Biotransformation of Coumarin by Rodent and Human Cytochromes P-450: Metabolic Basis of Tissue-Selective Toxicity in Olfactory Mucosa of Rats and Mice

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ABSTRACT

Coumarin was previously found to cause tissue-selective toxicity in the olfactory mucosa (OM) of rats and mice, with rats being the more sensitive species. The aim of this study was to explore the role of target tissue biotransformation in OM-selective toxicity and the metabolic basis of the species differences in coumarin toxicity. At least six coumarin metabolites were detected in OM microsomal reactions, with o-hydroxyphenylacetalddehyde (o-HPA) being the most abundant. Formation of o-HPA was inhibited by reduced glutathione, confirming its origin from a reactive intermediate. There were significant differences in the rates and metabolite profiles of coumarin metabolism in the livers of Wistar rats and C57BL/6 mice. The rates of metabolic activation of coumarin, as indicated by the formation of o-HPA, were comparable in OM microsomes of the two species but about 25- and 3-fold higher in OM than in liver microsomes of rats and mice, respectively. Thus, target tissue activation seems to play an important role in the tissue-selective toxicity, whereas differences in the rates of hepatic metabolism may be responsible for the species difference in olfactory toxicity. Purified, heterologously expressed mouse CYP2A5 and CYP2G1 produced 7-hydroxycoumarin and o-HPA as the predominant products, respectively. Kinetic analysis and immuno-inhibition studies indicated that the OM-specific CYP2G1 plays the major role in metabolic activation of coumarin. Furthermore, of 13 human cytochrome P-450s (P-450s) examined, five (CYP1A1, CYP1A2, CYP2B6, CYP2E1, and CYP3A4) were active in the metabolic activation of coumarin, suggesting a potential risk of coumarin toxicity in humans.

Coumarin (1,2-benzopyrone), a natural plant product, is widely used as a perfume additive in consumer products (Cohen, 1979; Opdyke, 1974). Coumarin was also used as a food additive, but this practice was discontinued because of potential hepatotoxicity (Hazelton et al., 1956; Hagan et al., 1967). In recent years, coumarin, combined with cimetidine, has been used in clinical trials for the treatment of malignant melanoma, metastatic renal cell carcinoma (Marshall et al., 1994), and prostatic carcinoma (Mohler et al., 1994).

Recent studies in this laboratory indicated that i.p. administration of coumarin at 25 to 50 mg/kg resulted in tissue-selective cytotoxicity in the olfactory mucosa (OM) of Wistar rats and C57BL/6 mice (Gu et al., 1997). In comparison, higher doses were needed to produce hepatotoxicity in rats (Lake, 1984; Lake et al., 1989). Dose-response analysis of the olfactory toxicity of coumarin indicated that Wistar rats were more sensitive than C57BL/6 mice (Gu et al., 1997). Similar species differences were also found in the sensitivity to coumarin-induced hepatotoxicity in earlier studies and were thought to be derived from species differences in the biotransformation of coumarin in the liver (Lake and Grasso, 1996; Ratanasavanh et al., 1996). In humans, 7-hydroxycoumarin is the predominant coumarin metabolite in vivo and in vitro in liver microsomes (Lake et al., 1992a; Moran et al., 1987). In rats, however, o-hydroxyphenylethylacetic acid and o-hydroxyphenylacetaldehyde (o-HPA) are the major in vivo and in vitro products, respectively (Cohen, 1979; Fentem et al., 1991; Lake et al., 1992b). Coumarin metabolism in mice was found to be strain dependent (Rauino et al., 1988; Wood and Taylor, 1979), and mice with low hepatic coumarin 7-hydroxylase activity were much more susceptible to coumarin-induced hepatotoxicity than those with high activity (Endell and Seidel, 1978).

High levels of coumarin 7-hydroxylase activity were observed in the OM in both rats and mice in previous studies.
Metabolic Activation of Coumarin

(Béreziat et al., 1995; Liu et al., 1996; Su et al., 1996), but little is known about the metabolic activation of coumarin in OM microsomes, the P-450 isoforms involved, the role of target tissue activation or detoxification in the OM-selective toxicity, or the basis of species differences in coumarin toxicity in the OM. In this study, coumarin metabolism in rat and mouse OM microsomes was analyzed and compared with that in liver microsomes using reverse phase high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS). The role of CYP2G1 and CYP2A5 [the nomenclature used in this report is according to Nelson et al. (1996)] in OM microsomal coumarin metabolism was examined immunochromically and by kinetic analysis of coumarin metabolism with purified, heterologously expressed mouse CYP2G1 and CYP2A5 in reconstituted systems. In addition, the activity of human P-450s in the metabolic activation of coumarin was examined using microsomes of SF9 cells heterologously expressing 13 individual human P-450 isoforms.

Materials and Methods

Chemicals. [4-14C]Coumarin, reduced glutathione (GSH), β-NADPH, β-NADP, D-glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co. (St. Louis, MO). Coumarin, 4-hydroxycoumarin, 7-hydroxycoumarin, 3,4-dihydrocoumarin, 2-hydroxyphenylacetic acid, 3-(2-hydroxyphenylpropionic acid), o-coumaric acid, and iodomethane were purchased from Aldrich (Milwaukee, WI). 3-Hydroxycoumarin, 6,7-dihydroxycoumarin, and 7,8-dihydroxycoumarin were obtained from Indofine Chemical Company (Somerville, NJ). o-HPA was kindly provided by Dr. Lois D. Lehman-McKeeman of the Procter & Gamble Company (Cincinnati, OH). Insect cell microsomes expressing recombinant human P-450 (Supersomes) were purchased from Gentest (Woburn, MA).

Determination of Catalytic Activity toward Coumarin. [4-14C]Coumarin (14.4 mCi/mmol), which was 99.7% pure based on HPLC radiocromatography, was used as substrate in all microsomal and reconstituted reaction systems unless indicated otherwise. Contents of various incubation mixtures are indicated in the legends to tables and figures. For metabolite determination, reactions were terminated by the addition of 1 volume of ice-cold methanol. After centrifugation at 12,000 rpm at 4°C for 10 min, an aliquot of the supernatant was analyzed by reverse phase HPLC as described below. Protein adduct formation was determined as described by Ding et al. (1996).

HPLC Analysis of Coumarin Metabolites. A Waters (Milford, MA) µBondapak C18 (3.9 × 150 mm) column was used to analyze coumarin metabolites with a Waters HPLC system consisting of a model 600S controller, a model 626 pump, a model 717 plus autosampler, and a model 996 photodiode array detector. A FLO-ONE A-500 radiochromatography detector (Packard; Downers Grove, IL) was used to detect radioactive metabolites in the HPLC effluent. The mobile phase consisted of 25% (v/v) tetrahydrofuran in water (A), water (B), and 1% (w/v) formic acid in water (C), and the metabolites were eluted according to Peters et al. (1991). On-line radioactivity detection and quantification of radiolabeled metabolites were performed as described previously (Ding et al., 1996).

GC-MS Analysis of Coumarin Metabolites. Microsomal reactions were carried out as described above with 0.5 mM unlabeled coumarin as substrate in a final volume of 250 µl. Reactions were stopped by the addition of 750 µl of ethyl acetate and organic compounds were extracted after the addition of 7-ethoxycoumarin as an internal standard. The organic phase was dried under nitrogen, dissolved in 100 µl of dry acetone, and derivatized with iodomethane based on the method described by de Vries et al. (1985). An isooctane solution (10 µl) containing derivatized and underivatized compounds was analyzed through a Hewlett-Packard (Wilmington, DE) GC-MS system consisting of a model 5890A gas chromatograph, with a model 7673A automatic sampler, interfaced to a model 5970 series mass selective detector. A 30 m × 0.25 mm DB-1 column (J & W Scientific; Folsom, CA) with 0.25-µm film thickness was used for separation of metabolites with helium as the carrier gas at a flow rate of 0.75 ml/min. The GC oven temperature was maintained at 80°C initially for 5 min, followed by a ramp of 10°C/min to 300°C, and then a hold at 300°C for 3 min. The mass spectrometer was operated in full scan mode (from m/z 65 to m/z 600 in 1-s intervals) or in selective ion monitoring (SIM) mode. The GC conditions afforded resolution of derivatized authentic standards including 3-, 4-, and 7-hydroxycoumarins; 6,7- and 7,8-dihydroxycoumarins; o-hydroxyphenylacetic acid; o-hydroxyphenylpropionic acid; and o-coumaric acid.

Other Methods. Microsomes were prepared from OM and liver of male C57BL/6 mice (about 20 g b.wt.) and Wistar rats (about 200 g b.wt.) according to Ding and Coon (1990). Purification of heterologously expressed mouse CYP2A5 and CYP2G1 and preparation of a polyclonal antibody to purified CYP2A5 have been described recently (Gu et al., 1998). The sources of purified rabbit hepatic NADPH-P-450 reductase and cytochrome b5 (b5) have been described elsewhere (Ding et al., 1996). Protein concentrations were determined using BCA protein assay reagents (Pierce, Rockford, IL) with bovine serum albumin as the standard. Microsomal P-450 concentrations were determined according to Omura and Sato (1964). Statistical significance was determined using Student's t test.

Results

Tissue Differences in Microsomal Coumarin Metabolism in Rats and Mice. The results of HPLC analysis of microsomal coumarin metabolites are shown in Fig. 1. The metabolites were identified, when possible, on the basis of comigration with authentic standards. In rats, several products were detected in OM microsomal reactions (Fig. 1A), including o-HPA, 7-hydroxycoumarin, 3-hydroxycoumarin, and three unidentified metabolites, designated X1, X2, and X3, with o-HPA being the most abundant. However, only one product, o-HPA, was detected in liver microsomal reactions (Fig. 1B), consistent with earlier reports that o-HPA is the predominant metabolite in rat liver (Lake et al., 1992b). In mice, the HPLC profile of OM microsomal coumarin metabolites (Fig. 1C) was very similar to that of rats, with no indication of a significant species difference in metabolism. With mouse liver microsomes, however, although o-HPA accounted for 78% of the total metabolites produced (Fig. 1D), several other metabolites, including X1, X2, X3, and 3-hydroxycoumarin, were also detectable, which is different from the results seen in rats. No metabolites were detected in control reactions in which NADPH was omitted. The 3-hydroxycoumarin peak was not well resolved from neighboring small peaks, which may also represent minor products. The HPLC system is capable of resolving available standards, including 3-, 4-, and 7-monohydroxycoumarins; 6,7- and 7,8-dihydroxycoumarins; 3,4-dihydroxycoumarin; and o-HPA.

Quantitative differences in the rates of microsomal coumarin metabolism in rats and mice are shown in Table 1. Rates of metabolic activation of coumarin, as indicated by the formation of o-HPA, were not significantly different in rat and mouse OM microsomes on a per-mg-of-microsomal-protein basis but were about 25- and 3-fold higher in OM than in liver microsomes of rats and mice, respectively. Similar
tissue differences in metabolic activation were also apparent in the rates of microsomal protein adduct formation (data not shown). The rate of total metabolite formation in rat liver microsomes was less than 0.2 nmol/min/mg protein, much lower than that seen with mouse liver microsomes (1.7 nmol/min/mg protein), which is consistent with previously reported species differences in the rates of hepatic metabolism and systemic clearance of coumarin. However, the rates of total metabolite formation in olfactory microsomes were not significantly different in the two species (6.2 and 6.6 nmol/min/mg protein in rats and mice, respectively). The rate of formation of 3-hydroxycoumarin was not quantified in the HPLC assay due to its low level and interference from neighboring peaks. Thus, although there were significant species differences in the rates of total metabolite formation in liver, comparable differences were not found in the OM of Wistar rats and C57BL/6 mice.

The tissue and species differences in coumarin metabolism were also evident when microsomal coumarin metabolites were extracted, derivatized, and analyzed by GC-MS. Five peaks potentially representing O-methyl derivatives of monohydroxylated coumarins were identified on inspection of the reconstructed-ion chromatograms obtained from full-scan analyses of the methylated OM microsomal metabolites.
Analysis of the derivatized microsomal extracts by SIM at m/z 176, the value for the molecular ion of the methoxycoumarins, clearly shows the five putative metabolite derivatives (Fig. 2). Two of these were identified as 3- and 7-methoxycoumarin, respectively, as their mass spectra and GC retention times (relative to an internal standard) were identical with those of 3- and 7-methoxycoumarin standards, respectively. 4-Hydroxycoumarin was not produced in any of these microsomal incubations because in no case was a peak observed at the same retention time as that of the 4-methoxycoumarin standard. The other three, designated M1, M2, and M3, respectively, did not comigrate with available standards, but their mass spectra showed fragmentation patterns consistent with those of monomethoxycoumarins, including characteristic peaks at m/z of 176, 148, 133, 90, and 77, representing M⁺, [M-CO]⁺, [M-CH₃CO]⁺, C₇H₆⁺, and C₆H₅⁺, respectively. Because 4-hydroxycoumarin was not detected as a metabolite, M1, M2, and M3 must be 5-, 6-, and 8-methoxycoumarin, for which standards were not available. Of the five monohydroxy metabolites, 7-hydroxycoumarin was the most abundant and 3-hydroxycoumarin was the least abundant in reactions with olfactory microsomes of either rats or mice. In contrast, M1 was the most abundant and 7-hydroxycoumarin was the least abundant hydroxy metabolite produced in reactions with mouse liver microsomes, and there was very little production of monohydroxy metabolites in reactions with rat liver microsomes, which was consistent with results from HPLC analysis. Derivatized metabolites from an approximately equal volume of reaction mixture were analyzed in each panel, and 7-ethoxycoumarin was added as the internal standard.

In other GC-MS experiments (not presented), very low levels of o-methoxyphenyl acetate methyl ester (m/z 180) were detected on analysis of methylated extracts of rat and mouse liver microsomal reactions but not of olfactory microsomal reactions. No additional metabolites were detected in analyses by SIM in which m/z 206, 194, and 192 were monitored, values corresponding to the molecular ions of dimethylated derivatives of dihydroxycoumarin, o-hydroxyphenylpropionic acid, and o-coumaric acid, respectively.

![Graph](image-url)

Fig. 2. GC-MS analysis of derivatized monohydroxycoumarins. Microsomal coumarin metabolism was carried out as described in the legend to Table 1 but using unlabeled substrate at a concentration of 500 μM. Reaction mixtures contained olfactory microsomal proteins (0.5 mg/ml) or liver microsomal proteins (2 mg/ml) in a final volume of 250 μl. Hydroxycoumarins formed by microsomal P450s were then extracted and methylated by reactions with CH₃I to form the methoxycoumarins. GC-MS analysis was performed in SIM mode (m/z 176) with methylated metabolites generated in reactions with rat OM (A), rat liver (B), mouse OM (C), and mouse liver microsomes (D). Five peaks were detected, including 3-methoxycoumarin (3-OH), 7-methoxycoumarin (7-OH), and three unidentified monomethoxycoumarins, designated M1, M2, and M3, which represent 5-, 6-, and 8-methoxycoumarins (5-, 6-, and 8-OH). Procedures for derivatization and GC-MS analysis are described in Materials and Methods. Note that the relative abundances are in different scales in each panel.
although the derivatized standards for these compounds were readily detectable under the same conditions. No efforts were made to detect o-HPA by GC-MS.

To determine which metabolites were derived from a reactive intermediate, microsomal metabolism of coumarin was assayed in the presence of a saturating level of GSH. As shown in Table 2, the addition of GSH resulted in 78 to 95% decreases in o-HPA formation and 88 to 97% decreases in X3 formation. Additional attempts to directly identify GSH conjugates of the coumarin metabolites by HPLC were unsuccessful. Nevertheless, the results shown in Table 2 are consistent with the previous finding that o-HPA is derived from a reactive coumarin epoxide (Born et al., 1997) and indicate that X3 is also derived from a reactive intermediate. For reasons not yet understood, small changes in the levels of the other metabolites were also observed with the addition of GSH, but the magnitude of decreases does not support their origin from a reactive intermediate.

**Coumarin Metabolism by Purified Mouse CYP2A5 and CYP2G1.** The ability was determined of purified CYP2A5 and CYP2G1, major P-450 isoforms in mouse OM, to metabolize coumarin. As shown in Fig. 3, both enzymes produced more than one metabolite. 7-Hydroxycoumarin was the predominant product generated by CYP2A5, but low amounts of o-HPA, X1, and X2 were also formed. The metabolite profile of mouse CYP2G1 is similar to that of mouse OM microsomes, with o-HPA being the primary product. Kinetic parameters of o-HPA and 7-hydroxycoumarin formation by purified mouse CYP2G1 and CYP2A5 were also determined (Table 3). Although CYP2A5 was much more active than CYP2G1 in 7-hydroxycoumarin formation, with a 10-fold higher catalytic efficiency and a 4-fold higher Vmax, it was much less active than CYP2G1 in the formation of o-HPA. With the substrate at 72 μM, the turnover numbers for o-HPA formation were 4.5 and 1.4 nmol/min/nmol of P-450 by CYP2G1 and CYP2A5, respectively (data not shown), and those for X1, X2, X3, and 3-hydroxycoumarin formations by CYP2G1 were 0.66, 0.32, 0.34, and 0.95 nmol/min/nmol of P-450, respectively. Kinetic parameters for o-HPA formation by CYP2A5 were not obtained because of difficulties in metabolite quantification at low substrate concentrations. Interestingly, CYP2G1 had a lower Km for o-HPA formation than for coumarin 7-hydroxylation, suggesting a preferential role of CYP2G1 in metabolic activation. The apparent Km values for mouse OM microsomal 7-hydroxycoumarin and o-HPA formations (3.1 μM and 2.7 μM, respectively; experiments not shown) were lower than the values found for the purified enzymes. This may be partly because of involvement of b3 because in other experiments not shown, the addition of b3 at a ratio of 4 nmol/nmol of P-450 increased the rates of coumarin metabolite formation by 30 to 36% with either CYP2G1 or CYP2A5 at a substrate concentration of 72 μM.

**Role of CYP2A and CYP2G1 in OM Microsomal Metabolism of Coumarin.** To determine whether mouse CYP2A5 and CYP2G1 play important roles in coumarin metabolism in the olfactory tissue, an antibody to mouse CYP2A5, which cross-reacts with mouse CYP2G1, was included in microsomal reaction mixtures. In experiments not shown, the antibody recognized heterologously expressed human CYP2A6 but did not recognize a number of other human P-450 isoforms on immunoblots, including CYP1A1, CYP1A2, CYP2B6, CYP2E1, CYP3A4, CYP1B1, CYP2C8, CYP2C9-Arg, CYP2C19, CYP2D6-Val, CYP3A5, and CYP4A11. Moreover, the antibody, when used at a level of 5 mg IgG/nmol of P-450, inhibited both 7-hydroxycoumarin and o-HPA formation by purified mouse CYP2G1 completely and by purified CYP2A5 by more than 90%. As shown in Fig. 4A, with anti-CYP2A5 IgG added at 1 mg/mg of mouse olfactory microsomal proteins and coumarin at 72 μM, formation of X3 and 7-hydroxycoumarin was totally inhibited. The addition of anti-CYP2A5 IgG also resulted in large decreases (64%) in o-HPA formation and similar decreases in X1 formation but no decreases in X2 formation. In other experiments (not presented), when coumarin concentration was reduced to 9 μM and the antibody was added at 3 mg/mg of OM microsomal proteins, a greater inhibition of microsomal o-HPA formation (75%) was observed, indicating that CYP2G1 plays a greater role in this coumarin metabolic activation at low than at high substrate concentrations.

Similar results were obtained when the immunoinhibition experiments were performed with rat OM microsomes, as shown in Fig. 4B, except that a small decrease (20%) in X2 formation was observed when the antibody was at the highest level used. The anti-CYP2A5 antibody is known to cross-react with rat CYP2A3 (data not shown) and presumably also cross-reacts with rat CYP2G1 because the two orthologous isoforms from rats and mice are 95% identical in deduced amino acid sequences (Hua et al., 1997). Similar to the mouse experiments, when coumarin concentration was reduced to 9 μM and the antibody was added at 3 mg/mg of OM microsomal proteins, a greater inhibition of microsomal o-HPA formation (77%) was observed, indicating that CYP2G1 plays a greater role in this coumarin metabolic activation at low than at high substrate concentrations.

**Table 2**

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<td>X2</td>
</tr>
<tr>
<td>Rat olfactory</td>
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<td>22 ± 2†</td>
</tr>
<tr>
<td>Rat liver</td>
<td>ND</td>
<td>5</td>
</tr>
<tr>
<td>Mouse olfactory</td>
<td>136 ± 29</td>
<td>13 ± 10</td>
</tr>
<tr>
<td>Mouse liver</td>
<td>107</td>
<td>20</td>
</tr>
</tbody>
</table>

ND, undetectable with or without the addition of GSH.

† Significantly lower than control group (p < .05).

§ Significantly lower than control group (p < .001).

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§ Significantly lower than control group (p < .001).
formation (85%) was observed (experiments not shown). In the liver, however, no inhibition (in rats) or only marginal (16%) inhibition of microsomal o-HPA formation was found when anti-CYP2A5 IgG was added at 3 mg/mg of microsomal proteins (Fig. 4C).

Coumarin Metabolism by Heterologously Expressed Human P-450s. Of 13 human P-450s examined, only six were active toward coumarin, including CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2E1, and CYP3A4; the other forms studied were CYP1B1, CYP2C8, CYP2C9-Arg, CYP2C19, CYP2D6-Val, CYP3A5, and CYP4A11 (data not shown). o-HPA was the only product detected in reactions with CYP1A1, CYP1A2, CYP2B6, and CYP2E1, whereas 7-hydroxycoumarin was the only product detected in reactions with CYP2A6. CYP3A4, however, produced relatively small amounts of X1 in addition to o-HPA. The activity of CYP2B6 was very low; product formation was detectable only after a 120-min reaction with reductase/P-450 (Fig. 4C). No metabolite was detected by HPLC analysis in reactions with microsomes from control SF9 cells.

Apparent kinetic parameters of o-HPA formation by CYP1A1, CYP1A2, CYP2E1, and CYP3A4 were determined and compared with those for 7-hydroxycoumarin formation by CYP2A6 (Table 4). For o-HPA formation, CYP2E1 had the lowest \( K_m \) and highest catalytic efficiency, whereas CYP1A1 had the highest \( V_{max} \). However, 7-hydroxycoumarin formation by CYP2A6 had a lower \( K_m \) and a higher catalytic efficiency than o-HPA formation by any of the human P-450 isoforms tested. It should be noted that the SF9 cell microsomal preparations contained different amounts of human NADPH-P-450 reductase for different P-450 isoforms and only some preparations contained coexpressed \( b_5 \) (as shown in the legend to Table 4); both factors could affect the accuracy of the kinetic parameters determined. Nevertheless, these results indicate that human P-450s are capable of metabolic activation of coumarin to the toxic intermediate and they identify the isoforms potentially involved.

Our previous study indicated that coumarin is a tissue-selective toxicant in rodent OM and that rats are more sensitive than mice to coumarin olfactory toxicity (Gu et al., 1997). However, it is not clear whether the differential sensitivity resulted from tissue and species differences in coumarin metabolism. Metabolic activation of coumarin is thought to involve the formation of the reactive coumarin 3,4-epoxide (Cohen, 1979; Fentem and Fry, 1993; Lake, 1984), which can react with GSH to form GSH conjugates or react with protein to form covalent adducts (Huwer et al., 1991; Lake et al., 1992a). Alternatively, the epoxide will undergo rapid degradation to form o-HPA (Born et al., 1997), which can also cause toxicity in the cell (Feron et al., 1991).

### Table 3

Kinetic analysis of coumarin metabolism by mouse CYP2A5 and CYP2G1

The kinetic parameters for the formation of o-HPA and 7-hydroxycoumarin by purified CYP2A5 and CYP2G1 were determined in reconstituted systems. The contents of reaction mixtures were the same as described in the legend to Fig. 3, with the substrate at 8 to 40 \( \mu \)M. The reactions were carried out at 37°C for 10 min. Values reported are mean ± S.D. (n = 3).

<table>
<thead>
<tr>
<th>Enzyme Preparation</th>
<th>7-Hydroxycoumarin Formation</th>
<th>o-HPA Formation</th>
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<tbody>
<tr>
<td></td>
<td>( K_m ) (( \mu )M)</td>
<td>( V_{max} ) (nmol/min/nmol P450)</td>
</tr>
<tr>
<td>CYP2A5</td>
<td>14 ± 4</td>
<td>15.5 ± 1.8a</td>
</tr>
<tr>
<td>CYP2G1</td>
<td>25 ± 7</td>
<td>3.3 ± 0.6</td>
</tr>
</tbody>
</table>

* Significantly greater than the \( V_{max} \) of CYP2G1 (p < .001).
* ND, not determined.
* Significantly lower than the \( K_m \) for 7-hydroxycoumarin formation by CYP2G1 (p = .014).
and the substrate at 5 to 72 m
6 the rate of product formation was linear with time. Values reported are mean
o-HPA and 7-hydroxycoumarin from coumarin. The results of
OM microsomes (Gu et al., 1998), and both can generate
activation or detoxification.
compound rather than because of differences in target tissue
difference in coumarin olfactory toxicity may be mainly be-
tion in OM microsomes of rats and mice. Thus, the species
metabolic activation or of total coumarin metabolite forma-
than rats, there was no significant difference in the rate of
coumarin metabolic rates, with mice being much more active
Although there were marked species differences in hepatic
role of target tissue activation in tissue-selective toxicity.
were much higher than those in the liver, consistent with the
OM microsomal preparations (per mg microsomal protein)
 Activation of coumarin in rat and mouse OM (A), rat OM (B), and rat or mouse liver (C). Reactions were carried out at 37°C for 10 min. The formations of X1, X2, X3, 7-hydroxycoumarin (7-OH), and o-HPA were determined for olfactory microsomal reactions, but only o-HPA was determined for liver microsomal reactions. The addition of preimmune IgG did not affect metabolic activity. The values presented are averages of two experiments with differences less than 15% of the mean.

Table 4
Kinetic analysis of coumarin metabolism by human P-450s
The kinetic parameters for the formation of o-HPA and 7-hydroxycoumarin were determined with microsomal preparations of SF9 cells expressing human CYP1A1 (reductase/P-450 = 19:1), CYP1A2 (reductase/P-450 = 7.3:1), CYP2A6 (reductase/P-450 = 0.9:1), CYP2E1 (reductase/P-450 = 2.6:1; b d P-450 = 1.5:1), and CYP3A4 (reductase/P-450 = 7.3:1; b d P-450 = 5:1). The contents of reaction mixtures were the same as described in the legend to Table 1 but with human P-450s added at 0.1 μM and the substrate at 5 to 72 μM. The reactions were carried out at 37°C for 10 min (for CYP1A1, CYP1A2, and CYP3A4) or 20 min (for CYP2A6 and CYP3A4), during which the rate of product formation was linear with time. Values reported are mean ± S.D. (n = 3).

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<tr>
<td></td>
<td>Km (μM)</td>
<td>Vmax (nmol/min/nmol P-450)</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>6.0 ± 1.2</td>
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<tr>
<td>CYP2E1</td>
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<td>ND</td>
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<td>CYP3A4</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a ND, product formation was not detected.
b Significantly higher than Vmax values for all other P-450 isoforms (p ≤ .010).
c Significantly lower than Km values for o-HPA formation by CYP1A1, CYP1A2, and CYP3A4 (p ≤ .010).
d Significantly lower than Km values for o-HPA formation by the other P-450 isoforms (p ≤ .020).

On the other hand, the formation of monohydroxy and dihydroxycoumarins represents detoxification (Gu et al., 1997; Lake et al., 1989). The results of this study indicate that the rates of metabolic activation of coumarin in rat and mouse OM microsomal preparations (per mg microsomal protein) were much higher than those in the liver, consistent with the role of target tissue activation in tissue-selective toxicity. Although there were marked species differences in hepatic coumarin metabolic rates, with mice being much more active than rats, there was no significant difference in the rate of metabolic activation or of total coumarin metabolite formation in OM microsomes of rats and mice. Thus, the species difference in coumarin olfactory toxicity may be mainly because of differences in the rates of systemic clearance of this compound rather than because of differences in target tissue activation or detoxification.

CYP2G1 and CYP2A5 are major P-450 isoforms of mouse OM microsomes (Gu et al., 1998), and both can generate o-HPA and 7-hydroxycoumarin from coumarin. The results of immunoinhibition assays and kinetic analyses suggest that in mice, CYP2G1 plays a major role in coumarin metabolic activation, whereas CYP2A5 plays a major role in coumarin detoxification in the OM. The incomplete inhibition of microsomal o-HPA formation by the anti-CYP2A5 antibody suggested that other P-450s, such as CYP2E1, CYP1A2, and CYP3A, which are known to be expressed in mouse OM (Genter et al., 1998), may also contribute to the metabolic activation, particularly at high substrate concentrations. Conversely, the lack of significant inhibition of hepatic coumarin metabolism by the anti-CYP2A5 IgG not only documents the specificity of the antibody but also confirms that the high activity in metabolic activation of coumarin in mouse olfactory microsomes is due to the presence of the tissue-specific CYP2G1.

The P-450 isoforms involved in coumarin metabolism in rat OM have not been as thoroughly studied. A previous report indicated that rat CYP1A1 and CYP2B1 are active in o-HPA formation from coumarin (Peters et al., 1991); these isoforms
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References


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