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Inhibition of Carbamyl Phosphate Synthetase-I and Glutamine Synthetase by Hepatotoxic Doses of Acetaminophen in Mice

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Abstract

The primary mechanisms proposed for acetaminophen-induced hepatic necrosis should deplete protein thiols, either by covalent binding and thioether formation or by oxidative reactions such as S-thiolations. However, in previous studies we did not detect significant losses of protein thiol contents in response to administration of hepatotoxic doses of acetaminophen *in vivo*. In the present study we employed derivatization with the thiol-specific agent monobromobimane and separation of proteins by SDS–PAGE to investigate the possible loss of specific protein thiols during the course of acetaminophen-induced hepatic necrosis. Fasted adult male mice were given acetaminophen, and protein thiol status was examined subsequently in subcellular fractions isolated by differential centrifugation. No decreases in protein thiol contents were indicated, with the exception of a marked decrease in the fluorescent intensity, but not of protein content, as indicated by staining with Coomassie blue, of a single band of approximately 130 kDa in the mitochondrial fractions of acetaminophen-treated mice. This protein was identified by isolation and N-terminal sequence analysis as carbamyl phosphate synthetase-I (CPS-I) (EC 6.3.4.16). Hepatic CPS-I activities were decreased in mice given hepatotoxic doses of acetaminophen. In addition, hepatic glutamine synthetase activities were lower, and plasma ammonia levels were elevated in mice given hepatotoxic doses of acetaminophen. The observed hyperammonemia may contribute to the adverse effects of toxic doses of acetaminophen, and elucidation of the specific mechanisms responsible for the hyperammonemia may prove to be useful clinically. However, the preferential depletion of protein thiol content of a mitochondrial protein by chemically reactive metabolites generated in the endoplasmic reticulum presents a challenging and potentially informative mechanistic question.

Acetaminophen is a widely used analgesic that appears to be safe when ingested in therapeutic doses, but causes marked hepatic damage in humans and experimental animals in larger doses (Mitchell *et al.*, 1973a; Black, 1984). Although there appears to be general agreement that the mechanisms of cell damage by acetaminophen involve alterations of biological molecules by chemically reactive metabolites of the parent drug (Mitchell *et al.*, 1973a), considerable disagreement persists regarding the relative contributions of different types of interactions (Nelson and Pearson, 1990). Alkylation or, somewhat more restrictively, arylation of hepatic proteins by a reactive metabolite(s) of acetaminophen was found to correlate with incidence and severity of injury (Jollow *et al.*, 1973). It is reasonable to expect that the structure and/or functions of a protein would be affected adversely by the covalent attachment of a xenobiotic residue, but the question of the manner and extent to which covalent binding contributes to cellular injury has not been resolved (Smith *et al.*, 1985a). Although covalent binding can occur in the absence of substantive cytotoxicity

(Corcoran *et al.*, 1985; Birge *et al.*, 1988), marked hepatic necrosis *in vivo* caused by acetaminophen has not been observed without measurable covalent binding, usually in the range of 1 nmol of metabolite bound per milligram of hepatic protein (Corcoran *et al.*, 1985).

Chemical alterations other than alkylation also have been implicated in the expression of acetaminophen-induced toxicity (Wendel *et al.*, 1979; Gerson *et al.*, 1985; Jaeschke, 1990; Tirmenstein and Nelson, 1990). The proposed alternative mechanisms generally involve oxidative reactions. *N*-Acetyl-*p*-benzoquinone imine (NAPQI), which is thought by many investigators to be the chemically reactive intermediate generated by metabolism of acetaminophen (Dahlin *et al.*, 1984), oxidizes thiols and forms thioethers through Michael addition reactions (Hoffmann *et al.*, 1985; Nicotera *et al.*, 1990). Although increases in hepatic glutathione disulfide (GSSG) concentrations and decreases in protein thiol contents in acetaminophen-treated animals have been reported (Jaeschke, 1990; Tirmenstein and Nelson, 1990), we have not observed measurable increases in thiol oxidation in response to administration of acetaminophen *in vivo*, nor have we observed decreases in hepatic protein thiol content with hepatotoxic doses of acetaminophen (Smith and Mitchell, 1985; Smith and Jaeschke, 1989).

Because 1,3-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) inhibits glutathione reductase, but has not been found to affect other enzymes to any comparable extent (Frischer and Ahmad, 1977), reports of exacerbation of acetaminophen toxicity by administration of BCNU (Gerson *et al.*, 1985; Kyle *et al.*, 1988; Nakae *et al.*, 1988; Farber *et al.*, 1988) have been interpreted as indicating significant contributions of oxidative mechanisms to acetaminophen-induced liver injury. The most straightforward mechanisms through which inhibition of glutathione reductase might potentiate injury would be through enhanced accumulation of products of protein S-thiolation reactions and the consequent changes in molecular structures and functions (Gilbert, 1990). However, the studies with BCNU have not revealed significant accumulations of GSSG, as would be expected if the potentiation of acetaminophen-induced hepatic damage was mediated by inhibition of glutathione reductase. In contrast, we observed no increase in GSSG in response to acetaminophen in BCNU-pretreated rats, and we found no enhancement of acetaminophen-induced hepatic injury, despite decreases in hepatic glutathione reductase activities to less than 30% of control (Smith and Mitchell, 1985).

Nonetheless, there are many reasons to suspect thiol modification as an important mechanism of acetaminophen toxicity. The present investigations were prompted by the hypothesis that the important damage done by acetaminophen metabolites might involve S-thiolation of and/or thioether formation with particular proteins. The selective loss of a limited number of protein thiol (PSH) groups would not be detected readily by the simple measurements of total PSH content, such as those employed in previous studies. Therefore, we sought to determine whether selective loss of one or more protein thiols could be detected in animals treated with hepatotoxic doses of acetaminophen by labeling the thiol groups with monobromobimane (mBBBr), with visualization of the fluorescent derivatives following separation of the proteins by SDS-PAGE (Birge *et al.*, 1991; Weis *et al.*, 1992).

In the present studies we observed no general loss of protein thiol content, but did observe a selective disappearance from mice given hepatotoxic doses of acetaminophen of thiol-derived fluorescence from a 130-kDa protein that we have identified as carbamyl phosphate synthetase-I (CPS-I). Hepatotoxic doses of acetaminophen also decreased hepatic activities of CPS-I and of glutamine synthetase. The effects on glutamine synthetase are in agreement with the recent report by Bulera *et al.* (1995) of inhibition of glutamine synthetase in cultured hepatocytes exposed to acetaminophen. Acetaminophen-induced decreases in activities of CPS-I and glutamine synthetase, as assayed in hepatic homogenates *in vitro*, were accompanied by increases in plasma concentrations of ammonia, thus indicating that CPS-I and/or glutamine synthetase were inhibited *in vivo* to an extent sufficient to compromise ammonia clearance. This hyperammonemia might contribute to the evolving hepatocellular injury, and the time courses, dose/response relationships, and interindividual variations support causal contributions, but further study will be needed to delineate the toxicological significance of the inhibition of CPS-I and glutamine synthetase, and the consequent hyperammonemia.

MATERIALS AND METHODS

Chemicals and reagents

mBBr was purchased from Calbiochem (La Jolla, CA). Reagents for electrophoresis were obtained from Bio-Rad (Hercules, CA). Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore (Bedford, MA). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Houston, TX) and were of the highest purity available from the respective sources.

Animals

Male ICR mice (6–10 weeks of age, weighing 20–40 g) were obtained from Harlan (Houston, TX) and maintained by the Baylor Animal Program in air-conditioned rooms with 12:12 hr light:dark cycle. The animals were kept in polycarbonate cages with wire lids, air filter bonnets, and 100% Aspen wood chip bedding. The animals were allowed free access to Purina 5001 rodent diet and tap water. The animals were adapted for at least 3 days before study. The mice were fasted 18 hr prior to administration of 0 to 400 mg/kg of acetaminophen in normal saline or of equal volumes (1 ml per mouse) of saline alone, ip. At 2, 4, or 6 hr post dose, the animals were anesthetized with pentobarbital and blood was obtained by cardiac puncture for isolation of plasma. Livers were removed and animals were killed by exsanguination, while under deep anesthesia.

Isolation of subcellular fractions

The livers were rinsed in saline, and 10% homogenates were prepared in 0.25 M ice-cold sucrose using 10 strokes of a Dounce homogenizer. The homogenates and successive supernatant fractions were centrifuged at 700g (10 min), 12,700g (20 min), and 105,000g (60 min) to separate nuclear, mitochondrial, and microsomal fractions, respectively, leaving the soluble fractions as the final supernatants. The pelleted subcellular fractions were resuspended with addition of 1 ml of 0.25 M sucrose, which provided protein concentrations

in the range of 20–40 mg protein per milliliter. Protein concentrations were determined by the method of Lowry *et al.* (1951).

Derivatization with mBBR

In final concentrations, 0.25 M *N*-ethylmorpholine, 0.3 mM EDTA, and 8 mM mBBR in acetonitrile (30% of final volume) were added to approximately 1 mg of protein. The mixtures were vortexed and allowed to react at room temperature for at least 5 min (Weis *et al.*, 1992).

Electrophoresis

SDS/2-mercaptoethanol/polyacrylamide slab gel electrophoresis was carried out in the buffer system described by Laemmli (1970). The stacking and the resolving gels contained 5% (w/v) and 12.5% (w/v) of polyacrylamide, respectively. After running, the gels were placed in methanol:acetic acid:water (45:10:45, v/v/v). The mBBR-derived fluorescence of the proteins was visualized with a TS-15 transilluminator (UVP, Inc., San Gabriel, CA) equipped with a 254-nm light source and photographed with a Polaroid camera using Kodak Wratten gelatin filter No. 15.

Electroblotting

Electroblotting was carried out in the Bio-Rad transfer electrophoresis unit (Bio-Rad Laboratories) at 30 V for 12 hr. After transfer, the blots were washed with deionized water for 5 min, stained with 0.1% Ponceau-S in 0.1% acetic acid (v/v) for 4 min and destained with water for 2–3 min. The bands were excised using a surgical blade as fine strips cut from the center portion of the bands, in an effort to minimize potential contamination from other proteins eluting near or with the protein of interest. The blots were dried, and the peptide band (130 kDa) was cut out. The protein was eluted from the blots, checked for purity by gel electrophoresis, and sequenced using an automated pulsed liquid phase sequencer (Applied Biosystems Model 477A protein sequencer with an in-line 120-A PTH analyzer). The sequence obtained was checked for homology to known proteins (Gene Bank data base).

Elution of proteins from PVDF membranes

The 130-kDa bands were cut from the blots and placed in 1-ml centrifuge tubes containing 500 µl of elution buffer (50 mM Tris-HCl, pH 9.0, 2% SDS (w/v), and 1% Triton X-100), so as to completely submerge the membranes. After addition of the eluant, the tubes were shaken at room temperature for 1 hr and centrifuged at 12,700g for 10 min, and the supernatant solutions were removed. The tubes and membranes were then rinsed with 250 µl of fresh elution buffer and centrifuged again for 5 min, and the supernatant materials were retained.

Determination of plasma alanine aminotransferase (ALT) activities

Plasma ALT activities were determined using Sigma assay kit (Procedure No. 59-UV), from the ALT-mediated conversion of alanine to pyruvate, and the reduction of pyruvate with lactate dehydrogenase, with detection of the coupled oxidation of NADH.

Determination of plasma ammonia concentrations

Plasma ammonia concentrations were determined by Sigma assay kit by reductive amination of 2-oxoglutarate, using glutamate dehydrogenase and following the decrease in absorbance at 340 nm due to the oxidation of NADPH (Sigma Procedure No. 471-UV).

Enzyme preparation

Liver homogenates (10% w/v) were prepared in buffer containing 0.25 M sucrose, 50 mM triethanolamine (pH 7.8), 1 mM dithiothreitol (DTT), 7 mM magnesium acetate, 20% glycerol, and 0.3% Triton. The homogenates were centrifuged at 12,700g for 20 min, and the supernatants were used for measurements of enzyme activities. The supernatants stored at -20°C for up to 1 week showed no loss of activity from assays conducted on freshly prepared supernatants. All data were collected within that time frame.

Determination of CPS-I activities

CPS-I activities were assayed at room temperature by coupling ADP formation to NADH oxidation with pyruvate kinase and lactate dehydrogenase (Fahien and Cohen, 1964) and measuring the decrease in absorbance at 340 nm. The assay mixtures contained the enzyme source, 2.5 mM phosphoenolpyruvate, 0.2 mM NADH, 10 mM NH₄Cl, 100 mM KHCO₃, 5 mM ATP, 10 mM MgSO₄, 10 mM *N*-acetylglutamate, 10 U/ml pyruvate kinase, 12.5 U/ml of lactate dehydrogenase, and 50 mM glycylglycine, pH 7.6, which was found to be the optimum pH for enzyme activity. The reaction could be started by the addition of either acetylglutamate or enzyme source. The initial velocity of the reaction was directly proportional to the CPS-I activity. One unit of CPS-I activity corresponds to the oxidation of 1 μmol of NADH/min at room temperature.

Determination of glutamine synthetase activities

Glutamine synthetase activities were measured by coupling the production of ADP in the biosynthetic assay to the oxidation of NADH, using the addition of phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase in excess, as described by Kingdon *et al.* (1968). A substrate mixture for 10 assays may be made from the stock reagents as follows: 0.3 ml of 1.67 M MgCl₂, 0.9 ml of 1.0 M KCl, 0.3 ml of 1.0 M sodium glutamate (pH 7.0), 0.5 ml of 1.0 M NH₄Cl, 0.5 ml of 1.0 M imidazole-HCl buffer (pH 7.0), and 1.5 ml water. The reactions were carried out at room temperature as follows, with the decrease in absorbance at 340 nm providing the measure of the rate of NADH oxidation: 0.4 ml of substrate mixture, 60 μl of 60 mM sodium ATP, 30 μl of 10 mg/ml NADH, 10 μl of 100 mM phosphoenolpyruvate (pH adjusted to 5.8 with KOH), and 25 μl of coupling enzyme mixture (10 U/ml pyruvate kinase and 12.5 U/ml of lactate dehydrogenase). The reaction mixtures were incubated at room temperature for 10 min, then enzyme and enough water were added to make final volumes of 1.0 ml. The absorbance recording was started within 20 sec of the enzyme addition. One unit of glutamine synthetase corresponds to the oxidation of 1 μmol of NADH/min.

Statistical analyses were performed by analysis of variance with Student-Newman-Keuls, using SPSS for Windows, Version 6.0 (Norusis, 1993; Zar, 1984). Data are presented as means ± SEM, and significant differences are noted at $p < 0.05$.

RESULTS

The SDS–PAGE analyses of mBBr-derivatized subcellular fractions of mouse liver 2 hr post dose showed no marked differences in the protein fluorescence intensities between the control and acetaminophen-treated animals (Fig. 1A). The mBBr-derivatized hepatic subcellular fractions of acetaminophen-treated animals at 6 hr after drug administration (Fig. 1B) also were remarkably indistinguishable from those of saline-treated control animals by SDS–PAGE, with the exception of the striking decrease in fluorescence in a protein band at about 130 kDa in the mitochondrial fractions. Figure 2 presents the SDS–PAGE of mBBr-derivatized mitochondrial fractions of other mouse livers at 2 and 6 hr post dose, showing the depletion of the thiol content in the 130-kDa band observed 6 hr after administration of acetaminophen. There were no appreciable differences between control and acetaminophen-treated mice in the abundance of the 130-kDa protein in these fractions, as seen by transferring the proteins to PVDF membranes and staining with Ponceau-S (Fig. 3). Coomassie-stained gels showed similar patterns, indicating no loss of content of this protein (data not shown).

The 130-kDa band was excised from blots prepared from samples obtained from saline-treated control animals, and the protein eluted as described under Materials and Methods. To assess the purity of the protein thus isolated, we analyzed the combined material eluted from 20 bands from blots from gels loaded with 80 µg of total protein per lane, by SDS–PAGE with detection by staining with Coomassie blue, which showed a single band at 130 kDa (Fig. 4). The apparent molecular weight of the protein was calculated from interpolation of the R_f -value of the band relative to the corresponding values measured from the protein standards, with logarithmic transformation (data not shown). The single band seen in Fig. 4 does not prove that the material we isolated was a single pure protein, but the figure shows that any contaminating material of significant quantity would have to coelute very closely. Furthermore, the fact that we obtained a single N-terminal sequence indicates that any contaminating protein would have to be N-terminally blocked or share the same N-terminal sequence as the substance we interpret as being CPS-I.

The N-terminal sequence of the mouse liver mitochondrial protein is presented in Table 1, along with the N-terminal sequence of the human and rat CPS-I proteins (Haraguchi *et al.*, 1991; Nyunoya *et al.*, 1985). In the mouse liver protein, the N-terminal sequence of the first 15 amino acids was identical with that of rat and human CPS-I, except that a glutamine-to-lysine substitution was observed in the fifth position. The triplet codon of these two amino acids differ only by a single A-to-C substitution. The N-terminal sequence of CPS-I starts at serine 40, as the enzyme is modified post-translationally in the mitochondrial matrix after being transported into the mitochondria (Nyunoya *et al.*, 1985).

Interestingly, in this series of animals the hepatic CPS-I activities were decreased significantly by doses of acetaminophen of 200 mg/kg or greater (Fig. 5), whereas significant decreases in hepatic activities of glutamine synthetase (Fig. 6) and increases in plasma ammonia concentrations (Fig. 7) were observed only at 300 or 400 mg/kg. Elevation of plasma ALT activities were observed at doses of 300 or 400 mg/kg, but not at 200 mg/kg or below (Fig. 8). Figure 9 shows the time-dependent changes in hepatic CPS-I activities of

mice treated with 400 mg/kg of acetaminophen. Although the hepatic CPS-I activities tended to be lower in the treated mice than in controls at 2 hr, the two groups were not different statistically. Hepatic CPS-I activities were markedly lower in the acetaminophen-treated mice 4 and 6 hr post dose. Hepatic glutamine synthetase activities in these mice were less than half of the activities observed from corresponding control animals (Fig. 10), and plasma ammonia levels were increased (Fig. 11) within 2 hr of drug administration. In studies subsequent to the ones used for the data presented in Fig. 5, we found that a measurable fraction of the CPS-I activity indicated by the assay employed was attributable to NADH oxidation that is independent of the presence of *N*-acetylglutamate. In these animals the onset of significant inhibition of CPS-I activities preceded increases in plasma ALT activities (Fig. 12), which were not elevated until 6 hr. In additional experiments, correction of estimates of CPS-I activities for this NADH oxidation in the absence of added *N*-acetylglutamate indicated greater than 90% inhibition of CPS-I by 400 mg/kg of acetaminophen, which appears to be more consistent with the loss of fluorescence staining of the 130-kDa band by this dose of drug (Figs. 1 and 2).

Decreased CPS-I activities and increased plasma ammonia levels were observed in two of the six animals examined 2 hr after administration of 400 mg/kg of acetaminophen. The other four animals given acetaminophen showed plasma ammonia levels that were elevated slightly, but the hepatic CPS-I activities in these four animals clearly were within the range of the activities observed in the control animals (data not shown). The animals examined 4 hr after drug administration were clearly different from controls in both parameters, and the decrease in enzyme activity and the accumulation of ammonia progressed through 6 hr.

A similar examination of individual glutamine synthetase activities and plasma ammonia levels in these same mice showed that glutamine synthetase activities were uniformly lower in all treated animals than in any control animals as early as 2 hr after administration of drug. The inhibition of glutamine synthetase and the elevations in plasma ammonia concentrations were greater in the two animals that also showed lower CPS-I activities at 2 hr after drug. Interestingly, plasma ALT activities were elevated in these same two animals, whereas ALT activities were not increased in the seven acetaminophen-treated animals examined at 4 hr, despite the diminished hepatic activities of glutamine synthetase and CPS-I observed in the seven animals examined at 4 hr.

DISCUSSION

The almost indistinguishable patterns of protein thiols revealed by electrophoresis of mBBBr-derivatized subcellular fractions from control and acetaminophen-treated mice (Fig. 1) are not consistent with the extent of depletion of protein thiols that Moore *et al.* (1985) reported from studies with isolated hepatocytes treated with NAPQI. The present observations are in agreement with our studies of PSH status in response to hepatotoxic doses of acetaminophen, in which we observed no measurable depletion of protein thiols (Smith and Mitchell, 1985). Hepatic necrosis caused by excessive doses of acetaminophen *in vivo* is associated with covalent binding at levels around 1 nmol of drug bound per milligram of protein (Corcoran *et al.*, 1985). Hoffmann *et al.* (1985) have estimated that alkylation of protein thiols accounts for about 70% of the measured levels of binding *in vivo*. With

approximately 16 nmol of protein thiols per milligram of liver and 0.2 mg of protein per milligram of hepatic tissue, binding to thiols at a level of 1 nmol/mg of protein would decrease protein thiol content less than 2% (Smith *et al.*, 1985b; Corcoran *et al.*, 1985). Whether or not covalent binding contributes to the biological manifestations of injury, even with complete specificity for protein thiols, this level of alkylation would decrease total protein thiol contents by amounts that would lie within experimental variation of the measurements. In addition, thiol depletion of this magnitude would not be expected to exhibit observable biological effects, unless considerable site specificities were expressed.

Protein thiols also could be altered by oxidative mechanisms, but similar quantitative limits have not been placed on these alterations. Oxidant stress-induced increases in steady state concentrations of GSSG, leading to increased protein S-thiolations, would appear to be the most likely mechanisms for oxidative protein thiol depletion (Gilbert, 1990), but we have not observed even a measurable increase in thiol oxidation in response to acetaminophen metabolism *in vivo* (Smith and Mitchell, 1985; Smith and Jaeschke, 1989). Increases in hepatic GSSG accumulation have been observed during the evolution of acetaminophen-induced hepatic damage (Jaeschke, 1990; Tirmenstein and Nelson, 1990), but these increases were only observed relatively late (6–9 hr post dose) and the contributions of these changes to initiation of injury are not certain. Some of the apparent differences in the results of these studies could be due to differences in the animal models, such as the use of phenobarbital-induced mice (Tirmenstein and Nelson, 1990), whereas our experiments did not employ phenobarbital-treated mice. However, acetaminophen-induced hepatic necrosis can be accompanied by a profound swelling of the liver caused by a vascular engorgement, which also might contribute to apparent decreases in hepatic PSH content expressed per gram of liver or per milligram of protein (Corcoran *et al.*, 1985; Smith and Mitchell, 1985). The absence of measurable increases in GSSG production during peak metabolism of acetaminophen or of decreases in hepatic PSH content in acetaminophen-treated animals, even in animals in which over 70% of hepatic glutathione reductase was inhibited by pretreatment with BCNU, is not consistent with the significant expression of the oxidative capabilities of NAPQI or other products of acetaminophen metabolism *in vivo* (Smith and Mitchell, 1985).

We also observed no potentiation of acetaminophen-induced hepatotoxicity in BCNU-pretreated animals (Smith and Mitchell, 1985). Other investigators have reported potentiation of acetaminophen toxicity in BCNU-pretreated cells and animals (Kyle *et al.*, 1988; Farber *et al.*, 1988; Nakae *et al.*, 1988; Gerson *et al.*, 1985), which they attributed to inhibition of glutathione reductase and interpreted as evidence of a significant oxidative component in acetaminophen-induced hepatotoxicity. Although we observed no depletion of PSH by hepatotoxic doses of acetaminophen, even in animals pretreated with BCNU, the selective depletion of PSHs would not have been precluded by our earlier studies. Hepatotoxic doses of acetaminophen do deplete tissue GSH (Mitchell *et al.*, 1973b), which could decrease the GSH/GSSG ratios and proportionately shift PSH/PSSX ratios through thiol–disulfide exchange equilibration reactions (Gilbert, 1990), but we are not aware of direct support for such transformations. The results of our present studies offer no support for substantive depletion of protein thiols by hepatotoxic doses of acetaminophen, with the exception of the 130-kDa protein (Figs. 1 and 2).

One significant difference between our study and the studies of Farber and his co-workers is that we allowed 18 hr between treatment with BCNU and administration of acetaminophen, whereas they employed a 2-hr interval. The inhibition of glutathione reductase by BCNU is rapid and sustained, and the extent of inhibition of reductase activities was comparable in both models. The longer lag time we employed might have resulted in decreased metabolic activation of acetaminophen, but depletion of biliary GSH after acetaminophen treatment was not affected, indicating that the generation of the reactive metabolite(s) in these animals was adequate. However, the cytotoxic effects of BCNU are correlated more closely with DNA alkylation than with protein carbamoylation, which appears to be responsible for inhibition of glutathione reductase (Bodell *et al.*, 1986; Davis *et al.*, 1993; Lemoine *et al.*, 1991). It is therefore reasonable to suspect that the greater hepatotoxicity of acetaminophen 2 hr after exposure to BCNU may be due to the contributions from DNA alkylation by the BCNU. Although BCNU is cleared rapidly, the repair of DNA interstrand crosslinks caused by BCNU requires 6–12 hr (Lemoine *et al.*, 1991). An interesting implication of this working hypothesis regarding the difference between the early and late effects of BCNU pretreatment on acetaminophen hepatotoxicity is the corollary that DNA alterations may play a significant role in acute cell death caused by acetaminophen.

It is conceivable that structural changes in a protein might inhibit thiol reactivity with mBBBr, but the solubility and diffusibility of mBBBr and its high reactivity with thiols make protein thiol S-alkylation or S-thiolation more likely causes of the observed loss of fluorescence. The loss of thiol-derived fluorescence associated with the 130-kDa protein appears to be more consistent with alkylation than with S-thiolation. The decreases in CPS-I activities we observe are not reversed by incubation of tissue preparations with DTT *in vitro*, and in our studies to date we have not observed a restoration of thiol status in the 130-kDa protein by incubation of mitochondrial fractions of acetaminophen-treated animals with DTT and/or GSH prior to protein derivatization with mBBBr, whereas restoration of thiol content and possibly of enzyme activity would be expected if inhibition were due to protein S-thiolation. The protein itself is not lost upon treatment with acetaminophen (Figs. 2 and 3). Hepatotoxic doses of acetaminophen lead to time-dependent decreases in CPS-I activities (Fig. 9) that also exhibit dose-threshold responses (Fig. 5) that parallel hepatic injury (Fig. 8). Studies published to date on the identification of the proteins alkylated by reactive metabolites of acetaminophen have focused primarily on a 56- to 58-kDa selenium binding protein and on a 44-kDa cytosolic protein recently identified as glutamine synthetase (Bartolone *et al.*, 1988; Birge *et al.*, 1988, 1991; Pumford *et al.*, 1992; Bulera *et al.*, 1995). Although not discussed, the Western analyses presented in the publications by these groups often show an immunoreactive band in the region of 130 kDa, though these studies employed microsomal or cytosolic fractions. The profile of immunoreactive proteins from mitochondrial fractions reported by Halmes *et al.* (1996) offers more direct evidence for formation of an alkylated species migrating at 130 kDa, which supports the hypothesis of alkylation of CPS-I more strongly. Although the covalent binding of acetaminophen metabolites is observed at greatest levels in the centrilobular hepatocytes (Jollow *et al.*, 1973; Roberts *et al.*, 1991), the alkylation indicated by the immunohistochemical studies reported by Roberts *et al.* was reported to extend through the midzonal hepatocytes. The autoradiographic methods used by Jollow *et al.* demonstrated bound radioactivity throughout all hepatocytes, but concentrated

in the central vein regions. Roberts *et al.* reported more intense immunostaining 1–2 h after dosing, which is earlier than we observe maximal binding using radiochemical methods (Corcoran *et al.*, 1985). Although some of the difference may be due to differences in the animal models studied, it is also possible that the antibodies employed in the studies reported to date are not uniformly responsive to all adducted species. The data available at present are consistent with the hypothesis that the 130-kDa protein indicated by the Western analyses to contain bound acetaminophen is CPS-I, but this hypothesis needs to be tested directly.

The decreases in activities of both CPS-I and glutamine synthetase are likely to contribute to the observed increased plasma concentrations of ammonia in the acetaminophen-treated animals (Figs. 5–7). In addition, Halmes *et al.* (1996) have shown that acetaminophen metabolites bind to glutamate dehydrogenase, which also would be expected to compromise a mechanism of ammonia clearance. Hepatic glutamine synthetase is located in perivenous hepatocytes, in layers one to three cells thick surrounding terminal venules (Haussinger, 1983; Gebhardt and Mecke, 1983; Gebhardt and Reichen, 1994) and is isolated with the microsomal fraction by differential centrifugation (Bulera *et al.*, 1995). In contrast, CPS-I is located in the mitochondrial matrix of hepatocytes lying outside this limited perivenous zone, extending through the midzonal hepatocytes through the perivenous hepatocytes (Gaasbeek Janzen *et al.*, 1985). The rat CPS-I mRNA encodes a polypeptide of 1500 amino acids containing a N-terminal presequence of 38 or 39 amino acids (Nyunoya *et al.*, 1985). These investigators reported two unambiguous NH₂-terminal sequences displaced by a single amino acid. The first sequence started with leucine 39, and the second with serine 40. The sequence indicated by our data (Table 1) starts with serine 40. The precursor form of CPS-I is synthesized on free polysomes in the cytosol and is transported to the mitochondria, where it is converted to the mature enzyme by proteolytic processing in the mitochondrial matrix, which involves removal of the mitochondrial targeting sequence and refolding of the protein (Mori *et al.*, 1981a,b).

Hepatic glutamine synthetase removes ammonia at concentrations below 40 μ M, whereas CPS-I is less effective at low concentrations of ammonia (Haussinger, 1983). The present data show that 2 hr after 400 mg/kg of acetaminophen, glutamine synthetase activities are decreased by 50%, while CPS-I activities are not decreased significantly (Figs. 9 and 10), suggesting that the hyperammonemia observed in mice 2 hr after acetaminophen is more likely to be due to the decrease in glutamine synthetase activities than to decreases in CPS-I activities, although the relative contributions of inhibition of glutamate dehydrogenase inhibition reported by Halmes *et al.* (1996) were not evaluated in the animals we studied. Halmes *et al.* (1996) report 20% inhibition of hepatic glutamate dehydrogenase activities 1 hr after 400 mg/kg of acetaminophen and 30% inhibition after 600 mg/kg. However, the possible contributions of decreased CPS-I activities to the hyperammonemia we observed are not disproved by the data presently available. The fact that we observed a significant decrease in hepatic CPS-I activities in mice treated with 200 mg/kg of acetaminophen (Fig. 5), but find no decrease in glutamine synthetase activities (Fig. 6), or increases in plasma ALT activities (Fig. 8) or ammonia concentrations (Fig. 7) in these same animals, suggests an even greater sensitivity of CPS-I to the hepatotoxic effects of acetaminophen. However, we would not suggest that too much be made of this statistical distinction at the present time.

The studies of Khairallah and Cohen and their coworkers offer strong evidence that the inhibition of glutamine synthetase by cytotoxic doses of acetaminophen is associated closely with alkylation of this protein (Bulera *et al.*, 1995). Our studies also are consistent with alkylation as the primary mechanism of inhibition of CPS-I and glutamine synthetase *in vivo*.

In conclusion, the results of the present studies indicate that marked depletion of PSH is not a necessary event in acetaminophen-induced hepatic necrosis *in vivo*. However, we did observe the clear loss of thiol-derived fluorescence in a single mitochondrial protein, which we have identified as CPS-I. More subtle changes in protein thiol status, such as the possible increase in contents or thiol reactivities of several proteins in the cytosolic fractions of acetaminophen-treated mice (Fig. 1) may be appreciated as the techniques and concepts of the present experimental approach are refined. With the data available at the present time, the decreases in hepatic activities of CPS-I and glutamine synthetase caused by hepatotoxic doses of acetaminophen are best interpreted as results of alkylation of the respective proteins. The hyperammonemia that is observed in animals given hepatotoxic doses of acetaminophen probably is a consequence of the inhibition of key enzymes in the two major routes for clearance of ammonia. In addition to the direct clinical relevance of acetaminophen toxicity, the mechanisms that mediate injury by acetaminophen have been investigated intensively, and the extensive background of information that has been developed provides a framework from which basic questions on the fundamental mechanisms of cell damage by reactive intermediates can be studied. The marked alteration of thiol status of the mitochondrial protein CPS-I by reactive metabolites of acetaminophen that presumably are generated in the endoplasmic reticulum is a most intriguing observation and suggests intracellular transport mechanisms that are not fully appreciated at the present time.

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FIG. 1.

Hepatic protein thiols in subcellular fractions 2 hr (A) or 6 hr (B) after acetaminophen. Subcellular fractions were isolated by differential centrifugation (nuclear, NUC; mitochondrial, MITO; microsomal, MICRO; soluble, SOL) after intraperitoneal administration of 400 mg/kg of acetaminophen (AP) or equal volumes of saline to controls (CO). The fractions were treated with mBBR and the proteins were separated by one-dimensional SDS-PAGE as described under Materials and Methods. The fluorescent bands reflect derivatization of the proteins with the thiol-selective reagent. The electrophoretic

mobility of the molecular mass standards is indicated on the left. The arrow denotes the 130-kDa protein band.

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FIG. 2.

Hepatic protein thiols in mitochondrial fractions 2 hr (lanes 1–4) and 6 hr (lanes 5–8) after acetaminophen. Subcellular fractions were isolated by differential centrifugation after intraperitoneal administration of 400 mg/kg of acetaminophen (AP) or equal volumes of saline to controls (CO). The fractions were treated with mBBBr and the proteins were separated by one-dimensional SDS–PAGE as described under Materials and Methods. The fluorescent bands reflect derivatization of the proteins with the thiol-selective reagent. The electrophoretic mobility of the molecular mass standards is indicated on the left. The arrow denotes the 130-kDa protein band.

FIG. 3.

Ponceau-S-stained gel pattern of the mitochondrial fractions 2 hr (lanes 1–4) and 6 hr (lanes 5–8) after acetaminophen. Mitochondrial fractions shown in Fig. 2 were separated on one-dimensional SDS–PAGE, electroblotted onto PVDF membranes, and stained with Ponceau-S, as described under Materials and Methods. No differences are evident in the intensities of the protein bands at 130 kDa (arrow). Similarly, examination of gels from these fractions stained with Coomassie blue showed no difference in intensities of this protein (not shown). The differences in band intensity shown in Fig. 2 are therefore less likely to be due to loss of this protein than to loss of thiol content by the protein.

FIG. 4.

Coomassie blue-stained gel of the 130-kDa protein isolated for characterization. The protein band at 130 kDa was isolated from a gel after one-dimensional SDS-PAGE of a mitochondrial fraction as described under Materials and Methods. The eluted protein was subjected to a second one-dimensional SDS-PAGE separation and stained with Coomassie blue. Molecular weight standards are on the left.

FIG. 5.

Dose-dependent inhibition of hepatic CPS-I by acetaminophen in mice. Livers were collected from mice treated with saline or acetaminophen and homogenized, and CPS-I activities were determined as described under Materials and Methods. Data are means $\pm$ SEM, $n = 7-10$ per group. *CPS-I activities in mice given 200 mg/kg or more of acetaminophen were different from activities in the control group by one-way ANOVA, with Student–Newman–Keuls, $p < 0.05$. In addition, the activities in the animals treated with 200, 300, or 400 mg/kg were different from each other.

FIG. 6.

Dose-dependent inhibition of hepatic glutamine synthetase by acetaminophen in mice.

Livers were collected from the mice described in the legend to Fig. 5 and homogenized, and glutamine synthetase activities were determined as described under Materials and Methods.

Data are means $\pm$ SEM, $n = 7-10$ per group. *Activities in mice given 300 or 400 mg/kg of acetaminophen were different from activities in the control group by one-way ANOVA, with Student-Newman-Keuls, $p < 0.05$, but were not different from each other.

FIG. 7.

Dose-dependent hyperammonemia caused by acetaminophen in mice. Ammonia concentrations of plasma samples collected from the mice described in the legend to Fig. 5 were measured as described under Materials and Methods. Data are means $\pm$ SEM, $n = 7-10$ per group. *Ammonia concentrations in mice given 300 or 400 mg/kg of acetaminophen were different from levels in the other groups by one-way ANOVA, with Student–Newman–Keuls, $p < 0.05$, but were not different from each other.

FIG. 8.

Dose-dependent hepatic injury by acetaminophen in mice. Plasma was collected from anesthetized animals 6 hr after dosing, and ALT activities were determined as described under Materials and Methods. Data are means $\pm$ SEM, $n = 7-10$ per group. *Different from respective control group by one way ANOVA, with Student–Newman–Keuls, $p < 0.05$.

FIG. 9.

Time course of acetaminophen-induced inhibition of hepatic CPS-I. Male ICR mice were fasted 18 hr prior to intraperitoneal administration of 400 mg/kg of acetaminophen in saline (AP) or equal volumes of saline (CONTROL). Livers were collected from anesthetized animals 2, 4, or 6 hr after dosing and homogenized, and mitochondrial fractions were separated by differential centrifugation. CPS-I activities were determined as described under Materials and Methods. Data are means $\pm$ SEM, $n = 6-10$ per group. *Different from respective control group by ANOVA Newman-Keuls, $p < 0.05$.

FIG. 10.

Time course of acetaminophen-induced inhibition of hepatic glutamine synthetase. Glutamine synthetase (GS) activities in livers obtained from the acetaminophen or saline-treated animals described in the legend to Fig. 9 were measured as described under Materials and Methods. Data are means $\pm$ SEM, $n = 6-10$ per group. *Different from respective control group by ANOVA Newman-Keuls, $p < 0.05$.

FIG. 11. Time course of acetaminophen-induced hyperammonemia in mice. Plasma ammonia levels were measured in mice treated with acetaminophen or saline, as described in Fig. 9. Data are means $\pm$ SEM, $n = 6$ –10 per group. *Different from respective control group by ANOVA Newman–Keuls, $p < 0.05$.

FIG. 12.

Time course of acetaminophen-induced hepatic injury. Plasma was obtained from the animals treated with acetaminophen or saline as described in the legend to Fig. 9, and ALT activities were determined. Data are means $\pm$ SEM, $n = 6-10$ per group. *Different from respective control group by ANOVA Newman-Keuls, $p < 0.05$.

TABLE 1

N-Terminal Amino Acid Sequence of the Mouse Liver Mitochondrial Protein of 130 kDa: Comparison with the N-Terminal Amino Acid Sequence of Rat and Human Liver Mitochondrial Carbamyl Phosphate Synthetase I

Mouse liver mitochondrial 130 kDa protein
S V K A <u>K</u> T A H I V L E D G T
Rat and human liver mitochondrial carbamyl phosphate synthetase-I
S V K A <u>Q</u> T A H I V L E D G T

Note. The mouse hepatic mitochondrial protein isolated as in Fig. 4 was sequenced as described under Materials and Methods. The rat and human N-terminal sequences are those reported previously (Nyunoya *et al.*, 1985; Haraguchi *et al.*, 1991). The fifth amino acid in the 130-kDa protein isolated in this study is different from the published structure of rat and human liver CPS-I.