HUMAN DRUG METABOLISM
From Molecular Biology to Man

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CRC Press
Boca Raton  Ann Arbor  London  Tokyo
Chapter 6

COVALENT BINDING OF ACETAMINOPHEN ESTIMATED FROM PROTEIN ADDUCTS*

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TABLE OF CONTENTS

I. Introduction ................................................................. 44

II. Toxicity of Acetaminophen ............................................. 44
    A. Mechanism of Toxicity ............................................. 44
    B. Immunological Approach .......................................... 45

III. Development of Immunoassays to Recognize Protein-Bound Acetaminophen .............................................. 45

IV. Results and Discussion ................................................ 47

V. Conclusions .............................................................. 50

References ........................................................................... 50

* Presented in part at the Symposium on "Human Drug Metabolism" at the Federation of Societies of Experimental Biology and Medicine, April 24, 1991, Atlanta, Georgia.
Although acetaminophen-induced hepatotoxicity has been studied extensively in animals and much information is available concerning the metabolic events that lead to toxicity, the mechanism(s) that cause cell death remain undetermined. The covalent binding theory of NAPQI remains unrefuted as the primary working hypothesis two decades after it was put forward. However, other mechanisms of acetaminophen-induced cytotoxicity may be important. For example, NAPQI is an oxidizing agent\textsuperscript{20} and may induce oxidative stress.\textsuperscript{21} Thus oxidative mechanisms may play a role in acetaminophen-induced hepatotoxicity.\textsuperscript{22} From a quantitative point of view the relative importance of oxidative stress, peroxidative damage, and covalent binding remain to be determined. Nonetheless, covalent binding of acetaminophen has emerged as the most reliable biomarker of acetaminophen toxicity.

B. IMMUNOLOGICAL ASSAY APPROACH

We perceived the need for sensitive assays that do not rely on the use of radioisotopes or extensive analytical methodology and that could accurately detect protein-bound acetaminophen in biological fluids in the presence of unbound acetaminophen. To this end, a sensitive avidin-biotin-amplified ELISA (A-B ELISA) and a particle concentration fluorescence immunoassay (PCFIA), that use antiserum specific for the major acetaminophen-protein adduct associated with toxicity have emerged.\textsuperscript{16,17,23-26} These assays are new tools to study the relationship between formation of the 3-Cys-A protein adduct and acetaminophen-induced toxicity and can be applied to any species and any biological material. In addition to their use in basic toxicological research, such tools make it possible to address unresolved basic clinical questions. Examples include:

- What is the optimum $N$-acetyl cysteine dosage schedule to treat acetaminophen poisoning?
- Is a delay of treatment waiting for Prescott’s risk group classification\textsuperscript{18} hazardous to the patient?
- Is prolonged $N$-acetyl cysteine treatment beneficial due to repair of proteins modified by covalent binding of NAPQI?
- Can cytochrome P450 inhibitor(s) improve the treatment schedule of acetaminophen overdose?
- Is NAPQI formed in extrahepatic tissues with cytochrome P450 activity, e.g., olfactory nasal region, lung, kidney, testes?
- Is chronic acetaminophen use associated with end-stage renal failure?
- Are chronic alcoholic patients at higher risk if abstaining, and are alcohol-intoxicated patients at lower risk?

III. DEVELOPMENT OF IMMUNOASSAYS TO RECOGNIZE PROTEIN-BOUND ACETAMINOPHEN

Previous assays for acetaminophen covalently bound to protein required the use of radiolabeled acetaminophen that was detected after extensive solvent extraction of the protein to remove unbound radioactivity and subsequent quantification of the radiolabel associated with the protein. Since acetaminophen binding to protein is primarily via cysteine residues,\textsuperscript{14,15} an immunogen was synthesized that contained the acetaminophen-cysteine adduct. Synthetic NAPQI was reacted with $N$-acetylcysteine to produce 3-($N$-acetylcystein-S-yl)acetaminophen(NAC-acetaminophen), which was purified by HPLC. The conjugate was subsequently coupled to an immunogenic carrier protein, keyhole limpet hemocyanin (KLH), using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride as a coupling reagent (Figure 1). Rabbits were immunized with the resulting KLH-NAC-acetaminophen.\textsuperscript{17}
An antigen was also synthesized for use as a solid-phase coating antigen in the A-B ELISA and as particle-bound antigen in the PCFIA. The requirement was for an immobilized antigen that contained acetaminophen bound to cysteiny1 groups in protein, as occurs in acetaminophen toxicity. Metallothionein was selected because it contained a high molar content of free cysteine sulfhydryl groups and it reacted directly with synthetic NAPQI to yield the relevant 3-(cystein-S-yl)acetaminophen adduct.

To select the antisera best suited for detection of 3-cystein-S-yl-substituted acetaminophen (3-Cys-A) adducts in the presence of free acetaminophen, the relative inhibitory potencies of NAC-acetaminophen and acetaminophen were compared for nine antisera in competitive A-B ELISA. For all responding rabbits, NAC-acetaminophen was a more efficient inhibitor than free acetaminophen. Inhibition curves using polyclonal anti-3-Cys-A from one of the rabbits, at a dilution of 1:4308, demonstrated that NAC-acetaminophen was 6300 times more potent an inhibitor than acetaminophen.

The competitive A-B ELISA was used in initial work to characterize the epitope and to assay samples from acetaminophen-dosed animals. In subsequent work the assay was adapted to a competitive PCFIA format and this assay was used to quantify 3-Cys-A protein adducts in plasma from acetaminophen overdose cases. The PCFIA has advantages over ELISA including: a covalently coupled solid phase, shorter incubation times, and a more flexible assay format. It utilizes a fluorimeter and specially designed assay plates (Baxter Healthcare Corp., Mundelein, IL).

Since our intent was to use the competitive A-B ELISA to quantify 3-(cystein-S-yl)acetaminophen adducts in biological samples at unknown and perhaps variable levels of protein modification, it was important to determine the effect of adduct substitution levels on quantitation. The capability of the competitive immunoassay to accurately quantify acetaminophen covalently bound to protein, regardless of substitution level, was demonstrated
using standards of known substitution levels prepared by derivatizing 9000 g liver supernatants with various concentrations of [\^H]NAPQI.\textsuperscript{25}

This highly characterized antiserum specific for the acetaminophen-protein adduct was also used to correlate the immunohistochemical localization of 3-Cys-A protein adducts with the development of cell injury following acetaminophen overdose. In animals, microwave fixation is the optimum method for preservation of the antigen.\textsuperscript{16} Preliminary evidence has been presented that the antigen is also well preserved in human biopsies fixed by formaldehyde and embedded in paraffin by standard histological processing.\textsuperscript{27}

IV. RESULTS AND DISCUSSION

The relationship between formation of 3-Cys-A adducts in protein and the development of hepatotoxicity has been established in mice overdosed with acetaminophen. As demonstrated earlier by many groups, serum levels of alanine aminotransferase (ALT), an index of hepatotoxicity, increased with the dose of acetaminophen, indicating increasing hepatotoxicity. At doses below 200 mg/kg body weight no formation of 3-Cys-A adducts were observed in the liver.

The finding that 3-Cys-A protein adducts were also observed in the serum of mice that received hepatotoxic doses of acetaminophen was serendipitous.\textsuperscript{26} In a time course study (Figure 2), immunohistochemically demonstrable 3-Cys-A adducts in the liver protein reached maximum levels 2 h after a 400 mg/kg dose of acetaminophen. By 6 to 8 h, the levels decreased to approximately 10% of the maximal level. In contrast, 3-Cys-A adducts in serum protein were delayed, reaching a sustained maximum 6 to 12 h after dosing. The correlation between the appearance of serum aminotransferases and 3-Cys-A adducts in serum protein, and the temporal correlation between the decrease in 3-Cys-A adducts in liver protein and the appearance of adducts in serum protein, are consistent with a hepatic origin of the adducts detected in serum protein.

It was thus hypothesized that the adducts appearing in serum were of hepatic origin, derived from injured hepatocytes during the development of drug-induced hepatotoxicity, and postulated that serum 3-Cys-A protein adducts are a specific biomarker that can be used to study acetaminophen hepatotoxicity. Subsequently, the hepatic origin of the 3-Cys-A protein adducts in serum was confirmed by comparison of adducts detected in SDS-PAGE immunoblots of serum and hepatic protein of B6C3F1 mice at various times after acetaminophen dosing.\textsuperscript{25} More than 15 proteins containing 3-Cys-A adducts were detected in 10,000 g liver supernatants. The most prominent protein containing 3-Cys-A adducts in the hepatic 10,000 g supernatants had a relative molecular mass (M_r) of 55 kDa. Serum proteins containing 3-Cys-A adducts had molecular masses similar to those found in the liver 10,000-g supernatants (55, 87, and approximately 102 kDa). Recently, using liver homogenates from acetaminophen dosed mice, Pumford et al.\textsuperscript{28} have isolated and sequenced the major 55 kDa protein containing 3-cys-A, and demonstrated that it has 97% amino acid sequence homology with the 56 kDa selenium binding protein identified by Bansal et al.\textsuperscript{29} The possible role of this selenium binding protein in acetaminophen toxicity has not been determined. However, it is interesting to note that Western blots of serum and liver homogenates from acetaminophen overdose patients also contain a prominent 3-cys-A containing protein with a M_r corresponding to approximately 55 kDa. Collectively, these data indicate that liver adducts are released into the serum following lysis of hepatocytes.

Using immunohistochemistry,\textsuperscript{16} it was recently demonstrated that the formation, distribution, and concentration of the specific 3-Cys-A adduct in livers of acetaminophen-overdosed mice correlated with cell injury as a function of time and dose. Within the liver lobule, immunohistochemically demonstrable adduct occurred in a temporally progressive, central-to-peripheral pattern, and there was concordance between immunohistochemical staining
FIGURE 2. Time course for acetaminophen-induced hepatotoxicity and the immunochemical localization of 3-(cystein-5-sulphonic acid)acetaminophen (3-Cys-A) protein adduct in liver. Mice were treated with 400 mg/kg acetaminophen and sacrificed 0.5, 1, 2, 4, 6, and 8 h after dosing. Controls were saline-treated mice sacrificed 2 h after dosing. Liver histopathology is symbolically represented where "-" indicates no pathology (control), "1+" indicates minimal necrotic area, "2+" indicates moderate necrotic area, "3+" indicates extensive necrotic area, and "4+" indicates very extensive necrotic area. The intensity and relative density of immunohistochemically localized 3-Cys-A protein adduct is represented as "-", indicating no adduct (control), and circular symbols in which diameter and darkness of shading symbolically represent relative differences in the area and intensity of 3-Cys-A binding around representative centrilobular regions. The immunochemical quantitation of the 3-Cys-A protein adduct in hepatic 10,000-g supernates and in serum, determination of hepatic glutathione concentrations, and serum concentrations of ALT are described in Ref. 18. Data points represent the mean ± standard error for 3 animals per point, except at the 4- and 8-h times, where there were 2 animals per point. (From Roberts, D. W., Bucci, T. J., Benson, R. W., Warbritton, A. R., McRae, T., Pumford, N. R., and Hinson, J. A., Am. J. Pathol., 138, 359, 1991. With permission.)

and quantification of the adduct in hepatic 10,000-g supernatants. Furthermore, in time course experiments, adducts were detected before the appearance of centrilobular necrosis. Drug-protein binding occurred in hepatocytes at subhepatotoxic doses and before depletion of total hepatic glutathione. Intriguingly, it was also demonstrated that drug binding occurred in the hepatocellular nucleus and that adducts occurred in metabolically active and dividing cells. Finally, debris in macrophages stained positively for adduct.

The rapid disappearance of 3-Cys-A from mouse liver and the prolonged appearance of the adduct in mouse plasma is another interesting observation that has led us to hypothesize that the maximum plasma concentration of 3-Cys-A is a reflection of the total amount of covalent binding of acetaminophen in the liver. This rests on the assumption that the volume of distribution of 3-Cys-A is the blood, which can be assumed valid for proteins of this size. It should also be noted that breakdown of the protein in plasma probably is without
### TABLE 1
The Concordance Between Hepatotoxicity and Acetaminophen Protein Adducts

<table>
<thead>
<tr>
<th>Group</th>
<th>Time to NAC (h)</th>
<th>Max ALT (IU/l)</th>
<th>3-Cys-A protein adducts (nmol/mg protein)</th>
<th>Plasma acetaminophen (μmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (n = 5)</td>
<td>4 (1–7)</td>
<td>20 (10–50)</td>
<td>0</td>
<td>1.8 (0.09–2.8)</td>
</tr>
<tr>
<td>II (n = 6)</td>
<td>41 (13–74)</td>
<td>8400 (4400–14000)</td>
<td>1.9 (0.09–4.1)</td>
<td>0.4 (0–1.5)</td>
</tr>
<tr>
<td>III (n = 3)</td>
<td>10 (4–15)</td>
<td>40 (10–80)</td>
<td>0</td>
<td>1.1 (0.2–1.8)</td>
</tr>
<tr>
<td>IV (n = 16)</td>
<td>10 (2–56)</td>
<td>20 (10–60)</td>
<td>0</td>
<td>0.6 (0.04–1.3)</td>
</tr>
</tbody>
</table>

Note: Values are means; ranges are given in parentheses.

importance, since the epitope will be picked up by the assay regardless of cleavage of the protein. If these assumptions hold true, the implications are that the total amount of covalent binding of acetaminophen to hepatic proteins in man can be estimated by sequential quantification of plasma 3-Cys-A adducts. Several observations are needed to support this hypothesis in man:

1. The occurrence of 3-Cys-A in human liver following acetaminophen intoxication must be demonstrated;
2. The disappearance of hepatic adducts late in the intoxication must be established;
3. The plasma 3-Cys-A adduct time course in man must be established; and
4. Evidence must be provided that the acetaminophen-protein adducts that appear in plasma are identical to the adducts initially formed in the liver.

Assuming that such evidence is provided, maximum plasma levels might underestimate the total amount of covalent binding in the liver of nonsurviving patients if 3-Cys-A adducts are maintained in the liver at the time of death.

At present we have presented evidence that acetaminophen toxicity in humans was mediated by mechanisms similar to experimental animals.\(^{23}\) We have also reported preliminary results on identification of 3-Cys-A in human liver by immunohistochemistry.\(^{27}\) We looked for the occurrence of 3-(cystein-S-yl)acetaminophen protein adducts in plasma samples from patients who had taken an overdose of acetaminophen. The patient histories indicated that all patients, except one presenting 58 h after overdose, were immediately treated with N-acetyl cysteine antidote (NAC) i.v. at 300 mg/kg body weight. Plasma obtained at admission was assayed for serum ALT activity and acetaminophen concentration. Of the 30 patients, 11 were at high risk for developing severe liver damage according to Prescott's classification (a nomogram relating risk as a function of time and plasma acetaminophen concentration\(^{18}\)), 5 patients (Group I) were treated with N-acetyl cysteine within 8 h after overdose, 5 patients were treated later than 8 h, and one was untreated (Group II), 3 patients were at moderate risk for developing hepatotoxicity (Group III), and 16 patients were at low risk for developing hepatotoxicity (Group IV in Table 1). All patients who had liver damage, as indicated by elevated plasma ALT, had immunochemically detectable 3-Cys-A adducts in their plasma. In contrast, adducts were not found in the plasma of patients who did not show evidence of hepatotoxicity. The relationship between plasma ALT and levels of 3-Cys-A protein adducts at the time of admission is the first direct evidence of a mechanism involving 3-Cys-A adducts in acetaminophen-induced liver toxicity in man. Figure 3 depicts the observed close relationship between plasma 3-Cys-A and ALT at the time of admission.
of patients at severe risk for developing liver damage. In one patient, the risk could not be established exactly (open square) because there was no acetaminophen in the plasma. However, the urine at admission contained considerable amounts of acetaminophen metabolites, suggestive of high risk (unpublished observations).

V. CONCLUSIONS

Immunological approaches have been developed to supplement radiolabeling methods to determine covalent binding of acetaminophen to protein after overdose. These methods are applicable to the human overdose situation and give direct evidence for an identical toxic mechanism in experimental animals and man. At present it is hypothesized that the total amount of covalently bound acetaminophen in the liver can be estimated from the maximum plasma level in experimental animals as well as man. This hypothesis requires further confirmation. The primary target for covalent binding appears to be the 56 kDa selenium binding protein; however, the relevance of binding to this or to other proteins is unknown.

These new immunological tools have the advantage of specificity for the 3-(cystein-S-yl)-acetaminophen protein adduct associated with acetaminophen toxicity, and show promise for gaining further insight regarding the processes that ultimately lead to acetaminophen-induced cellular necrosis. Hopefully, such techniques and the insight they provide can be extended to improve understanding of cellular damage from other arylating agents.

REFERENCES


