory control ratio (State 3/State 4), but there was a dramatic decrease in the respiratory control ratios of mitochondria from the livers of the control mice treated with acetaminophen [190]. Thus, JNK translocation to mitochondria may be an important mechanism leading to MPT with alterations of mitochondrial respiration and increased oxidant stress (by ROS and RNS).

The apoptosis regulator BAX is another cytosolic protein reported to translocate to hepatic mitochondria following a toxic dose of acetaminophen [191,192]. BAX is a member of the BCL-2 family, which comprises both pro- and antiapoptotic proteins: BAX is a proapoptotic protein that is activated by various cellular stresses, whereas BCL-2 is an antiapoptotic protein [193,194]. The balance between these is an important factor in initiation of events leading to cell lethality. BAX activation results in its translocation to mitochondria, where it inserts into the outer membrane and forms oligomeric pores or channels [195,196]. These channels release apoptotic proteins, including apoptosis-inducing factor (AIF), cytochrome *c*, endo G (endonuclease G), and Smac (Diablo homolog). These proteins are normally in the intermembrane space in inactive forms, but when released they can translocate to the nucleus and/or initiate protease cascades leading to cell death by apoptosis or necrosis [197].

Although activation of BAX is frequently associated with apoptosis, histopathological evidence indicates that acetaminophen does not cause apoptosis in vivo [11,15,16]. However, BAX activation appears to play an important role in acetaminophen-induced necrosis. Bajt et al. [198] reported that two important hepatic mitochondrial intermembrane proteins, AIF (allograft inflammatory factor; a protein that causes chromatin condensation) and endo G, translocated to the nucleus in cultured mouse hepatocytes treated with acetaminophen. This resulted in the previously observed nuclear fragmentation, assessed by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), a phenomenon interpreted by a number of investigators as evidence of apoptosis. Moreover, BAX knockout mice had delayed toxicity and nuclear fragmentation compared to wild-type mice, but by 12 h there were no differences in toxicity and nuclear fragmentation [199]. In addition, protein nitration was not delayed. Thus, BAX is important for the translocation of AIF and endo G to the nucleus, but in its absence other mechanisms result in AIF and endo G translocation. The importance of endonucleases in acetaminophen toxicity was clearly shown by Napirei et al. [200]. These investigators found that deoxyribonuclease I (DNase I) knockout mice were much less sensitive to acetaminophen hepatotoxicity than were wild-type mice, and there was significantly less nuclear fragmentation in the livers of the acetaminophen-treated mice. Subsequently, Bajt et al. [201] found that Harlequin mice deficient in AIF were much less sensitive to hepatic necrosis and had much less nuclear fragmentation, as determined by the TUNEL assay. These data indicate a role for endonucleases (endo G and DNase I) and AIF, a DNA-binding protein that promotes endo G activity, in acetaminophen hepatotoxicity.

The importance of necrosis versus apoptosis in acetaminophen-induced hepatotoxicity may be related to a rapid onset of MPT and thus a dramatic decrease in the ability of the hepatocyte to produce ATP. The relative amount of ATP appears to be an important factor for determining whether hepatocytes die by necrosis or apoptosis: low ATP levels are associated with necrosis, whereas adequate ATP levels favor apoptosis [202]. With acetaminophen, Kon and coworkers [13] showed that cultured mouse hepatocytes died primarily by necrosis. However, the inclusion of fructose, an ATP-generating glycolytic substrate that does not depend on mitochondrial activity, and glycine, a membrane stabilizer, in the media decreased necrosis and promoted apoptosis. Figure 19-5 presents a postulated mechanism of how cell signaling and MPT may cause acetaminophen toxicity.

HEPATIC INFLAMMATION

Acetaminophen-induced hepatic necrosis occurs with an inflammatory response (sterile inflammation)

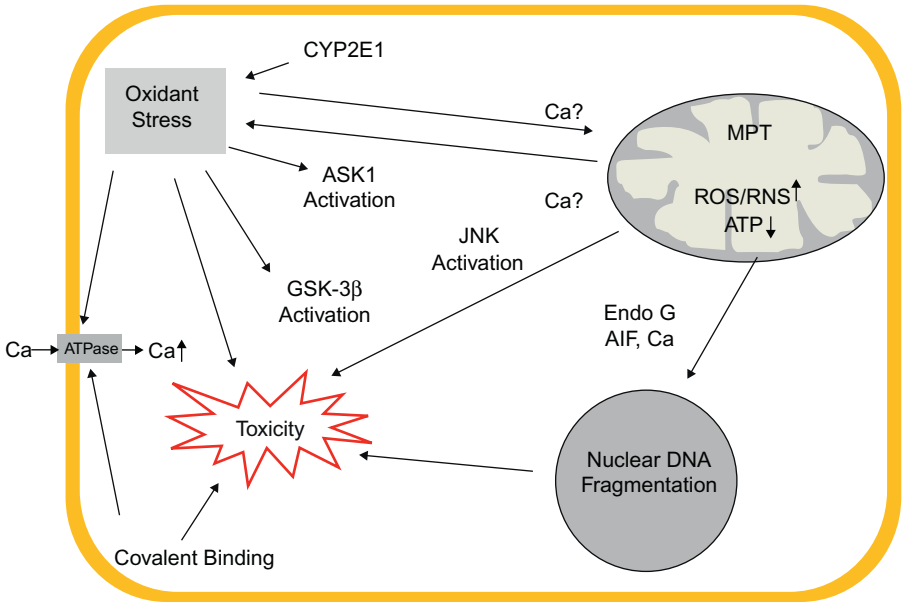

FIGURE 19-5 **Mechanisms important in phase ii of acetaminophen toxicity.** A critical factor contributing to the increased oxidative stress is the depletion of glutathione by NAPQI (shown in Fig. 19-1). AIF, apoptosis-inducing factor; ASK1, apoptosis signal-regulating kinase 1, or MAP3K5; Ca, calcium; CYP2E1, cytochrome P450 2E1; Endo G, endonuclease G; GSK-3β, glycogen synthase kinase-3 beta; JNKs, c-Jun *N*-terminal kinases; MPT, mitochondrial permeability transition; ROS, reactive oxygen species; RNS, reactive nitrogen species.

[203,204]. The importance of cytokines in immunity, inflammation, cell proliferation, differentiation, and cell death is well described [203]. In acetaminophen toxicity, the role of inflammation was initially investigated by Laskin and coworkers [125]. They reported that acetaminophen toxicity was associated with activation of KCs (which are hepatic macrophages). TNF-α increases oxidative stress and recruits and activates other inflammatory cells [205,206]. Blazka and coworkers reported a dramatic increase in serum TNF-α and IL-1α levels in mice following toxic doses of acetaminophen [207]. Treatment of mice with either anti-TNF-α or anti-IL-1α antibodies partially prevented acetaminophen hepatotoxicity [208]. Moreover, in multiple laboratories the KC inactivators, gadolinium chloride and dextran sulfate, were reported to decrease acetaminophen toxicity in the rat [124] and the mouse [126,209,210]. These data suggested that KC-mediated proinflammatory responses play an important role in acetaminophen toxicity. However, subsequent research indicated that KC activation was not mechanistically important in acetaminophen toxicity. Ju and coworkers [211,212] treated mice with liposome-encapsulated clodronic acid [(dichloromethylene)bisphosphonic acid]. This treatment was more efficient at eliminating KCs from the liver than the previously utilized gadolinium chloride or dextran sulfate. Treatment of these mice with acetaminophen resulted in reduced levels of *Clq* (encoding a complement C1q subcomponent subunit), *Il6*, *Il10*, *Il18bp* (encoding interleukin-18-binding protein), *Pgts2* (encoding prostaglandin G/H synthase 2), and *Tnf* mRNAs and resulted in an increase in liver toxicity. These findings suggest alternative roles for KCs in the toxicity. Moreover, the increase in toxicity suggests that KCs may have a beneficial role in the toxicity, such as recruitment of circulating macrophages leading to increased liver repair [213].

The importance of TNF-α in the development of acetaminophen toxicity has been investigated in genetically altered mice. Wild-type and TNF-α knockout mice had equal sensitivity to acetaminophen [214], treatment with anti-TNF-α antibody or soluble TNF receptor did not alter acetaminophen toxicity in mice [215], and there was no difference in toxicity between TNFR-1 knockout mice and wild-type mice [216]. Thus, the role that TNF-α may have in acetaminophen toxicity remains unclear.

Other proinflammatory cytokines such as IL-1β and interferon gamma (IFN-γ) are also induced in acetaminophen toxicity [205,207,217]. Ishida et al. [218] and Liu et al. [219] reported that *Ifng* mRNA was induced in livers of acetaminophen-treated mice; however, IFN-γ knockout mice were shown to have reduced acetaminophen toxicity and lethality compared to wild-type mice [218].

IL-6 is another cytokine that is induced during acetaminophen toxicity. Masubuchi et al. reported that mice lacking IL-6 had increased susceptibility to acetaminophen toxicity [220]. Moreover IL-6 knockout mice had reduced expression of heat shock proteins (HSPs) 25, 32, and 40, as well as of inducible HSP70. These results suggest that IL-6 may protect the liver from injury, at least in part by upregulating the hepatic expression of several cytoprotective HSPs.

The role of antiinflammatory cytokines in acetaminophen hepatotoxicity has been examined. IL-10, IL-11, and IL-13 are antiinflammatory cytokines that are known to modulate the proinflammatory response in

hepatic injuries [121,221–224]. The liver is a major source of IL-10, which is produced by activated KCs. Bourdi et al. reported that serum levels of IL-10 and IL-13 were increased during acetaminophen toxicity [121]. The authors found that IL-10 knockout mice had increased toxicity to acetaminophen compared to wild-type mice. Moreover, IL-10 knockout mice had increased mRNA transcripts for the proinflammatory cytokines TNF-α and IL-1 and for iNOS. The antiinflammatory cytokine IL-11 is known to be protective in a number of organ toxicities [225–227] and may decrease proinflammatory cytokine production or decrease macrophage activation. Trepicchio et al. [226] found that pretreatment of mice with recombinant human IL-11 lowered serum ALT and TNF-α levels by approximately half compared to non-IL-11-treated mice. In addition, histopathological measures of hepatotoxicity were improved. IL-13 has been examined for its hepatoprotective role in acetaminophen toxicity. Pretreatment of mice with IL-13 antibody exacerbated acetaminophen liver injury. Additionally, IL-13 knockout mice were more sensitive to the toxic effects of acetaminophen than were the corresponding wild-type mice [224].

The roles that chemokines may have in acetaminophen hepatotoxicity have also been examined in acetaminophen-induced toxicity. Low molecular weight cytokines were initially recognized for their role in the chemotaxis of lymphocytes. In addition, some classes of chemokines may have angiogenic, antiinflammatory, cell proliferative, or wound healing properties. Multiple laboratories have shown that chemokines are upregulated in acetaminophen toxicity [228–232].

The prototype chemokine, CXCL2/MIP-2 (C-X-C motif chemokine 2), is produced by many cell types in response to the proinflammatory cytokines, TNF-α and IL-1β. It is a neutrophil chemoattractant and activator [203]. Jaeschke's laboratory showed that chemokine upregulation (CXCL2 and KC) and neutrophil accumulation occurred after development of acetaminophen toxicity in mice [233]. Hepatocytes exposed to acetaminophen developed toxicity in the absence of neutrophils [66,234]. Mouse strains with differing degrees of neutrophil accumulations had similar toxicities to acetaminophen, suggesting that neutrophils are not mechanistically important in the development of acetaminophen toxicity [128]; however, this remains controversial [235]. While it has been postulated that the primary role of neutrophil influx in acetaminophen toxicity is the removal of damaged cells and cellular debris [233], a study using the anti-Gr-1 antibody (RB6–8C5), which specifically detects neutrophils, showed that toxicity was significantly attenuated by neutrophil depletion in acetaminophen-treated mice [181]. Evidence suggests a role for the CXCL2 chemokine as a hepatoprotective factor in acetaminophen toxicity. Hogaboam's laboratory [228] reported that treatment with CXCL2 was more effective than the antidote NAC when given as a *late therapy* to acetaminophen-treated mice. In vitro data showed that CXCL2 maintained hepatocyte proliferation in cells exposed to acetaminophen. In addition, adenoviral vector delivery of CXCL2 reduced toxicity in acetaminophen-treated mice [228,236].

The mechanism of the protective effects of the CXC chemokines in acetaminophen toxicity is poorly understood. Hogaboam and coworkers have suggested that CXCL2 may cause increased nuclear localization of the transcription factor signal transducer and activator of transcription 3 (STAT3), which is important in hepatocyte regeneration [228,237,238]. Another CXC chemokine, IP-10 (C-X-C motif chemokine 10), does not have a direct mitotic effect in vitro, but can induce hepatocyte growth factor (HGF), a known mitogen [239]. Bone-Larson et al. [230] found that IP-10 was protective in acetaminophen toxicity and that protection was associated with induction of the CXCL2 receptor CXCR2 on hepatocytes. These data suggest that that CXCL2 and IP-10 are important in cell proliferation (i.e., hepatocyte regeneration). One study suggested that chemokines may also dampen proinflammatory cytokine production in acetaminophen toxicity. Mice deficient in the primary receptor for the chemokine MCP-1 (C-C motif chemokine 2) were found to have increased levels of TNF-α and IFN-γ. Neutralization of these proinflammatory cytokines resulted in attenuation of acetaminophen toxicity [229].

Prostaglandin G/H synthase 2 (COX-2) may play a role in acetaminophen toxicity [240]. Whereas COX-1 is constitutively expressed and produces low levels of prostaglandins, COX-2 is inducible and plays a role in antiinflammatory processes. While COX-2-derived prostaglandin metabolites play important roles in bone resorption, female reproduction, mucosal defense, and renal function, they have also been reported to be protective in various hepatotoxicities [241]. Reilly and coworkers showed that COX-2, but not COX-1, was induced in livers of acetaminophen-treated mice. Moreover, acetaminophen was more toxic with increased lethality in COX-2 knockout mice compared to wild-type mice. Also, the COX-2 inhibitor celecoxib (Celebrex) increased acetaminophen toxicity [240]. Thus, COX-2 may have an antiinflammatory role in acetaminophen-induced hepatotoxicity.

High mobility group B1 protein (HMGB1) may be an important activator of inflammatory cells in acetaminophen hepatotoxicity. HMGB1 is an intracellular DNA-binding protein important in chromatin remodeling, which promotes protein assembly on DNA targets. It is passively released from necrotic cells, but actively

released from inflammatory cells. It binds with high affinity to RAGE (advanced glycosylation end product-specific receptor) and other receptors, resulting in proinflammatory gene activation [242]. A number of reports indicate that HMGB1 is released in acetaminophen-induced hepatotoxicity [242–245]. Dragomir et al. recently reported that HMGB1 is released from acetaminophen-treated hepatocytes. When culture medium from treated hepatocytes was used to treat macrophages, the induction of *CAT*/CAT (catalase) and *HMOX1*/HO-1 mRNAs and proteins was observed; induction was blocked by ethyl pyruvate, an antiinflammatory agent that inhibits release of HMGB1 from cells [246]. A large increase in RAGE was also observed. Thus, HMGB1 appears to be a critical factor in the activation of inflammatory cells in acetaminophen toxicity.

GENOMICS, PROTEOMICS, AND METABOLOMICS

Genomics, metabolomics, and proteomics are innovative approaches to understanding toxicological problems. Whereas classical methods have yielded a great deal of information, these new approaches may add a new dimension to our understanding of toxicology. Defining how cells respond to insults by altering mRNA, proteins, and critical metabolic processes in acetaminophen-induced hepatotoxicity is expected to yield new clues to help our understanding of the toxicology. Genomic and proteomic analyses have been examined in mice treated with toxic doses of acetaminophen and a large number of changes have been documented [247–249]. Welch et al. examined the ratio of hepatic mRNAs [248] and proteins [249] that were induced in SJL mice sensitive to the toxic effects of acetaminophen (300 mg/kg) versus C57Bl/6 mice that are relatively insensitive to acetaminophen. mRNA and protein expression was determined after acetaminophen administration. A large number of proteins (1,632) were identified, of which 247 were expressed at significantly different levels in the two strains of mice, and with 161 of these proteins being more abundant in the more sensitive SJL mice. Table 19-2 lists the major mRNAs that are induced (>threefold increase) after 3 h and the major proteins induced (>threefold increase) after 6 h: there was not an excellent correlation between patterns of mRNA and protein induction. Metabolomic studies have also yielded some interesting data. Chen et al. reported that following toxic doses of acetaminophen there were large increases in serum levels of palmitoylcarnitine, triglycerides, and free fatty acids [250]. Thus, an alteration in fatty acid oxidation occurs in acetaminophen hepatotoxicity and acylcarnitines may be biomarkers of this effect.

TABLE 19-2 Genomic and Proteomic Alterations Reported in Livers of Acetaminophen-Sensitive Mice versus Nonsensitive Mice

Genomic Alterations (3 h) [248]
Ratios of Major mRNAs Induced in Acetaminophen-Sensitive Mice over Nonsensitive Mice

Protein Encoded by mRNA	Ratio
Plasminogen precursor	13.4
38 kDa FK-506 binding protein homolog	11.3
BAG-family molecular chaperone regulator-3	6.4
High-density lipoprotein-binding protein	6.1
RIKEN cDNA B430201G11	5.7
NADPH-cytochrome P450 reductase	4.7
Putative serine-rich protein	4.5
Valyl-tRNA synthetase 2	3.7
Iron responsive element-binding protein	3.6
Δ-Aminolevulinic acid dehydratase	3.2
Calcium/calmodulin-dependent protein kinase type II γ-chain	3.0
SON protein	3.0

Proteomic Alterations (6 h) [249]
Ratio of Some Major Proteins Induced in Acetaminophen-Sensitive Mice versus Nonsensitive Mice

Protein Name	Ratio
Ubiquitin-like 2 activating enzyme E1B	10.0
Complement C5 precursor	7.1
Prostaglandin G/H synthase 1 (COX-1) precursor	5.5
Peroxiredoxin 1	5.1
Hsp70-binding protein	4.3
GST μ-2	3.2
Senescence marker protein-30 or regucalcin	3.1

These data are the major mRNAs and proteins induced after a toxic dose of acetaminophen at the indicated times. The data are the ratio of induction in a sensitive strain of mice (SJL) to induction in a nonsensitive strain of mice (C57BL/6) following 300 mg/kg dose of acetaminophen. GST, glutathione *S*-transferase.

CONCLUSIONS

Even though acetaminophen-induced hepatotoxicity has been an active area of research since the 1970s, there are still many unanswered questions about how it produces liver toxicity. The metabolic aspects of acetaminophen toxicity are well defined (Fig. 19-1): the reactive metabolite NAPQI is formed by CYP enzymes. The specific CYP enzymes important in acetaminophen conversion to its reactive metabolite NAPQI have been isolated and clearly shown to catalyze the reaction. NAPQI reacts with GSH, leading to its depletion in the

liver. NAPQI also covalently binds to protein and specific protein adducts have been identified, although the role of covalent binding in toxicity is unclear.

Acetaminophen toxicity occurs in two phases: a metabolic phase is followed by a toxicity phase. The toxicity phase is characterized by increased oxygen/nitrogen stress and MPT, which occurs with a loss of ability of the hepatocyte to produce ATP; however, mechanisms occurring in the toxicity phase are poorly understood. The cause of MPT is yet to be determined. In general, MPT is known to be caused by increased oxidative stress or calcium and leads to a large increase in oxidative stress. In acetaminophen studies, there are credible data to support both oxidative stress and calcium initiation of MPT. Iron chelators, which inhibit Fenton-mediated oxidant stress, have been shown to delay the toxicity in both acetaminophen-treated hepatocytes and mice. Also, in acetaminophen-treated hepatocytes there is an early increase in free ferrous ions that occurs with disruption of lysosomes. These data suggest that iron-catalyzed oxidative stress may occur early in acetaminophen toxicity and that this may be a mechanism leading to MPT. Likewise, there are credible data supporting a role for calcium in the initiation of MPT. Cellular calcium concentrations have been shown to increase in acetaminophen-treated hepatocytes, and multiple investigators have shown that calcium chelators can block acetaminophen toxicity in hepatocytes. Plasma membrane calcium ATPase has been reported to decrease in acetaminophen toxicity. One possible mechanism by which calcium could initiate MPT is activation of nNOS. nNOS is known to be activated by calcium via a calmodulin binding site, and this site can be inhibited by the MPT inhibitor, trifluoperazine, which also inhibits acetaminophen toxicity. Moreover, activation of nNOS leading to increased peroxynitrite may be mechanistically important in MPT and increased oxidant stress. Lastly, another possible mechanism for MPT activation may be through activation of JNK. JNK is activated in acetaminophen toxicity and translocates to the mitochondria. It is known to activate a number of genes, including those important in cell death, and its activation may be important in acetaminophen-induced MPT and toxicity. Downstream of MPT, endo G and AIF are released from the mitochondria and nuclear DNA fragmentation occurs. Endo G knockout mice and DNase I knockout mice have less acetaminophen toxicity than wild-type mice. Thus, it appears that in acetaminophen hepatotoxicity there are multiple pathways leading to hepatocyte death. Mechanisms important in acetaminophen toxicity are presented in Figure 19-5.

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