IMMUNOCHEMICAL COMPARISON OF 3’-HYDROXYACETANILIDE AND ACETAMINOPHEN BINDING IN MOUSE LIVER

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ABSTRACT:
The hepatotoxicity of the analgesic acetaminophen is believed to be mediated by covalent binding to critical proteins. Radiolabeled 3’-hydroxyacetanilide, a regiosomer of acetaminophen, covalently binds to proteins at levels similar to those of acetaminophen, but without toxicity. Covalent binding has recently been detected by Western blot to a 50-kDa microsomal protein that comigrated with CYP2E1 and was accompanied by a loss of the CYP2E1 activity. However, radiolabel studies previously indicated that a significant amount of the radiolabel is lost during electrophoresis. In the present study, 3’-hydroxyacetanilide covalent binding was detected immunohistochemically in liver using an anti-acetaminophen antiserum. 3’-Hydroxyacetanilide (1000 mg/kg, ip) administration to mice resulted in panlobular immunostaining in liver, with the single layer of hepatocytes surrounding the central veins having the greatest intensity of staining. Staining was most intense at 1 hr and somewhat decreased at 3 and 6 hr. In contrast, immunohistochemical staining indicated that covalent binding of acetaminophen (250 mg/kg, ip) was confined to the centrilobular hepatocytes, the area of the ensuing necrosis. Cobaltous chloride pretreatment decreased the total intensity of the panlobular immunostaining following 3’-hydroxyacetanilide. The CYP2E1 inhibitor diallyl sulfide decreased the intensity of immunostaining in the central vein area only. Western blot analysis indicated diallyl sulfide also eliminated binding to the microsomal 50-kDa protein. These data are consistent with centrilobular binding of 3’-hydroxyacetanilide, mediated in part by CYP2E1, and panlobular binding, mediated by other P450 enzymes.

The analgesic APAP is safe at therapeutic doses but, in an overdose, produces centrilobular hepatic necrosis. Toxicity is believed to result from metabolism of APAP to the reactive metabolite NAPQI. NAPQI is detoxified by glutathione at therapeutic doses, but following toxic doses hepatic glutathione is depleted, allowing this metabolite to covalently bind to proteins. Covalent binding to critical proteins with loss of function has been postulated to be the mechanism of toxicity (Hinson et al., 1995; Pumford and Halmes, 1997).

AMAP, a regiosomer of APAP, covalently binds to proteins at levels similar to that of APAP but is not hepatotoxic (Roberts et al., 1990; Tirmenstein and Nelson, 1989). One possible explanation for the absence of hepatotoxicity of AMAP is that covalent binding of its metabolites occurs with noncritical proteins. In an effort to better understand binding to critical vs. noncritical proteins, the metabolism and toxicity of APAP and AMAP have been studied and compared. Tirmenstein and Nelson (1989) showed that APAP covalently binds to mitochondrial proteins to a greater extent than AMAP and postulated that mitochondrial binding is important in the toxicity. It was envisioned that the relative amount of binding to mitochondria may be a result of differences in the chemical properties of the respective metabolites, with the APAP metabolite less reactive than those from AMAP. Thus, it was conceived that the AMAP metabolite(s) may bind at sites more proximal to formation, whereas the APAP metabolite may diffuse into mitochondria and arylate proteins of the mitochondrial matrix (Pumford and Halmes, 1997; Rashed and Nelson, 1989; Rashed et al., 1990; Tirmenstein and Nelson, 1989).

The specific proteins to which AMAP covalently binds have also been investigated. Myers et al. (1995) used radiolabeled AMAP and APAP to compare covalent binding of the two regioisomers to mouse hepatic proteins. SDS-PAGE analysis of hepatic proteins showed a number of protein adducts from APAP, consistent with previously reported Western blot data (Bartolone et al., 1987; Pumford et al., 1990a). AMAP binding was also observed, albeit weakly, to cytosol and mitochondrial proteins. AMAP seemed to bind primarily to a 56-kDa cytosolic protein. Interestingly, the finding that AMAP covalent binding to protein was significantly decreased by ultrafiltration and by SDS-PAGE suggested more than one type of binding (Myers et al., 1995).

Subsequently, Matthews et al. (1997), using an antiseraum raised against 4-acetamidobenzoic acid (anti-APAP) and Western blots, showed that there was a major 50-kDa protein adduct in hepatic microsomes from APAP-treated mice. Significant levels of adducts were not detected in other fractions. A cytosolic adduct at 56 kDa was detected but seemed to be minor. Further studies (Halmes et al., 1997) revealed that the microsomal 50-kDa protein comigrated with CYP2E1 in vivo and in vitro, and binding was associated with a loss of 4-nitrophenol hydroxylase, an activity of this enzyme. Thus, it was postulated that AMAP was a suicide inactivator of CYP2E1.

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1 Abbreviations used are: APAP, acetaminophen; NAPQI, N-acetyl-p-quinone imine; AMAP, 3’-hydroxyacetanilide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DAB, 3,3’-diaminobenzidine; DAS, diallyl sulfide.

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In this work, we have used the same anti-APAP antiserum to analyze AMAP covalent binding using immunohistochemical analysis and the use of selective P450 enzyme inhibitors. These data indicate that AMAP covalently binds to more than one site in the liver, whereas APAP binds specifically in the centrolobular regions of the liver.

**Materials and Methods**

Chemicals. APAP, AMAP, DAB, SKF-525A, clotrimazole, indomethacin, dialyl sulfide, and cobalt chloride were purchased from Sigma.

Animals and Treatments. Adult B6C3F1 male mice (Harlan Sprague-Dawley, Indianapolis, IN) weighing 22–25 g, were used. Mice were housed on corn cob bedding in temperature- and humidity-controlled animal quarters with a 12-hr light/dark cycle and were allowed free access to water before and during the experiments. Mice were fasted for 16 hr before the APAP or AMAP dose and then given food for the duration of the experiments. The following pretreatment regimens were used to inhibit cytochrome P450 4+ activity in the liver before treatment with APAP or AMAP: cobalt chloride, 60 mg/kg ip, 24 and 48 hr before treatment; clotrimazole, 30 mg/kg ip, 1 hr before treatment; SKF-525A, 50 mg/kg ip, 30 min before treatment; or DAS, 200 mg/kg po, 2, 24, and 48 hr before treatment. Indomethacin, 10 or 30 mg/kg ip, was administered 30 min before treatment with APAP or AMAP to inhibit prostaglandin synthetase activity. After pretreatment, mice were administered a single ip dose of 250 mg/kg APAP or 1000 mg/kg AMAP in warm saline. The dose for each compound was determined in preliminary experiments to be the maximum tolerated dose that allowed survival for the duration of the experiments (24 hr). APAP, cobalt chloride, and SKF-5252A were dissolved in saline and given with an injection volume of 10 ml/kg body weight. AMAP was dissolved in saline and given with an injection volume of 20 ml/kg, as its solubility precluded using a smaller injection volume. Clotrimazole and DAS were dissolved in corn oil and given with an injection volume of 5 ml/kg body weight. Indomethacin was dissolved in 2% sodium bicarbonate and given with an injection volume of 10 ml/kg body weight. Mice were killed by carbon dioxide asphyxiation 0, 1, 3, 6, or 24 hr after AMAP or APAP administration.

**Immunohistochemical Detection of AMAP and APAP Adducts In Mouse Liver.** AMAP and APAP adducts were detected by immunohistochemistry as described elsewhere (Salminen et al., 1997) with the following modifications. Briefly, sections from formaldehyde-fixed and paraffin-embedded liver sections were cut sequentially so that liver morphology and APAP or AMAP immunostaining could be compared. The sections were deparaffinized and incubated with blocking solution (25% v/v normal bovine serum and 3% w/v purified bovine serum albumin diluted in TBS (20 mM Tris, 500 mM sodium chloride, pH 7.5)) at 37°C for 1 hr. The anti-APAP antiserum (rabbit polyclonal) used in this study was raised against 4-acetamidobenzoic acid-keyhole limpet hemocyanin, and analysis of epitope specificity of the antiserum has been reported previously (Matthews et al., 1996). The anti-APAP antiserum was diluted 1:100 in blocking solution, placed on the appropriate slides, and incubated at 37°C for 1 hr and then for 18 hr at 24°C. Primary antibody binding was localized using a biotinylated secondary antibody followed by incubation with streptavidin-linked horseradish peroxidase and the chromogenic substrate, DAB. Using the above procedure, no binding of normal rabbit serum (used as a negative control) was observed, and secondary antibody-only treated slides exhibited no binding. As a specificity control, the anti-APAP antibody exhibited no binding in livers from mice given an acute necrogenic dose of carbon tetrachloride, bromobenzene, or cocaine. Preincubation of the anti-APAP antiserum with 1 mM APAP at 37°C for 1 hr, before placing on the slides, prevented the binding of the antibody to the livers from APAP- or AMAP-treated mice.

**Western Blot Detection of AMAP Adducts on Microsomal Liver Protein.** Microsomal liver protein was isolated 3 hr after dosing from mice administered saline (control) or AMAP (1000 mg/kg, ip). The livers were homogenized and subjected to differential centrifugation to isolate microsomes as previously described (Pumford et al., 1990b). AMAP adducts on microsomal protein were detected by Western blot analysis using an anti-APAP antibody (Matthews et al., 1996). Briefly, microsomal liver protein was resolved by electrophoresis on a 7.5% discontinuous one-dimensional SDS-PAGE (30 μg protein/lane). The resolved proteins were electroblotted onto nitrocellulose membrane and then probed with the anti-APAP antibody at a 1:2,000 dilution. Bound primary antibody was detected by treatment with peroxidase-conjugated sheep anti-rabbit IgG (Boehringer Mannheim) followed by exposure of the blot to a lumino-based enhanced chemiluminescence detection agent (Amersham, UK).

**Results**

Immunohistochemical detection of AMAP (1000 mg/kg) and APAP (250 mg/kg) binding in situ was measured 1, 3, 6, and 24 hr after the dose. Similar to previous observations (Roberts et al., 1991), APAP was irreversibly bound in the centrolobular and midzonal hepatocytes of the liver at all the time points (fig. 1). No binding was observed in the perportal region. APAP immunostaining was maximal at 3 and 6 hr with staining diminished, but still extensive at 24 hr (data not shown). APAP also produced extensive morphological changes (i.e. cell swelling) in the centrolobular hepatocytes beginning at 3 hr with maximal damage and necrosis observed at 24 hr. The loss of adducts, which occurs late in the progression of the toxicity, has been shown previously to be a result of hepatocyte lysis and release of the cytosolic adducts into serum (Roberts et al., 1991). In contrast, AMAP immunostaining was not restricted to a particular region of the liver. Staining was uniform throughout the liver with the exception of...
the single layer of hepatocytes surrounding the central veins, which contained the highest levels of staining (fig. 2). In addition, some portal regions of some mice, but not all, seemed to have slightly increased levels of AMAP immunostaining. AMAP immunostaining was maximal at 1 hr with only a moderate decrease in staining at 3 and 6 hr. By 24 hr, AMAP immunostaining was barely detectable. Unlike APAP, and consistent with previous observations (Tirmenstein and Nelson, 1989; Roberts et al., 1990), AMAP did not cause any morphological changes indicative of liver injury as observed by light microscopy (fig. 2).

Previously, Roberts et al. (1990) reported that radiolabeled-AMAP binding was inhibited by cobaltous chloride pretreatment. In the present study, pretreatment with cobaltous chloride completely prevented AMAP binding as detected immunohistochemically (fig. 3). Cobaltous chloride, which is an inducer of heme oxygenase (Netter, 1987). To further explore the role of P450 enzymes in AMAP bioactivation, other inhibitors were also tested. Pretreatment with SKF-525A had no effect on AMAP immunostaining. Pretreatment with the CYP2E1 inhibitor DAS produced a moderate decrease in AMAP immunostaining in the layer of cells immediately surrounding the central veins but no effect on staining elsewhere in the lobule (fig. 4). Also, the CYP3A inhibitor clotrimazole seemed to decrease immunostaining slightly in the central areas (data not shown).

To verify the effectiveness of the inhibitors at the doses employed in the AMAP experiments, the effects of these inhibitors on APAP immunostaining and toxicity were also assessed. APAP has been previously reported to be metabolically activated by CYP2E1, CYP1A2, and CYP3A4 (Patten et al., 1993). Pretreatment of mice with the CYP3A inhibitor clotrimazole or the CYP2E1 inhibitor DAS prevented APAP immunostaining as well as the development of hepatic necrosis (data not shown). SKF 525A was less effective and produced only a moderate reduction in immunostaining and severity of APAP-induced lesions.

It has been previously demonstrated by Western blot that the major hepatic adduct of AMAP is a 50-kDa protein (Matthews et al., 1997). Subsequently, it was determined that this 50-kDa protein comigrated in a Western blot with CYP2E1 and that treatment with AMAP both in vivo and in vitro resulted in decreased CYP2E1 activity (Halmes et al., 1997). To examine the relationship between the partial reduction in AMAP immunostaining by DAS and effects on AMAP binding to target proteins, hepatic proteins from AMAP-treated mice with and without DAS pretreatment were separated by SDS-PAGE and probed with the anti-APAP antibody in a Western blot. As shown in fig. 5, DAS pretreatment prevented binding of AMAP to this protein.

Lastly, the importance of prostaglandin synthetase on covalent binding of AMAP was determined. This enzyme has been implicated in the metabolism and toxicity of APAP (Ben-Zvi et al., 1990), and...
the cyclooxygenase activity of prostaglandin synthetase relies upon a functional heme moiety. The cyclooxygenase inhibitor indomethacin was ineffective in inhibiting AMAP immunostaining, suggesting that prostaglandin synthetase is not responsible for AMAP-reactive metabolite formation and binding (data not shown).

**Discussion**

Studies nearly 25 years ago demonstrated the relationship between hepatotoxicity of APAP and covalent binding (Jollow et al., 1973), and the mechanism of covalent binding of APAP has been extensively...
studied by many investigators. Data indicate that the toxicity is mediated by metabolic bioactivation of the drug by CYP2E1, CYP1A2, and CYP3A4 to NAPQI, which binds to protein (Patten et al., 1993). Covalent binding has been shown by immunohistochemical analysis to occur in the hepatocytes of the centrilobular and midzonal areas of the liver, and there is a high degree of correlation between the site of covalent binding and development of the necrosis (Bartolone et al., 1987; Roberts et al., 1991). In this study, we have shown that treatment of mice with the CYP2E1 inhibitor DAS decreases APAP covalent binding and toxicity. Also, the CYP3A4 inhibitor clotrimazole decreased binding and toxicity. These data further support the role of these enzymes in the metabolic activation of APAP.

The immunohistochemical staining of livers of mice treated with APAP was most interesting. The detection of panlobular binding of AMAP was unexpected. Previously, it was shown by Western blot that AMAP covalently bound both in vivo and in vitro to a microsomal enzyme that comigrated with cytochrome P450 2E1 (Halmes et al., 1997; Matthews et al., 1997). Also, it was shown that the CYP2E1 specific activity, 4-nitrophenol hydroxylase, was inhibited both in vivo and in vitro. Thus, binding was expected to be centrilobular (zone 3), and detection of binding over the central, midzonal, and the periportal areas (panlobular) of the liver indicated that more than one mechanism of binding was operative. This conclusion was supported by inhibition studies. Treatment of mice with the CYP2E1 inhibitor DAS was shown by Western blot to decrease binding to the 50-kDa microsomal protein and to decrease somewhat binding in the central areas of the liver (fig. 4). These data are consistent with one of the mechanisms of covalent binding being catalyzed by CYP2E1; however, this treatment did not substantially decrease the panlobular nature of the binding. Thus, the panlobular binding was definitely not a result of metabolism of AMAP by CYP2E1 to a metabolite that subsequently bound in the periportal region. CYP2E1-mediated formation of these reactive metabolites is also improbable because it would require retrograde diffusion of the metabolites from their site of formation in central regions of the lobule to periportal areas. However, the finding that cobaltous chloride treatment of mice decreased panlobular binding strongly suggests involvement of heme-containing proteins in this bioactivation. This treatment is known to suppress the levels of a number of P450 enzymes and has been previously shown to decrease covalent binding of radiolabeled AMAP. Also, it should be pointed out that although certain enzymes such as CYP2E1 and CYP3A are localized in the centrilobular areas of the liver, other enzymes such as CYP1A2, CYP2A, CYP2B, and CYP2C have been shown in humans to be expressed uniformly throughout the liver acinus (Palmer et al., 1992). Thus, there are a number of candidate cytochrome P450 enzymes that may be important in AMAP covalent binding.

The chemical structure of the AMAP covalently bound adducts is of interest. In a previous work, Halms et al. (1997) reported that AMAP, but not APAP, was a mechanism-based inhibitor of CYP2E1 and covalently bound to the enzyme. A mechanism-based inhibitor suggests that the reactive metabolite is formed at the active site of the enzyme but does not leave this site and covalently binds to structural or catalytically active groups at the site. Binding may occur to nucleophilic groups on the primary structure of the enzyme with or without the heme. Both mechanisms may result in enzyme inactivation. AMAP-reactive metabolites have been described that react with protein nucleophiles by Michael addition mechanisms (Rashed and Nelson, 1989). Alternatively, in the formation of these products, radical intermediates may be formed that may be captured by the heme prosthetic groups. The latter mechanism has been well described as a characteristic of most P450 mechanism-based inhibitors (Netter, 1987). Because the CYP2E1 adduct can be separated and assayed by Western blot, it seems likely that the adduct is associated with the primary structure of the enzyme and that this technique would not detect any heme adducts. However, it seems possible that in the immunohistochemical analysis, which is a much milder condition than the Western blot, both heme adducts and primary structure adducts may be detected. Also, this postulation may explain the radioactive data of Myers et al. (1995). These investigators showed that ultrafiltration or PAGE analysis of radioactive protein-AMAP adducts caused partial loss of radioactive adducts.

In conclusion, immunohistochemistry seems to be a useful tool to examine the binding of AMAP-reactive metabolites in liver and their relationship to toxicity. Observations using this approach indicate fundamental differences between AMAP and its regioisomer APAP in terms of the localization of reactive metabolite binding within the lobule and in the enzyme(s) responsible. Although the results here are consistent with a role for CYP2E1, there seems to be one or more other enzymes that are also responsible for AMAP bioactivation that have not as yet been identified. Most importantly, the data clearly indicate that the metabolism and mechanisms of covalent binding of AMAP are very different from that of APAP.

References