Liver Oxidant Stress Induced By Paracetamol Overdose

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Citation

Abstract
Acetaminophen (APAP, AAP) overdose can cause severe hepatotoxicity and even liver failure and hepatic centrilobular necrosis in experimental animals and humans. Despite substantial efforts over the last 30 years, the mechanism of APAP-induced liver cell injury is still not completely understood. In the initial phases of toxicity acetaminophen is metabolically activated by cytochrome P450 enzymes to a reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI) that depletes glutathione (GSH) and covalently bound to cellular proteins including a number of mitochondrial proteins. N-acetylcysteine has been currently used as antidote as it was shown that repletion of GSH prevented the toxicity. Although covalent binding has been shown to be an excellent correlate of toxicity, a number of other events have been shown to occur and are likely important in the initiation and repair of toxicity. Recent studies have demonstrated that nitrotyrosine residues, induced by rapid reaction of superoxide and nitric oxide (NO), occur in the necrotic cells following toxic doses of acetaminophen. Also, NO synthesis is dramatically increased following acetaminophen overdose earning a role in controlling lipid peroxidation. In addition, the significance of cytokines and chemokines (IL-1α, IL-10, macrophage inhibitory protein-2, monocyte chemotacttractant protein-1) in the development of toxicity and repair processes has been demonstrated by recent studies. This review reconciles many of the controversial findings of the past and provides a viable hypothesis for the mechanism of hepatocellular injury after APAP overdose.

INTRODUCTION
Overdoses of the analgesic and antipyretic acetaminophen represent one of the most common pharmaceutical product poisonings in the United States today (1). Although considered safe at therapeutic doses, in overdose, acetaminophen produces a centrilobular hepatic necrosis that can be fatal (2). Whereas the initial biochemical and metabolic events that occur in the early stages of toxicity have been well described, the precise mechanisms of hepatocyte death are poorly understood. Necrosis is recognized as the mode of cell death and apoptosis has been ruled out (3, 4).

Several recent excellent reviews on acetaminophen toxicity have been recently published (3, 4). The following manuscript will review the role of covalent binding in toxicity as well as other factors recently identified that contribute to the toxicity. These factors include oxidative stress, nitrotyrosine formation, inflammatory cytokines, and the possible importance of mitochondrial permeability transition.

METABOLIC ACTIVATION OF ACETAMINOPHEN
The metabolism in acetaminophen toxicity is very important. Acetaminophen is metabolically activated by cytochrome P450 to form a reactive metabolite that covalently binds to protein (5). The reactive metabolite was found to be N-acetyl-p-benzoquinone imine (NAPQI), which is formed by a direct two-electron oxidation (6). More recently, the cytochromes 2E1, 1A2, 3A4, and 2A6 (7,8,9) have been reported to oxidize acetaminophen to the reactive metabolite. NAPQI is detoxified by glutathione (GSH) to form an acetaminophen-GSH conjugate. After a toxic dose of acetaminophen, total hepatic GSH is depleted by as much as 90%, and as a result, the metabolite covalently binds to cysteine groups on protein, forming acetaminophen-protein adducts (5).

BIOCHEMICAL MECHANISMS OF TOXICITY
Events that produce hepatocellular death following the formation of acetaminophen protein adducts are poorly understood. One possible mechanism of cell death is that covalent binding to critical cellular proteins results in subsequent loss of activity or function and eventual cell death and lysis. Primary cellular targets have been postulated to be mitochondrial proteins, with resulting loss of energy production, as well as proteins involved in cellular ion...
control (12). Tirmenstein and Nelson (13) and Tsokos-Kuhn et al. (14) reported alterations of plasma membrane ATPase activity following toxic doses of acetaminophen.

A number of proteins bound to acetaminophen have been isolated and identified. A review of the individual proteins that were isolated and identified by individual analysis has been published (15–16). Subsequently, Qui et al. (17) used matrix-assisted laser desorption ionization mass spectrometry to identify 20 additional proteins containing covalently bound acetaminophen.

Although it is plausible that partial inhibition of a large number of enzymes may contribute to cell death, questions are generated relative to the validity of the hypothesis that covalent binding to critical proteins is the only mechanism of acetaminophen toxicity.

Loss of mitochondrial or nuclear ion balance has also been suggested to be a toxic mechanism involved in acetaminophen-mediated cell death since either of these losses can lead to increases in cytosolic Ca2+ concentrations, mitochondrial Ca2+ cycling, activation of proteases and endonucleases, and DNA strand breaks (12, 18–19). The effect of the addition of NAPQI on isolated mitochondria has been reported (20) and inhibition of mitochondrial respiration has been investigated as an important mechanism in acetaminophen toxicity (20).

EARLY RESEARCH ON OXIDATIVE STRESS

Oxidative stress is another mechanism that has been postulated to be important in the development of acetaminophen toxicity. Thus, increased formation of superoxide would lead to hydrogen peroxide and peroxidation reactions by Fenton-type mechanisms. It has been shown that NAPQI reacts very rapidly with GSH (k1 = 3.2 x 104 M−1 s−1 at pH 7.0) (22), and there are a number of potential mechanisms that have been suggested to play a role. Under conditions of NAPQI formation following toxic acetaminophen doses, GSH concentrations may be very low in the centrilobular cells, and the major peroxide detoxification enzyme, GSH peroxidase, which functions very inefficiently under conditions of GSH depletion (23), is expected to be inhibited. In addition, during formation of NAPQI by cytochrome P450, the superoxide anion is formed, with dismutation leading to hydrogen peroxide formation (24). Also, others have suggested that peroxidation of acetaminophen to the semiquinone free radical would lead to redox cycling between the acetaminophen and the semiquinone. This mechanism may lead to increased superoxide and toxicity (25).

A significant amount of evidence has pointed to the potential involvement of oxidative stress in acetaminophen toxicity. Nakae et al. (26) reported that administration of encapsulated superoxide dismutase decreased the toxicity of acetaminophen in the rat. Moreover, the iron chelator, deferoxamine, has been shown to decrease toxicity in rats (27). Schnellmann et al. showed that deferoxamine caused a delay in the rate of development of acetaminophen toxicity in mice, but after 24 h, the relative amount of toxicity was not affected (28). These data suggest that an iron-catalyzed Haber-Weiss reaction may also play a role in the development of oxidant stress and injury.

ROLE OF KUPFFER CELLS

Several laboratories have studied the role of macrophage activation (29) in acetaminophen toxicity. Kupffer cells are the phagocytic macrophages of the liver. When activated, Kupffer cells release numerous signaling molecules, including hydrolytic enzymes, eicosanoids, nitric oxide, and superoxide. Kupffer cells may also release a number of inflammatory cytokines, including IL-1, IL-6, and TNF-α (30), and multiple cytokines are released in acetaminophen toxicity (31–33). Laskin et al. (34) examined the role of Kupffer cells in acetaminophen hepatotoxicity by pretreating rats with compounds that suppress Kupffer cell function (gadolinium chloride and dextran sulfate). These investigators reported that rats pretreated with these compounds were less sensitive to the toxic effects of acetaminophen. Similar effects were reported in the mouse (35). Goldin et al. (36) showed that treatment of mice with liposomes containing dichloromethylene disphosphonate to knock out the Kupffer cells also decreased acetaminophen toxicity. Similarly, Hinson et al. reported that pretreatment of mice with gadolinium chloride or dextran sulfate decreased the toxic effects of acetaminophen (37). These studies suggested a critical role for Kupffer cells in the development of acetaminophen hepatotoxicity; however, recent work by Ju et al. (38) came to a different conclusion. They pretreated mice with gadolinium chloride and found that the number of Kupffer cells in the liver was only partially decreased. Consistent with previous reports, they showed decreased acetaminophen toxicity in the pretreated mice. However, treatment of mice with dichloromethylene diphosphonate completely depleted the liver of Kupffer cells, but toxicity was increased. These data raise questions relative to the importance of Kupffer cells in acetaminophen toxicity.
NITROTYROSINE AND ACETAMINOPHEN TOXICITY

Hinson et al recently showed that nitrotyrosine occurs in the centrilobular cells of the liver of acetaminophen-treated mice \((a)\). Immunohistochemical analysis of liver of acetaminophen-treated mice indicated that nitration occurred in the same cells that contained acetaminophen adducts and developed necrosis \((a)\). Nitrotyrosine was formed by a reaction of peroxynitrite with tyrosine. Nitration of tyrosine has been shown to be an excellent biomarker of peroxynitrite formation \((a)\). It is formed by a rapid reaction between nitric oxide and superoxide, and Mayeux et al. found that NO synthesis (serum levels of nitrate plus nitrite) was increased in acetaminophen toxicity \((a)\). Also, others reported the induction of hepatic iNOS in the acetaminophen-treated rat \((a)\). NO and superoxide react to produce peroxynitrite \((ONOO^-)\) at a rate near the diffusion-controlled limit \((7 \times 10^9 \text{ M}^{-1} \text{s}^{-1})\). Peroxynitrite is a species that not only leads to the nitration of tyrosine but is also a potent oxidant that can attack a wide range of biological targets \((a)\), and under conditions of reduced cellular oxidant scavenging capability, peroxynitrite is highly toxic \((a)\). The anion is relatively stable, but the acid rearranges to nitrate with a half-life of 1.9 s at pH 7.4; the pK is 7.5 \((a)\). Oxidation reactions can involve one or two-electron processes. Oxidation of lipids \((a)\), proteins, or DNA bases \((a)\) has been reported. Thus, peroxynitrite is an excellent candidate for a toxic species. Moreover, it is normally detoxified by GSH/GSH peroxidase, and GSH is depleted in acetaminophen toxicity \((a)\). GSH peroxidase is a key enzyme in this defense mechanism \((a)\). Thus, a normal detoxification mechanism for peroxynitrite is impaired. Also, even though acetaminophen itself will detoxify peroxynitrite \((a)\), the drug is metabolized rapidly in the mouse and concentrations are low at the time when nitration is observed. However, mechanisms other than peroxynitrite have been recently reported to lead to nitrotyrosine. Thus, myeloperoxidase has been shown to oxidize the nitrite ion to the NO2 radical, and this is a nitrating species \((a)\). Since myeloperoxidase is present in neutrophils and neutrophil recruitment is a late event in acetaminophen toxicity, this mechanism may not be important \((a)\). Also, nitrate may be oxidized by heme or free metals, leading to the NO2 radical \((a)\).

NITRATION OF TYROSINE VERSUS OXIDATIVE STRESS

The role of tyrosine nitration in acetaminophen toxicity was investigated using inhibitors of nitric oxide synthetase and mice that were genetically deficient in iNOS \((a)\). A comparison of the time course for development of acetaminophen toxicity in iNOS knockout mice and wild-type mice indicated no difference in the histological development of toxicity between the two groups of mice. Serum levels of nitrate plus nitrite (a measure of NO synthesis) were increased in the wild-type mice but not in the iNOS knockout mice and analysis of liver homogenates for tyrosine nitration indicated significant levels in the wild-type mice and a decreased amount in the knockout mice, possibly as a result of endothelial NOS. It was concluded that the major murine enzyme important in tyrosine nitration under normal wild-type conditions was iNOS. Interestingly, these experiments showed that acetaminophen caused a 3-fold increase in lipid peroxidation (oxidative stress) in the iNOS knockout mice but no increase in the wild-type mice. Thus, in the wild-type mice, toxicity was accompanied by tyrosine nitration, whereas in the iNOS knockout mice, toxicity was accompanied by oxidative stress. In a subsequent study \((a)\), was determined the effect of NOS inhibitors on acetaminophen toxicity in mice. The iNOS inhibitor aminoguanidine did not significantly alter acetaminophen toxicity, but it greatly decreased tyrosine nitration and increased lipid peroxidation. Thus, the iNOS inhibitor gave information similar to that obtained in the iNOS knockout mice. These data were interpreted to indicate that excess levels of superoxide were formed in acetaminophen toxicity. When NO is present, as in the wild-type mice, the superoxide preferentially reacts to form peroxynitrite, which nitrates proteins. In the absence of NO, superoxide leads to lipid peroxidation. These data indicate the importance of NO in the disposition of superoxide, leading to oxidative stress.

CYTOKINES AND OTHER INFLAMMATORY MEDIATORS IN ACETAMINOPHEN TOXICITY

Several laboratories have reported that inflammatory cytokines are increased in acetaminophen toxicity. Blazka reported the up-regulation of TNF-α and IL-1β in the acetaminophen-treated mouse \((a)\). In addition, it was shown that selective immunoneutralization of either TNF-α or IL-1β partially decreased toxicity for a period of time. In vitro studies by Kuo showed that IL-1β caused a dose-dependent up-regulation of NO in rat hepatocytes exposed to acetaminophen \((a)\). Consistent with these data, Hinson et al. recently found that IL-1β and NO up-regulation in the acetaminophen-treated mouse are temporally related \((a)\).
Although others have confirmed the up-regulation of TNF-α in acetaminophen toxicity, its role in the mediation of toxicity is somewhat controversial. TNF-α knockout mice were not protected from toxicity, and a recent report showed that immunoneutralization of TNF-α had no effect on toxicity. Studies in IL-10 knockout mice suggest that IL-10 is protective in APAP toxicity by controlling NO and iNOS formation. In addition, the increased susceptibility of IL-10 knockout mice to acetaminophen was associated with increased levels of TNF-α and IFN-β. Thus, it may be that proinflammatory cytokines contribute to the toxicity, and that they are regulated by anti-inflammatory cytokines, such as IL-10 and others.

Macrophage migration inhibitory factor (MIF) is a recently described protein that has characteristics of a cytokine, a hormone, and an enzyme. It may promote the up-regulation of other pro-inflammatory cytokines, adhesion molecules, matrix metalloproteinase-2 expression, NO release, and cyclooxygenase-2 and has counter-regulatory effects on endogenous glucocorticoids. Bourdi et al reported that MIF was up-regulated in acetaminophen toxicity. Moreover, MIF knockout mice were less susceptible to acetaminophen and had decreased IFN-γ production and increased heat shock protein expression. Heat shock proteins are considered to be a protective mechanism against various physiologic and environmental stressors. In addition, cyclooxygenase-2 has been reported to be hepatoprotective in acetaminophen, possibly due to up-regulation of heat shock proteins.

Various chemokines have also been reported to be up-regulated in acetaminophen toxicity. Chemokines were initially recognized for their role in leukocyte recruitment. However, Lawson et al. showed that neutrophils followed the development of toxicity and that administration of antibodies to integrins had no effect on toxicity. Chemokine up-regulation in acetaminophen toxicity appears to be hepatoprotective. Macphage migration inhibitory protein-2 (MIP-2) and other related chemokines promoted hepatocyte regeneration in acetaminophen-treated mice. In additional studies, viral vector delivery of MIP-2 to acetaminophen-treated mice was protective against acetaminophen toxicity. Studies in mice deficient in CCR2, the primary receptor for the chemokine MCP-1, showed these mice to have increased toxicity to acetaminophen and increased formation of TNF-α and IFN-β. Cumulatively, these studies suggest that cytokines play complex roles in the toxicity and that alterations in the equilibrium of pro- and anti-inflammatory cytokine formation contribute to the toxicity. Chemokines appear to facilitate hepatocyte regeneration following toxicity, likely by increasing the nuclear translocation of growth-regulatory transcription factors.

**SUPEROXIDE FORMATION AND MITOCHONDRIAL DYSFUNCTION IN ACETAMINOPHEN TOXICITY**

Superoxide may be formed via a number of mechanisms including formation from cytochrome P450 and other enzymes. The importance of activation of Kupffer cells, macrophages, or neutrophils (the so-called respiratory burst) in acetaminophen toxicity has been evaluated. This sudden excess utilization of oxygen by activated phagocytes is a result of increased activity of the enzyme, NADPH-oxidase. The result is release of superoxide anion at the outer surface of the plasma membrane. James et al utilized mice genetically deficient in Gp91 phox, a critical subunit of NADPH oxidase, and found that these mice and wild-type mice had comparable toxicity to acetaminophen. Also, both knockout mice and wild-type mice had the same degree of tyrosine nitration in the liver. An analysis of mitochondrial glutathione disulfide was found to be comparable in the two groups. These data suggest that the increased superoxide in acetaminophen toxicity is not from activated macrophages.

Available data suggest that mitochondrial dysfunction may be an important mechanism in acetaminophen-induced hepatotoxicity. It is known that mitochondrial permeability transition (MPT) occurs with formation of superoxide, and this may be the source of superoxide leading to peroxynitrite and tyrosine nitration. MPT represents an abrupt increase in the permeability of the inner mitochondrial membrane to small molecular weight solutes. Oxidants such as peroxides and peroxynitrite, Ca2+, and Pi promote the onset of MPT, whereas Mg2+, ADP, low pH, and high membrane potential oppose onset. Associated with the permeability change is membrane depolarization, uncoupling of oxidative phosphorylation, release of intramitochondrial ions and metabolic intermediates, and mitochondrial swelling. MPT mechanisms have been reviewed by Lemasters et al. In the late 1980s it was shown that cyclosporin A specifically blocked the onset of MPT in a saturable manner, implying a protein channel or pore. It has been subsequently recognized that this pore transports both anionic and cationic solutes of mass less than 1500 Da and is the previously identified...
multiple conductance channel. Of particular note is the fact
that oxidation of vicinal thiols in the pore promote an open
conductance state \(c_\text{p}\). Addition of NAPQI to isolated rat
liver mitochondria caused a decrease in synthesis of ATP
and an increase in release of sequestered Ca\(_{2+}\). This release
was blocked by cyclosporin A \(c_\text{o}\). These data are consistent
with the hypothesis that NAPQI causes MPT, as has been
reported about other quinones \(c_\text{q}\). This is presumably a
result of NAPQI-mediated oxidation of the vicinal thiols at
the MPT pore. NAPQI is known to be both an oxidizing
agent and an arylationating agent, and Tirmenstein and Nelson
\(c_\text{t}\) have reported that acetaminophen leads to oxidation of
protein thiols. Also, McLean and coworkers have reported
that inhibitors of MPT decrease acetaminophen toxicity in
rat liver slices and in vivo \(c_\text{v}\). Lastly, Grewal reported that
late addition of N-acetylcysteine to freshly isolated mouse
liver hepatocytes had no effect on the toxicity; however,
dithiothreitol decreased the further development of toxicity
\(c_\text{d}\). Also, in similar experiments with isolated hamster
hepatocytes, the late addition of dithiothreitol to cells
washed free of acetaminophen prevented the further
decrease in cell viability and appeared to reverse
morphological changes \(c_\text{m}\). Dithiothreitol is known to
reduce the vicinal thiols at the MPT pore and prevent the
further progression of toxicity \(c_\text{f}\). These data are consistent
with a mechanism whereby acetaminophen toxicity is by
NAPQI-mediated oxidation of thiol groups in mitochondria
leading to MPT. The toxicity is mediated by mitochondrial
dysfunction resulting in production of reactive
oxygen/nitrogen species.

**SUMMARY**

With the analgesic acetaminophen it was shown that the
hepatic necrosis was not a result of the parent compound.
Metabolism by cytochrome P450 enzymes to a reactive
metabolite was necessary for the production of toxicity.
After therapeutic doses of acetaminophen, the reactive
metabolite was efficiently detoxified by conjugation with
GSH. However, after toxic doses, GSH was depleted by the
conjugation reaction and the metabolite covalently bound to
protein. Covalent binding correlated with development of
toxicity. Treatment with cysteine to increase GSH
detoxification was shown to be an effective antidote against
toxicity. The latter finding led to the development of N-
acetylcysteine as the current clinical antidote. Since these
initial findings, many investigators have added additional
details relative to the concept of how acetaminophen is toxic,
and some of these are discussed in this review. Despite these
recent studies, the concept of metabolic activation as the
critical initiating event in the development of toxicity has
remained. Moreover, metabolic activation has proven to be a
general mechanism relevant for the development of toxicity
for many drugs and chemicals.

**ABBREVIATIONS**

NAPQI, N-acetyl-p-benzoquinone imine; GSH, glutathione;
IL, interleukin; TNF-\(\alpha\), tumor necrosis factor - \(\alpha\); NO, nitric
oxide; iNOS, inducible nitric oxide synthase; IFN-\(\gamma\),
interferon-\(\gamma\); MIF, macrophage migration inhibitory factor;
MPT, mitochondrial permeability transition.

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