Covalent Binding of Acetaminophen to N-10-Formyl-Tetrahydrofolate Dehydrogenase in Mice

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ABSTRACT

The analgesic acetaminophen is frequently used as a model chemical to study hepatotoxicity; however, the critical mechanisms by which it produces toxicity within the cell are unknown. It has been postulated that covalent binding of a toxic metabolite to crucial proteins may inhibit vital cellular functions and may be responsible for, or contribute to, the hepatotoxicity. To further understand the importance of covalent binding in the toxicity, a major cytosolic acetaminophen-protein adduct of 100 kDa has been purified by a combination of anion exchange chromatography and preparative electrophoresis. N-Terminal and internal amino acid sequences of peptides from the purified 100-kDa acetaminophen-protein adduct were found to be homologous with the deduced amino acid sequence from the cDNA of N-10-formyltetrahydrofolate dehydrogenase. Antiserum specific for N-10-formyltetrahydrofolate dehydrogenase and acetaminophen react in a Western blot with the purified 100-kDa acetaminophen-protein adduct. Administration of a toxic dose of acetaminophen (400 mg/kg) to mice resulted in a 25% decrease in cytosolic N-10-formyltetrahydrofolate dehydrogenase activity at 2 hr. The covalent binding of acetaminophen to proteins such as N-10-formyltetrahydrofolate dehydrogenase and the subsequent decreases in their enzyme activity may play a role in acetaminophen hepatotoxicity.

The commonly used analgesic acetaminophen in overdose can result in a fulminating hepatic necrosis in humans and animals (Hinson et al., 1995). The hepatotoxicity is dependent on the biotransformation of acetaminophen by cytochromes P450 to a reactive metabolite, N-acetyl-p-benzoquinone imine (Hinson et al., 1995). N-acetyl-p-benzoquinone imine covalently binds to hepatic proteins, and this binding correlates with the hepatotoxicity (Bartolone et al., 1987; Pumford et al., 1990).

Using antibodies specific for acetaminophen-protein adducts, the covalent binding of acetaminophen has been shown to occur to certain target proteins located in various subcellular fractions (Pumford et al., 1990). The covalent modification and change in function of one or more of these proteins is believed to be involved in the mechanism of acetaminophen hepatotoxicity. In an attempt to better understand covalent binding in acetaminophen toxicity, we previously isolated two acetaminophen-protein adducts. One protein, the major adduct, was identified as the 56-kDa selenium binding protein (Bartolone et al., 1992; Pumford et al., 1992). This is a protein of unknown function that has been previously shown to interact with selenium. Recently, we have shown that the mitochondrial protein glutamate dehydrogenase also becomes covalently adducted at an early time following administration of toxic doses of acetaminophen to mice (Halms et al., 1996). Moreover, the activity of this enzyme is significantly decreased in mice treated with hepatotoxic doses of acetaminophen.

In this work we have purified and characterized a 100-kDa protein that we previously showed to be a major cytosolic target for the reactive metabolite of acetaminophen (Pumford et al., 1990). This protein is N-10-formyltetrahydrofolate dehydrogenase, a protein of importance in one carbon metabolism. The identification of this protein and the assessment of its function after hepatotoxic treatment with acetaminophen will give a better understanding of the role of covalent binding in acetaminophen hepatotoxicity.

Methods

Materials. Acetaminophen, sucrose, N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid), ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride, leupeptin, pepstatin, and diethylaminoethyl (DEAE)-Sepharose fast flow anion exchange packing material were from Sigma Chemical Co. (St. Louis, MO).

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ABBREVIATION: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.
Centricon-10 and centrprep-10 concentrators were from Amicon (Beverly, MA). Polyvinylidene difluoride membrane was obtained from Schleicher & Schuell (Keene, NH). All other chemicals and reagents were of the highest quality and purity available.

**Purification of the 100-kDa protein.** Male B6C3/F1 mice (20 animals, 8 wk old) were treated with acetaminophen (400 mg/kg in 40°C 0.9% NaCl, 25.5 ml/kg). After 2 hr, livers were removed and homogenized in 5 vol 0.01 M tris-HCl (pH 7.4), containing 0.25 M sucrose, 1 mM EDTA, 0.25 mM phenylmethylsulfonyl fluoride, 0.001 mM pepstatin and 0.001 mM leupeptin. Cytoisol was prepared as previously reported (Pumford et al., 1990). Briefly, the liver homogenate was centrifuged at 1,000 × g at 4°C for 10 min and the resulting supernatant was centrifuged at 10,000 × g for 20 min. The 10,000 × g supernatant was then centrifuged 105,000 × g for 90 min, and the supernatant (cytosol) was frozen at −80°C. Before purification the cytosol was dialyzed against two 1-liter changes of 0.01 M sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl and concentrated using Amicon centriprep-30 centrifugal ultrafiltration units. Dialyzed cytosol (1.2 g) was applied in six fractions at a flow rate of 5 ml/min to a DEAE-Sepharose fast flow anion exchange column (20 × 1 cm) using a Waters (Bedford, MA) 600E high performance liquid chromatography system that had been equilibrated with 20 mM Tris-HCl, pH 7.5. Proteins were eluted using a linear NaCl gradient. Fractions were analyzed by SDS-PAGE for their protein content and immunoblotted as reported previously with antisera specific for 3-(cystein-S-yl)acetaminophen adducts (Pumford et al., 1992). Protein concentration was determined according to the method of Bradford with bovine serum albumin as the standard (Bradford, 1976). This fraction was then concentrated approximately 10-fold using Amicon concentrsep-10 concentrators and further separated by preparative electrophoresis (model 491 Prep Cell, Bio-Rad Laboratories, Hercules, CA) by loading 27.6 mg of protein on a 7% T polyacrylamide gel. Fractions were collected and analyzed for protein content and immunochemical reactivity with acetaminophen. The purified 100-kDa protein was separated by SDS-PAGE and tested for immunochemical reaction in a Western blot using anti-N-10-formyltetrahydrofolate dehydrogenase (Cook and Wagner, 1986).

**Amino acid sequence analysis.** Purified 100-kDa protein (100 μg) in 100 mM Tris-HCl buffer (pH 8.0), containing 2 M urea and 20 mM methylene was digested with 10 μg/ml trypsin overnight at 37°C. Peptides from the digest were isolated using reverse phase high performance liquid chromatography and sequenced by automated Edman degradation. In addition, an N-terminal sequence was obtained by microsequencing (Martin and Eliason, 1991). Briefly, 15 μg of purified 100-kDa acetaminophen-modified protein was separated by SDS-PAGE and then transferred to polyvinylidene difluoride membrane; the band containing the 100-kDa protein was excised and sequenced on a Beckman (Fullerton, CA) LF3600 protein sequencer equipped with a System Gold (Fullerton, CA) model 125 on-line PTH analyzer and examined with System Gold data analysis. Sequence homology (% identity) and sequence similarity (% similarity) of both the internal peptides and the N-terminal with known proteins was determined by searching protein database using a sequence analysis software package from Genetics Computer Group, Inc. (Madison, WI) (Devereux et al., 1984).

**Enzyme activity.** The 8-wk-old B6C3/F1 male mice were treated i.p. with either 400 mg/kg of acetaminophen in 0.1 M sodium-potassium phosphate-buffered (pH 7.2) containing 0.8% wt/vol NaCl and 0.02% KCl or phosphate-buffered saline alone and killed at 2 hr (five to six animals per group). Livers were surgically removed, rinsed in phosphate-buffered saline, and homogenized in 10 vol of 0.25 M sucrose buffer (pH 7.4) containing 10 mM HEPES. Cytosol was prepared as described above. N-10-Formyltetrahydrofolate was synthesized as previously described (MacKenzie and Tan, 1980). N-10-Formyltetrahydrofolate dehydrogenase activity was determined according to Min (Min et al., 1988). Statistical analysis was performed using a Student’s t test, with the results expressed as mean ± S.E.

**Results**

Previously, we reported that the major cytosolic acetaminophen-protein adducts in liver from acetaminophen-treated mice as 100- and 55-kDa proteins. We have isolated and identified the major 55-kDa cytosolic protein containing the most intensely immunochemically stained protein as a 56-kDa selenium binding protein (Pumford et al., 1992). The next most intensely staining band in the cytosol in a Western blot using antiacetaminophen was the 100-kDa protein fraction (fig. 1) (Pumford et al., 1990; Pumford et al., 1992). The 100-kDa acetaminophen-protein adduct fraction was isolated by initial separation using DEAE anion exchange chromatography of cytosol from B6C3/F1 mice treated with 400 mg/kg acetaminophen and killed at 2 hr. The 100-kDa acetaminophen-protein adduct eluted at a NaCl concentration between 0.26 and 0.35 M NaCl. Acetaminophen-protein adducts of 50 and 115 kDa were also eluted in this fraction (fig. 1). The anion exchange 100-kDa protein fraction was then separated using preparative electrophoresis under reducing conditions, and selected fractions were stained for total protein and acetaminophen-protein adducts (data not shown). The fractions containing the 100-kDa acetaminophen-modified protein were then pooled and concentrated. The ion-exchange fraction was enriched with the 100-kDa acetaminophen-protein adduct, and after preparative electrophoresis the 100-kDa fraction appears to be at least 97% pure based on Coomassie blue stained gels (fig. 1).

The purified 100-kDa fraction was then separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane, excised and an N-terminal amino acid sequence was obtained by microsequencing and found to be 100% homologous with the deduced amino acid sequence for rat liver N-10-formyltetrahydrofolate dehydrogenase (EC 1.5.1.6; ta-

**Fig. 1.** Purification of the 100-kDa cytosolic acetaminophen-protein adduct. Figure 1 represents the progressive purification of the 100-kDa acetaminophen-protein adduct after SDS-PAGE separation of the proteins. The purification was followed by total protein stain with Coomasie blue (CBB) and immunoblotting with antiacetaminophen (anti-APAP). The individual lanes were 1) preparative electrophoresis purified APAP 100-kDa protein (5 μg), 2) partially purified APAP 100-kDa protein by diethylaminoethyl Sepharose anion-exchange chromatography (50 μg) and 3) mouse liver cytosol after 400 mg/kg acetaminophen and killed 2 hr after dosing (50 μg).
induced hepatotoxicity has been hindered by lack of methods understanding of the role of covalent binding in acetaminophen toxicity (Jollow et al., 1991). The antiserum was specific for N-10-formyltetrahydrofolate dehydrogenase as indicated by immunoreactivity with only one protein in cytosol (fig. 2). The ion exchange fraction was enriched with a protein immunochemically reactive with the rat liver N-10-formyltetrahydrofolate dehydrogenase. In addition, the preparative electrophoretically purified 100-kDa acetaminophen-protein adduct was immunochemically reactive with the rat liver anti-N-10-formyltetrahydrofolate dehydrogenase antiserum (fig. 2).

To determine if covalent binding of a reactive metabolite of acetaminophen to N-10-formyltetrahydrofolate dehydrogenase decreases the activity of the enzyme, mice were treated with a hepatotoxic dose of acetaminophen (400 mg/kg). Two hours after drug administration livers were removed and the cytosolic fraction analyzed for N-10-formyltetrahydrofolate dehydrogenase activity. As compared to controls, in acetaminophen-treated mice there was a significant decrease in N-10-formyltetrahydrofolate dehydrogenase activity (fig. 3).

**Discussion**

The mechanism of acetaminophen hepatotoxicity has yet to be identified although several mechanisms have been hypothesized including oxidative stress, disruption of calcium homeostasis, DNA fragmentation and Kuppfer cell activation. Covalent binding has also been postulated as a mechanism of acetaminophen hepatotoxicity (Mitchell, et al., 1973; Hinson, 1980; Hinson et al., 1995), and covalent binding of acetaminophen has been found to correlate with the hepato-toxicity (Jollow et al., 1973; Pumford et al., 1988). The major targets have been identified as glutamate dehydrogenase (Cook and Wagner, 1986). The individual lanes were 1) preparative electrophoresis purified acetaminophen 100-kDa protein from two different preparations (0.5 μg), 2) partially purified acetaminophen 100-kDa protein by DEAE Sepharose anion-exchange chromatography (25 μg) and 3) mouse liver cytosol after 400 mg/kg acetaminophen and killed 2 hr after dosing (100 μg). Lane 1 was developed for a longer period of time compared to lanes 2 and 3.

**TABLE 1**

<table>
<thead>
<tr>
<th>100-kDa Peptide Sequences</th>
<th>N</th>
<th>% Similarity with N-formyl THFD</th>
<th>% Identity with N-formyl THFD</th>
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<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SSW</td>
<td>3</td>
<td>100</td>
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</tr>
<tr>
<td>All Peptides</td>
<td>47</td>
<td>95</td>
<td>83</td>
</tr>
</tbody>
</table>

<sup>a</sup> THFD is tetrahydrofolate dehydrogenase.

<sup>b</sup> N-Terminal amino acid sequence.

**Fig. 2.** Immunoblot developed with anti-N-10-formyltetrahydrofolate dehydrogenase (Cook and Wagner, 1986). The individual lanes were 1) preparative electrophoresis purified acetaminophen 100-kDa protein from two different preparations (0.5 μg), 2) partially purified acetaminophen 100-kDa protein by DEAE Sepharose anion-exchange chromatography (25 μg) and 3) mouse liver cytosol after 400 mg/kg acetaminophen and killed 2 hr after dosing (100 μg). Lane 1 was developed for a longer period of time compared to lanes 2 and 3.

required to identify proteins covalently modified by acetaminophen. Recently, immunochemical methods have been developed that can be used to identify the major protein targets of acetaminophen (Bartolone et al., 1987; Roberts et al., 1987). The major cytosolic 55-kDa acetaminophen-protein adduct has been identified as a selenium binding protein (Bartolone et al., 1992; Pumford et al., 1992) and later an isomorph was identified and referred to as acetaminophen binding protein (Lanfear et al., 1993). The importance of acetaminophen binding to this protein is unknown, because the function of the protein is unknown (Bansal et al., 1990). Other proteins to which acetaminophen binds covalently have been identified in various subcellular fractions. Mitochondrial function is deceased early in the time course after a hepatotoxic dose of acetaminophen (Burcham and Harman, 1988) and appears to be an early target for a reactive metabolite of acetaminophen (Pumford et al., 1990). Two of the major targets have been identified as glutamate dehydrogenase (Halbes et al., 1996) and aldehyde dehydrogenase (Landin et al., 1996). In addition, the mitochondrial carbamyl phosphate dehydrogenase has recently been identified as a target of acetaminophen (Gupta et al., 1995). A microsomal 44-kDa acetaminophen-binding protein was found to be an early target of acetaminophen after administration of a hepatotoxic dose of acetaminophen and was identified as glutamine synthetase (Bulera et al., 1995). The activity of the mitochondrial and microsomal proteins were decreased after hepatotoxic treatment with acetaminophen. The decreases in activity of these critical enzymes may be important in acetaminophen hepatotoxicity.

We have isolated and identified N-10-formyltetrahydrofolate dehydrogenase as a target of the reactive metabolite of acetaminophen. Western blot analysis using antiacetaminophen antiserum indicated that this 100-kDa protein is a major cytosolic protein that contains covalently bound acet-
aminophen. This protein was purified by anion exchange chromatography followed by preparative electrophoresis. The sequences of the N-terminal and internal peptides indicated a high degree of homology (83%) with the deduced amino acid sequence of the previously identified rat N-10-formyltetrahydrofolate dehydrogenase. Moreover, there was more than 95% similarity in the 47 amino acids with that of the previously deduced composition of the rat protein. The small differences in sequence homology may be due to differences between rats and mice. Or alternatively, the 100-kDa acetaminophen protein adduct may be an isoenzyme of N-10-formyltetrahydrofolate dehydrogenase. Western blots of mouse liver protein separated by two-dimensional electrophoresis indicated at least two polypeptides at 100 kDa that were immunohemochromically reactive with anti-N-10-formyltetrahydrofolate dehydrogenase. Differences in isoelectric points could also be due to differences in phosphorylation. However, to be certain that the enzyme was N-10-formyltetrahydrofolate dehydrogenase, an antibody raised against the rat enzyme was used (Cook et al., 1991) and shown to be immunohemochromically reactive with the purified mouse enzyme (fig. 2), further supporting the identity of the 100-kDa acetaminophen-protein adduct.

N-10-Formyltetrahydrofolate dehydrogenase is an enzyme that is important in one-carbon metabolism. It is a major determinant in the regulation of formate oxidation in the liver. N-10-Formyltetrahydrofolate catalyzes the oxidation of formyl groups to CO₂ and has an essential cytochrome (cyt-707) at the active site of the enzyme (Cook and Wagner, 1995). N-Ethylmaleimide, a sulphydryl reagent, inhibits enzyme activity as does disulfiram. Even though the specific site of binding of acetaminophen to the enzyme has not been determined, the finding that anti-3-(cystein-S-yl)acetaminophen antiserum recognizes the enzyme indicates that the acetaminophen reactive metabolite does bind to sulphydryl groups. Moreover, analysis of hepatic N-10-formyltetrahydrofolate dehydrogenase activity indicates that after administration of a hepatotoxic dose of acetaminophen, the enzyme activity was decreased by approximately 25%. Presumably, this decrease is a result of derivatization of the critical sulphydryl group by the reactive metabolite, and the activity decrease is directly proportional to sulphydryl arylation.

The inhibition of N-10-formyltetrahydrofolate dehydronase after acetaminophen overdose may result in pathological changes within the cell. Although it has been established that mice lacking this enzyme are not compromised with respect to survival (Champion et al., 1994), the acute effects of enzyme inhibition may be entirely different. It has been suggested that inhibition of N-10-formyltetrahydrofolate dehydrogenase could result in metabolic acidosis by interfering with hepatic folate metabolism (Cook et al., 1991). Clinical case reports indicate that acidosis is a consequence of acetaminophen overdose in humans (Black et al., 1982), and this may be related to the acute inhibition of N-10-formyltetrahydrofolate dehydrogenase, which we have demonstrated in mice after treatment with a toxic dose of acetaminophen.

How covalent binding may produce the observed liver toxicity is unclear. A central hypothesis has been that covalent binding to sulphydryl groups on critical enzymes is an initiating event in development of the toxicity. We have now isolated three proteins to which acetaminophen covalently binds. We previously reported that acetaminophen covalently bound to the 55-kDa selenium binding protein (Pumford et al., 1992). This is a protein of unknown function, so it has been impossible to determine how its activity or function is altered. Recently we showed that the mitochondrial enzyme glutamate dehydrogenase is also a target of the reactive metabolite of acetaminophen (Halmes et al., 1996). At 1 hr after acetaminophen (400 mg/kg) its activity was decreased approximately 20% and further decreased to 35% at a dose of 600 mg/kg. It is interesting to note that the decrease in activities of glutamate dehydrogenase and N-10-formyltetrahydrofolate dehydrogenase are similar after the same dose of acetaminophen (400 mg/kg). From these data one could conclude that acetaminophen hepatotoxicity may be occurring when approximately 25% of the available sulphydryl groups are derivatized. Sulphydryl group derivatization presumably would lead to a proportional decrease in enzyme activities. If this is the case and it is assumed that the necrosis is a result of loss of enzyme activity after arylation, then it would appear that the critical enzyme (or enzymes) may not have to be completely inhibited for the toxicity to develop. Conversely, inhibition of the enzyme activity of one protein may not directly lead to cell death; the inhibition of many enzymes or critical pathways may be required for toxicity. Thus, how covalent binding may lead to develop-
ment of the toxicity remains a critical question. The identification of potentially critical acetaminophen-protein adducts and the demonstration of alterations in their function brings us a step closer to understanding the role of covalent binding of acetaminophen to proteins and the ensuing toxicity.

References


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