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Role of CYP2A5 and 2G1 in Acetaminophen Metabolism and Toxicity in the Olfactory Mucosa of the *Cyp1a2(−/−)*Mouse

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ABSTRACT. Acetaminophen (AP) is a widely-used analgesic agent that has been linked to human liver and kidney disease with prolonged or high-dose usage. In rodents, the target organs that are affected include liver, kidney, and the olfactory mucosa. AP toxicity requires cytochrome P450(CYP)-mediated metabolic activation, and the isozymes CYP1A2, 2E1, and 3A are known to activate AP in the human. In the present study, we determined that olfactory mucosal toxicity of AP was not different between the *Cyp1a2(+/+)* wild-type and the *Cyp1a2(−/−)* knockout mouse, whereas the hepatic toxicity of AP was significantly diminished in *Cyp1a2(−/−)* mice. Western blots of olfactory mucosa revealed that CYP2E1 and CYP3A levels are similar between untreated *Cyp1a2(+/+)* and *Cyp1a2(−/−)* mice. Diallyl sulfide (DAS), a known inhibitor of CYP2E1 and of CYP2A10/2A11 (the rabbit orthologue of mouse CYP2A5), completely eliminated olfactory toxicity of AP in both the *Cyp1a2(−/−)* and wild-type mouse olfactory mucosa. We found that heterologously expressed mouse CYP2A5 and CYP2G1 enzymes (known to be present in olfactory mucosa) form 3-hydroxyacetaminophen (3-OH-AP) and 3-(glutathion-S-yl)acetaminophen (GS-AP); CYP2A5 is considerably more active than 2G1. Addition of GSH caused increases in GS-AP proportional to decreases in 3-OH-AP, suggesting that these two metabolites arise from a common precursor or are formed by way of competing pathways. We also found that both CYP2A5 and CYP2G1 are inhibitable by DAS *in vitro*. These studies provide strong evidence that, in addition to CYP2E1, CYP2A5 and 2G1 are important in AP bioactivation in the mouse olfactory mucosa and that CYP1A2 appears to be of minor importance for AP olfactory toxicity. BIOCHEM PHARMACOL **55**;11: 1819–1826, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. olfactory mucosa; CYP2A5; *Cyp1a2(−/−)* knockout mouse; acetaminophen; CYP2G1; metabolism

AP** is a widely-used analgesic agent which has been linked epidemiologically to human liver and kidney disease following prolonged or high-dose usage, and exhibits toxicity to several organ systems in rodents [1–4]. Among the target organs affected by AP in the rodent are the liver, kidney, and the olfactory mucosa [5–7]. The toxicity of AP is associated with CYP-mediated metabolic formation of several tissue-reactive metabolites, including a quinone imine [8] which binds to tissue macromolecules and glutathione [9–10]. Conditions associated with increased hepatic glutathione (e.g. administration of prednisolone, S-adenosylmethionine, or clofibrate) are associated with decreased AP toxicity and fewer AP metabolites [11–13].

Three major P450 forms—CYP3A, CYP2E1 and CYP1A2—are known to have significant metabolic activity toward AP [9, 14]. Administration of an inhibitor of CYP2E1 resulted in decreased hepatotoxicity of AP [15]. It has also been reported that CYP2E1 and an AP-binding protein colocalize by immunohistochemistry in olfactory mucosa [16]. In addition, CYP2A10/11, expressed predominantly in the rabbit olfactory mucosa (low levels are also present in the liver), has been shown to metabolize phen-

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** *Abbreviations:* AP, acetaminophen; CYP, cytochrome P450; DAS, diallylsulfide; GS-AP, 3-(glutathion-S-yl)acetaminophen; GSH, reduced glutathione; H&E, hematoxylin and eosin; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; NADPH, β-Nicotinamide adenine dinucleotide phosphate (reduced); 3-OH-AP, 3-hydroxyacetaminophen; SDS, sodium dodecyl sulfate.

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acetin, which is structurally-related to AP [17–19]. CYP2A10/11, originally purified from rabbit olfactory mucosa, appears to correspond to CYP2A3 in the rat [17, 20] and CYP2A5 in the mouse [21]. To date, CYP2G1 expression has been reported only in the olfactory mucosa [22–24]; this enzyme has activity toward various substrates, including testosterone and progesterone [24–25]. CYP2A5 and CYP2G1 are abundant isoforms in the mouse olfactory mucosa and may together account for more than 30% of the total P450 in this tissue [26].

The role of CYP1A2 in the metabolism of various xenobiotics has recently been evaluated in a novel model system, the *Cyp1a2(−/−)* knockout mouse [27]. These mice lack detectable *Cyp1a2* mRNA and protein, yet exhibit normal development, viability, and fertility [27]. In these mice, metabolism of the muscle relaxant zoxazolamine, a known substrate for CYP1A2, is markedly decreased [27].

The purpose of this report is to evaluate AP toxicity in two target tissues, the liver and the olfactory mucosa. Whereas we demonstrate a significant decrease in liver toxicity of AP in the *Cyp1a2(−/−)* knockout mouse, compared with the *Cyp1a2(+/+)*, the toxicity of AP in the olfactory mucosa is not different between the *Cyp1a2(−/−)* and *Cyp1a2(+/+)* mice. This suggests that CYP1A2 may only be a minor route of activation of AP in the nasal cavity. *In vitro* studies with heterologously expressed mouse CYP2A5 and 2G1 revealed that both of these enzymes are active in AP metabolism. Taken together with previous CYP2E1 studies, these observations suggest that three CYPs—CYP2E1, 2G1, and 2A5—are more important than CYP1A2 in AP bioactivation in the mouse olfactory mucosa.

MATERIALS AND METHODS

Chemicals

Uniformly ring-labeled[^{14}C]-AP (6.3 Ci/mol), unlabeled AP, and DAS were obtained from Sigma. [Glycine-2-^{3}H]-GSH (44.8 Ci/mmol) was purchased from DuPont/NEN.

Animals

Cyp1a2(+/+) wild-type homozygotes and *Cyp1a2(−/−)* knockout homozygotes were littermates generated by crossing the *Cyp1a2(+/−)* heterozygote females and males. These mice were bred in the Nebert mouse colony at the University of Cincinnati Department of Laboratory Animal Medicine and identified by PCR amplification of DNA obtained from tail clips and Southern blot analysis as previously described [27]. Mice were housed 2–3 per cage in standard shoebox cages with microisolator tops and were maintained on a 12 hr light:dark cycle. They were allowed free access to rodent chow and tap water.

Twenty-one *Cyp1a2(+/+)* wild type and 20 *Cyp1a2(−/−)* knockout mice (ages 8–16 weeks) were randomly assigned to treatment groups and administered one of the following dosing regimens: a) AP (600 mg/kg); b) DAS (500 mg/kg), followed 4 hr later by AP; c) DAS alone; d) vehicle(s) only. AP was administered by i.p. injection in warm physiological saline, and DAS was administered by i.p. injection in corn oil (final volume of either vehicle was 100 μl per 25 g of body weight in each case). Mice were killed 24 hr later by carbon dioxide asphyxiation.

Histopathology

Nasal cavities were fixed by retrograde perfusion with 10% neutral buffered formalin (NBF), followed by immersion fixation in the same fixative. Samples of liver were similarly fixed in NBF. After 24 hr, liver samples were placed in 70% ethanol, while nasal cavities were decalcified in 7.5% formic acid for 5 days. After washing overnight in cold running tap water, nasal cavities were sectioned as previously described [28]. Level 2, 3, and 4 nasal cavity sections, as well as liver samples, were embedded in paraffin using standard techniques, sectioned at 5 μm, and stained with H&E for light microscopic evaluation.

Western Blot Analysis of Microsomal Proteins

Cyp1a2(+/+) wild type and *Cyp1a2(−/−)* knockout mice (two of each) were killed for preparation of microsomal proteins from the olfactory mucosa, liver, and kidney as previously described [29]. Protein concentrations were determined using the BioRad protein reagent. Microsomal proteins (3 μg/lane) were separated on 10% SDS-polyacrylamide minigels under denaturing conditions. Separated proteins were transferred to nitrocellulose and visualized with Ponceau S to verify equivalent loading across lanes. Western blot analysis was performed using three antibodies: polyclonal goat anti-CYP2E1 and polyclonal rabbit anti-CYP3A4 (obtained from Oxford Biomedical Research) and goat anti-CYP1A1/1A2 (Gentest). HRP-conjugated secondary antibodies (Dako) and ECL Chemiluminescent System (Amersham) were used for visualization.

Assay for AP Metabolism

Baculovirus-mediated heterologous expression of mouse CYP2G1 in insect cells has been described recently [24]. The bacterial expression assay for CYP2A5 was performed as described [30]. Recombinant CYP2A5 and CYP2G1 have been purified to electrophoretic homogeneity, with specific contents of approximately 6.8 (CYP2A5) and 6.7 (CYP2G1) nmol P450/mg of protein [26]. Purification of rabbit liver microsomal NADPH-P450 reductase, rabbit CYP2E1, and rabbit CYP1A2 has been described previously [20, 25]. Purified rat CYP1A1 was a gift from Dr. Laurence Kaminsky of the Wadsworth Center [31]. The activity of recombinant P450 isoforms in AP metabolism was determined essentially as previously described [32], except that a higher concentration of reduced glutathione (10 mM of

TABLE 1. Metabolism of acetaminophen by purified mouse CYP2A5 and CYP2G1

Substrate concentration (mM)	GSH addition (mM)	Rate of product formation (nmol/min/nmol P450)			
		CYP2A5		CYP2G1	
		3-OH-AP	GS-AP	3-OH-AP	GS-AP
0.2	0.0	8.8	<0.1	2.2	<0.1
0.2	10.0	6.7	22.5	1.7	9.2
0.5	0.0	12.9	<0.1	3.4	<0.1
0.5	10.0	9.3	30.0	2.2	13.4
2.0	0.0	18.8	<0.1	4.6	<0.1
2.0	10.0	13.9	40.4	3.1	17.2

Reaction mixtures contained 50 mM of potassium phosphate buffer (pH 7.6), a reconstituted system containing 0.2 μM of P450, 0.6 μM of NADPH-P450 reductase, 30 μg/ml of phospholipid, and [^{14}C]AP and GSH at the concentrations indicated. Reactions were initiated by the addition of NADPH to 1.0 mM. After incubation at 37° for 10 min, reactions were stopped and metabolites were analyzed by HPLC. Reactions were carried out in duplicate and values reported are the averages of 2–4 determinations, with differences <10% of the mean.

GSH) was used. Substrate concentrations were selected based on previously-derived K_m values [32]. Contents of individual reaction mixtures are indicated in the legend to Table 1. Reactions were initiated by the addition of NADPH to a final volume of 250 μL and, after incubation at 37° for 10 min, were terminated by the addition of 125 μL of 3 M of perchloric acid. HPLC analysis of AP metabolites was performed with a Waters μBonadpak C_{18} column, according to Harvison *et al.* [33], with a radiometric HPLC system as described recently [20]. Metabolites were detected by absorbance at 250 nm and by radioactivity measurements with [^{14}C]AP as the substrate. Metabolites were identified on the basis of comigration with standards generated in enzymatic reactions with CYP1A1 in a reconstituted system (data not shown), which was previously shown to metabolize AP to 3-OH-AP and GS-AP [33]. Quantitation of 3-OH-AP and GS-AP was carried out by measuring radioactivity of individual peaks with an online radioactivity detector, and the amounts were calculated based on percent recovery of total radioactivity.

RESULTS

Olfactory Toxicity of AP in Cyp1a2*(+/+) Wild-Type and* Cyp1a2*(−/−) Knockout Mice*

Histopathologic evaluation (Fig. 1) revealed that acetaminophen-induced damage was observed in centrilobular hepatocytes in the *Cyp1a2(+/+)* wild-type mice. In contrast, the *Cyp1a2(−/−)* knockout mice exhibited no evidence of centrilobular necrosis (Fig. 1). Both the wild-type and the CYP1A2-deficient mice suffered similar damage to the olfactory mucosa covering the nasal turbinates. Interestingly, the nasal mucosal damage varied extensively in its severity. Approximately half of the affected mice (both *Cyp1a2(+/+)* and *Cyp1a2(−/−)*) displayed extremely focal degenerative lesions, occurring primarily in three regions in the nasal cavity: a) the junction of respiratory and olfactory mucosa on the nasal turbinates and the nasal septum, appearing to involve short sections of both olfactory and respiratory epithelium; b) the olfactory epithelium lining the dorsal medial meatus of the nasal cavity in level 3 and 4 nasal cavity sections [28] (Fig. 2); and c) the olfactory epithelium covering the second ethmoturbinate [34] and the adjacent lateral wall (Fig. 2). The other half of the acetaminophen-treated mice (both *Cyp1a2(+/+)* and

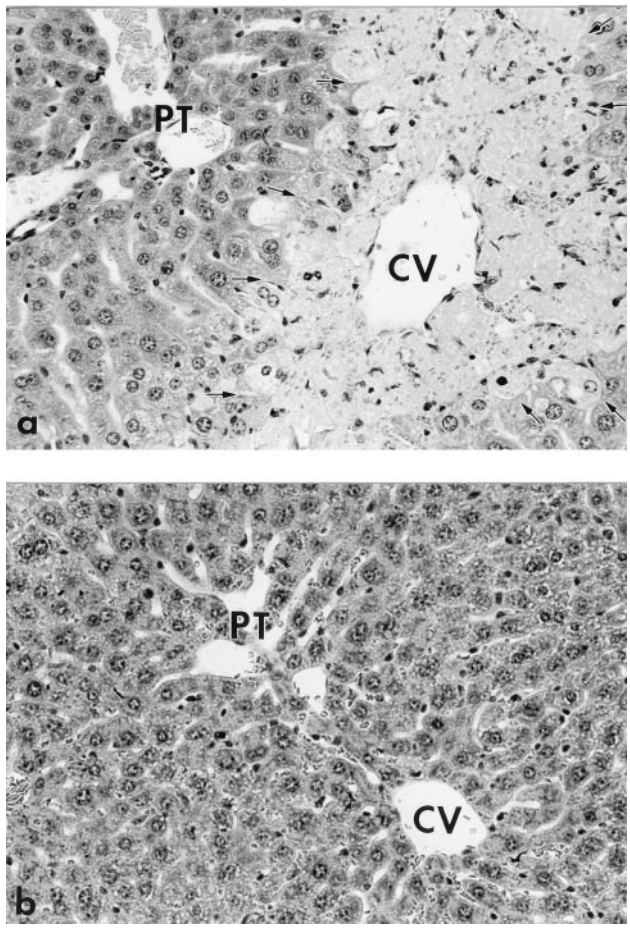

FIG. 1. Light micrographs of wild-type (a) and *Cyp1a2(−/−)* (b) liver sections following administration of AP (600 mg/kg i.p.). Note cellular necrosis in a centrilobular distribution (arrows) in the wild-type mouse, while the knockout is protected from AP-induced toxicity. Hepatocytes surrounding the portal triad (PT) appear unaffected in both mice. CV = central vein. H&E, 67×.

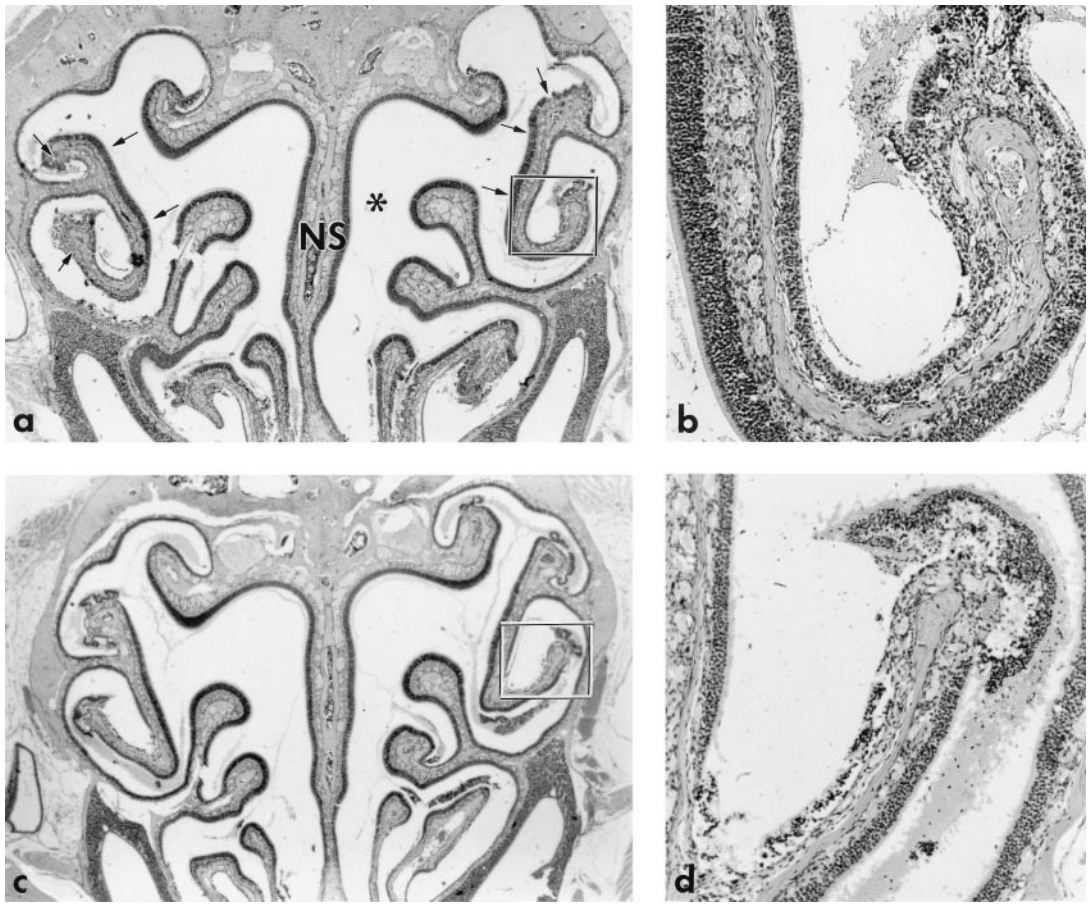

FIG. 2. (a) Low power photomicrograph of a frontal section through a wild-type mouse nasal cavity 24 hr after administration of AP (600 mg/kg i.p.). In both wild-type and knockout mice, lesions most often affected the 2′ ethmoturbinate (arrows). The area enclosed in the box is enlarged in (b). (b) Sloughing of the olfactory mucosa of the 2′ ethmoturbinate following AP treatment. (c) Low power photomicrograph of a frontal section through a *Cyp1a2(−/−)* knockout mouse nasal cavity 24 hr after administration of AP (600 mg/kg i.p.). The area enclosed in the box is enlarged in (d). (d) Sloughing of the olfactory mucosa of the 2′ ethmoturbinate in the knockout mouse is virtually identical to that seen in the wild-type mice. Asterisk (*) marks nasal airways; NS = nasal septum. H&E, (a) and (c) 7.2×, (b) and (d) 35×.

Cyp1a2(−/−)) displayed degenerative lesions involving 100% of the olfactory mucosa (Fig. 3). Pretreatment with DAS completely eliminated the olfactory toxicity of AP in both wild-type and knockout mice (Fig. 3).

Western Blots

Western blot analysis revealed the presence of CYP3A (detected with an anti-CYP3A4 antibody), CYP2E1, and CYP1A2 in the olfactory mucosa and liver of *Cyp1a2(+/+)* wild-type mice. CYP3A and CYP2E1, but not CYP1A2, were likewise present in the liver of the *Cyp1a2* knockouts at levels similar to those in the wild-type mice (Fig. 4). We did not detect CYP3A or 1A2 in the kidney of *Cyp1a2(+/+)* or *Cyp1a2(−/−)* mice (Fig. 4). The percentage of CYP1A2 per unit of P450 was not determined for either liver or olfactory mucosal samples. However, based on the data in Fig. 4, there is unlikely to be more than a two-fold difference in CYP1A2 content between liver and olfactory mucosal microsomes.

Metabolic Activation of AP by Mouse CYP2A5 and CYP2G1

The rates of formation of 3-OH-AP and GS-AP were determined in a reconstituted system at three different AP concentrations (Table 1). The levels of NADPH-P450 reductase and GSH added to the reconstituted system were saturating. Both CYP2A5 and 2G1 were active in 3-OH-AP formation, although CYP2A5 was much more active than 2G1. Formation of 3-OH-AP was not dependent on GSH (Table 1), whereas formation of GS-AP, as confirmed by its detection by using either [^{3}H]GSH or [^{14}C]AP (data not shown), was dependent upon GSH addition. Addition of GSH to the reaction mixture resulted in formation of GS-AP at rates that are much higher than for 3-OH-AP formation at all AP concentrations. Intriguingly, we found in all reactions that increases in GS-AP formation were accompanied by decreases in 3-OH-AP formation. In other experiments not presented, purified rabbit CYP2E1 metabolized AP to GS-AP at a rate of 1.4 nmol/min/nmol of P450 with substrate at 2 mM and purified rabbit CYP1A2

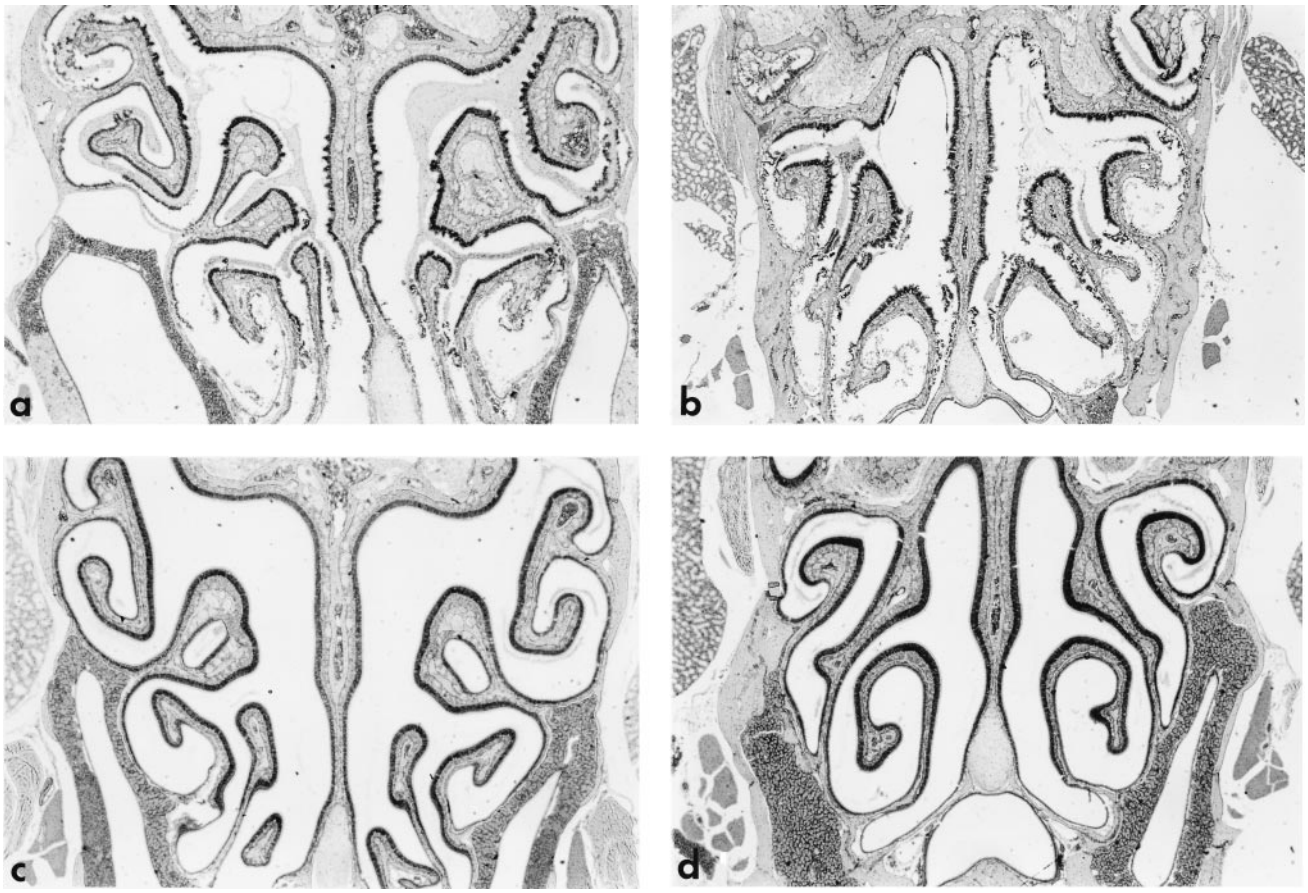

FIG. 3. More severe olfactory mucosal damage induced by AP in (a) *Cyp1a2(−/−)* and (b) wild-type mice. Note sloughing of nearly 100% of the olfactory epithelium into the airway. (c) *Cyp1a2(−/−)* mouse pretreated with DAS (500 mg/kg) 4 hr prior to AP administration (600 mg/kg i.p.). (d) Wild-type mouse pretreated with DAS (500 mg/kg) 4 hr prior to AP treatment (600 mg/kg i.p.). Note that the olfactory mucosa appears completely intact in DAS pretreated mice. H&E, 5×.

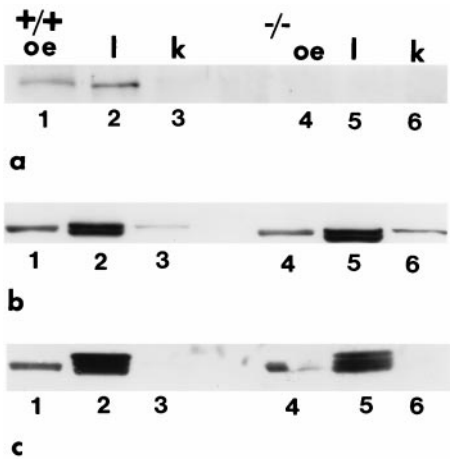

FIG. 4. Western blot analysis of olfactory, liver and kidney microsomes from wild-type mice (Lanes 1–3) and *Cyp1a2(−/−)* mice (last three lanes) using antibodies for (a) CYP1A2; (b) CYP2E1; and (c) CYP3A4. All three isozymes are present in olfactory and liver microsomes in the wild-type mice, but CYP3A and 1A2 appear to be absent from kidney. HRP-conjugated secondary antibodies and a chemiluminescent detection system were used for visualization.

metabolized AP to GS-AP at a rate of 3.1 nmol/min/nmol of P450 with AP at 10 mM.

Inhibition of AP Metabolism by DAS

We have already shown above that DAS completely blocks olfactory toxicity of AP in the intact mouse. Figure 5 illustrates that DAS inhibited both CYP2A5 and CYP2G1 activities in a dose-dependent manner. At the highest concentration of DAS tested (0.5 mM, equal molar mass with the substrate), the formation of both 3-OH-AP and GS-AP was almost completely abolished. Thus, it seems likely that the block in AP toxicity by DAS which was observed in the intact animal (Fig. 3) might reflect, at least in part, the inhibition of CYP2A5 and CYP2G1 activities.

DISCUSSION

The present study demonstrates that the liver toxicity of AP is decreased in the *Cyp1a2(−/−)* knockout mouse. It has been reported that CYP2E1 is important in AP-mediated liver toxicity at low AP doses, whereas CYP1A2

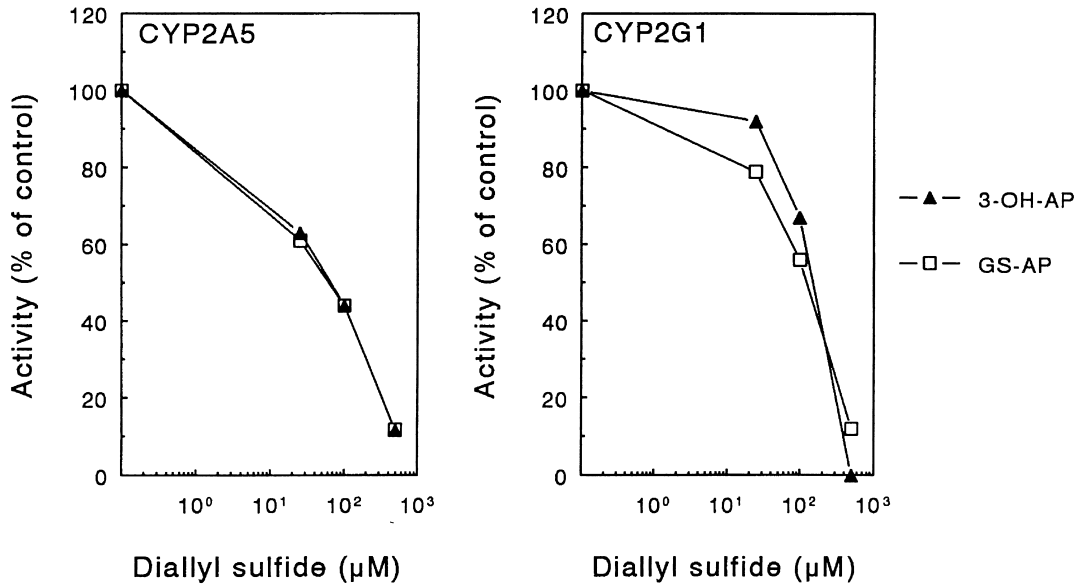

FIG. 5. Inhibitory effect of DAS on the formation of AP metabolites by CYP2A5 and 2G1. Assay conditions were the same as described in Table 1, with 0.5 mM of AP and 10 mM of GSH. DAS, ranging from 0.025 to 0.5 mM, added in 2.5 μL of methanol, or methanol alone (control) was added to the reaction mixture in a final volume of 250 μL. The values reported represent average of two determinations with differences <20% of the mean.

plays a larger role in hepatic toxicity at higher doses [35]. Because we used a high AP dose (600 mg/kg; Fig. 1), our data are consistent with those of Snawder *et al.* [35].

This study also demonstrates that the olfactory mucosal toxicity is not CYP1A2-dependent, suggesting that one or more other forms of P450 in the olfactory mucosa are active in AP bioactivation. In the olfactory mucosa, we found that, in addition to CYP1A2 seen only in the *Cyp1a2(+/+)* wild-type mouse, both CYP2E1 and CYP3A (as recognized by an anti-CYP3A4 polyclonal antibody) are present at similar levels in both the *Cyp1a2(+/+)* and *Cyp1a2(−/−)* mouse (Fig. 4). It should be noted that the mouse has three characterized *Cyp3a* genes—encoding CYP3A11, 3A13, and 3A16 [36]; whether the anti 3A4 antibody used (raised against human CYP3A4) recognizes one or more of these *Cyp3a* gene products, or another as yet uncharacterized *Cyp3a* gene, is not known. In addition, in this study we demonstrate for the first time the capacity of both the olfactory-specific CYP2G1, and the olfactory-predominant CYP2A5, to metabolize AP. Therefore, it appears that CYP1A2 plays a minor role in the bioactivation of AP in the mouse olfactory mucosa.

The presence of multiple enzymes capable of metabolic activation is likely to be important in the pathogenesis of AP-induced lesions. Tee *et al.* [37] determined that AP toxicity was correlated with the capacity to form the toxic quinone imine, and was unrelated to subsequent detoxification steps. Emeigh Hart and collaborators [16] reported colocalization of CYP2E1 and an AP-binding protein by immunohistochemistry in both liver and olfactory mucosa, suggesting that CYP2E1 plays a role in the nasal toxicity of AP. It is clear that both CYP2E1 and CYP1A2 play a role in hepatic AP bioactivation, as the toxicity of this drug is greatly diminished in the *Cyp1a2(−/−)* mouse (present study) and in the *Cyp2e1(−/−)* mouse [38]. In addition, there is indirect evidence that one or more metabolic enzymes present in the olfactory mucosa but not in the liver may contribute to AP bioactivation. For example, the structurally-related compound phenacetin was demonstrated to be an excellent substrate for O-deethylation by CYP2A10/2A11 [17–18].

When we found that DAS prevented AP-induced damage to the olfactory mucosa in the mouse (Fig. 3), our original interpretation was that DAS had inhibited CYP2E1, as has been previously demonstrated [39–40]. Subsequent *in vitro* metabolism studies (Fig. 5), however, demonstrated that both CYP2A5 and CYP2G1 can also be inhibited by DAS.

There are two novel findings in the present study. First, AP is a substrate for both the olfactory-predominant CYP2A5 and the olfactory-specific CYP2G1. Second, both of these enzymes can be inhibited by DAS, a finding which seriously undermines previous claims that DAS is a relatively specific inhibitor of CYP2E1. Formation of both GS-AP and 3-OH-AP represent the formation of reactive intermediates that can cause cytotoxicity [41]. Studying heterologously expressed CYP2A5 and 2G1 (Table 1), we found that the rates of formation of both 3-OH-AP and GS-AP are much higher than those reported for a number of rat [33], rabbit [32] and human [42–43] P450 enzymes. This observation and previous findings that orthologous CYP2A and CYP2G isoforms may account for more than 20% of total olfactory microsomal P450 in rabbits [18], strongly suggest an important role for CYP2A5 and 2G1 in AP-mediated olfactory toxicity. The levels of CYP2A5 and CYP2G1 in mouse olfactory microsomes have not been

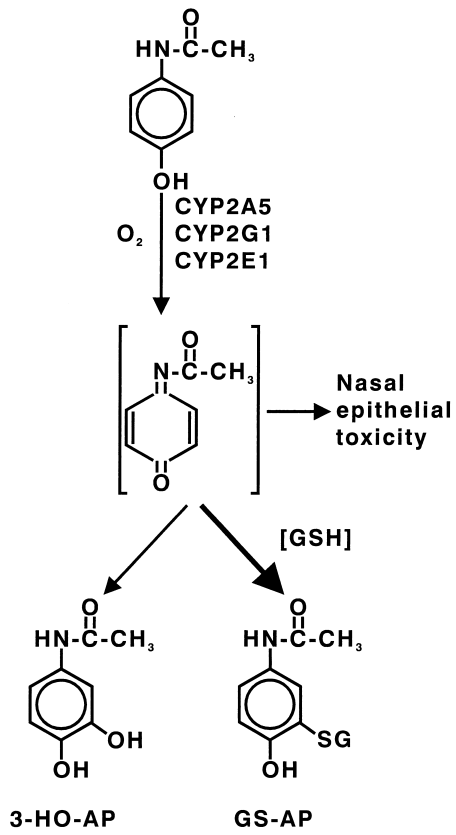

FIG. 6. AP metabolic pathway consistent with the results of the present study.

determined because monospecific antibodies to these mouse isoforms are not yet available. However, preliminary studies with a rabbit anti-CYP2A5 antibody that also recognizes mouse CYP2G1 suggest that the combined level of the two isoforms may account for at least 30% of total P450 in olfactory microsomes from C57BL/6 mice [26].

We also found that increases in the rate of GS-AP formation were accompanied by corresponding decreases in the rate of 3-OH-AP in all reactions (although GS-AP formation is 2- to 6-fold greater than 3-OH-AP formation). Our data suggest that the two metabolites are derived from a common precursor as has been previously hypothesized [44], or formed by way of competing pathways. This interpretation differs from that of other investigators, who have proposed that GS-AP is likely to be the product of the toxic quinone imine, whereas the catechol 3-OH-AP was felt to represent a distinctly different pathway [41, 45]. Our data are consistent with both the GS-AP and 3-OH-AP metabolites arising from the same P450-mediated unstable quinone imine intermediate (Fig. 6).

Finally, in contrast to the differences in CYP1A2-dependent AP toxicity found in mouse liver, the present study shows that AP caused both the wild-type and CYP1A2-deficient mice to suffer similar olfactory mucosal damage. Interestingly, we found that the olfactory mucosal damage varied extensively in its severity. Approximately half of the *Cyp1a2(+/+)* and half of the *Cyp1a2(−/−)* mice—which were littermates—suffered 100% olfactory mucosal degeneration, while the other half had very minor lesions. This observation is most likely due to the heterogeneous genetic background of this (and all) knockout mouse lines when they are first produced (reviewed in [46]). Because these mice were derived from the C57BL/6J and 129/Ola inbred strains [27], future work from our laboratories will examine this putative inbred strain-specific difference in AP-induced toxicity of the olfactory epithelium.

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