Short Communication

IDENTIFICATION OF THE CYTOCHROMES P450 THAT CATALYZE COUMARIN 3,4-EPOXIDATION AND 3-HYDROXYLATION

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ABSTRACT:

Coumarin, a widely used fragrance ingredient, is a rat liver and mouse lung toxicant. Species differences in toxicity are metabolism-dependent, with injury resulting from the cytochrome P450-mediated formation of coumarin 3,4-epoxide (CE). In this study, the enzymes responsible for coumarin activation in liver and lung were determined. Recombinant human and rat CYP1A forms and recombinant human CYP2E1 readily catalyzed CE production. Coinhibition with CYP1A1/2 and CYP2E1 antibodies blocked CE formation by 38, 84, and 67 to 92% (n = 3 individual samples) in mouse, rat, and human hepatic microsomes, respectively. Although CYP1A and 2E forms seem to be the most active catalysts of CE formation in liver, studies conducted with the mechanism-based inhibitor 5-phenyl-pentyne demonstrated that CYP2F2 is responsible for up to 67% of CE formation in whole mouse lung microsomes. In contrast to the CE pathway, coumarin 3-hydroxylation is a minor product of coumarin in liver microsomes from mice, rats, and humans and is catalyzed predominately by CYP3A and CYP1A forms, confirming that CE and 3-hydroxycoumarin are formed via distinct metabolic pathways.

Coumarin (cis-α-coumaric acid lactone) is a natural product used widely as a fragrance ingredient. Coumarin has also been used clinically at high dosages in humans in the treatment of high-protein lymphedemas (Jamal and Casley-Smith, 1989) and as an antineoplastic agent in the treatment of renal cell carcinoma (Marshall et al., 1994) and malignant melanoma (Marshall et al., 1989). Although reports of adverse effects in humans resulting from coumarin treatment are rare (Cox et al., 1989; Egan et al., 1990), administration of coumarin to rodents produces rat liver (Lake, 1984; Lake et al., 1989) and mouse lung toxicity (Born et al., 1998).

Species differences in toxicity are metabolism-mediated. Coumarin metabolism in humans occurs predominately via CYP2A6 (Yamano et al., 1990), which produces the nontoxic metabolite 7-hydroxycoumarin. Following an oral dose of coumarin to humans, 7-hydroxycoumarin and its glucuronide and sulfate conjugates may constitute 40 to 97% of urinary metabolites in most subjects (Shilling et al., 1969; Egan et al., 1990). Coumarin-mediated rat liver damage was also recognized as a cytochrome P450-dependent process (Lake, 1984), probably involving the generation of a coumarin 3,4-epoxide (CE\(^1\)) intermediate. Early efforts to define the metabolic fate of coumarin by Kaighn and Williams (1961) suggested that CE rearranged to form 3-hydroxycoumarin (3-HC), which hydrolyzed to form ring-opened metabolites. However, recent data obtained with authentic CE have demonstrated that CE rearranges directly to form o-hydroxyphenylacetaldehyde (o-HPA) and that 3-HC is not a product of the epoxidation pathway (Born et al., 1997).

\(^1\) Abbreviations used are: CE, coumarin 3,4-epoxide; 3-HC, 3-hydroxycoumarin; o-HPA, o-hydroxyphenylacetaldehyde; P450, cytochrome P450; CHZ, chlorzoxazone; 5-PP, 5-phenyl-1-pentyne; EROD, ethoxyresorufin O-dealkylase.

Materials and Methods

Reagents. Coumarin was purchased from Aldrich Chemical Company (Milwaukee, WI). Chlorzoxazone (CHZ) and ethoxyresorufin were purchased from Sigma Chemical Company (St. Louis, MO). 5-Phenyl-1-pentyne (5-PP) was purchased from Lancaster Synthesis (Pelham, NH). Polyclonal, anti-rat CYP2B1/2B2, and CYP3A1/3A2 antibodies were purchased from XenoTech LLC (Kansas City, KS). Polyclonal anti-rat CYP1A1/1A2, CYP2C11, polyclonal anti-human CYP2D6, and monoclonal anti-human CYP2E1 were purchased from GENTEST (Woburn, MA). As described in the product literature,
antibodies were specific to P450s within a subfamily and inhibited P450 activity strongly or very strongly (XenoTech, LLC and GENTEST). Recombinant human CYP1A1, CYP1A2, CYP2A6, CYP2C8, CYP2C9*1 (Arg144), CYP2C19, CYP2D6*1, CYP3A5, and rat CYP1A1 and CYP1A2 were coexpressed with NADPH-cytochrome P450 reductase and purchased as Super- somes from GENTEST. Recombinant human CYP2E1 and CYP3A4 were coexpressed with NADPH-cytochrome P450 reductase and cytochrome b5 and were also purchased as Supersomes from GENTEST. o-HPA was synthesized according to the method of Bruce and Creed (1970). 3-Hydroxycoumarin was synthesized according to the method of Rajalakshmi and Srinivasan (1978).

Animals. Female B6C3F1 mice (20–25 g) and male F-344 rats (210–220 g) were purchased from Charles River Laboratories (Portage, MI). Animals were housed in humidity- and temperature-controlled rooms and allowed free access to food (Purina Laboratory Rodent chow; Ralston-Purina, St. Louis, MO) and water.

Human Liver Samples. Microsomal fractions from 11 human livers were obtained from the International Institute for the Advancement of Medicine (IIAM, Exton, PA), XenoTech, LLC, or were provided as a gift from Dr. Brian Lake (BIBRA International, Surrey, UK). The characteristics of these samples have been described previously (Born et al., 2000).

Preparation of Liver and Lung Microsomes. Hepatic microsomes were prepared from untreated mice (n = 10/pool; five pools) and untreated rats (n = 12/pool; two pools). Lung microsomes were prepared from mice (n = 10/pool; three pools). Microsomes were prepared via differential centrifugation (Guengerich, 1989). The microsomal protein was determined by the method of Bradford (1976) with bovine serum albumin as standard.

Coumarin Metabolism by Recombinant P450s. The recombinant en- zymes (50–100 pmol) were incubated in a 1-ml reaction volume that contained 100 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA, 2 mM MgCl2, 5 mM glucose 6-phosphate, 1 IU/ml glucose-6-phosphate dehydrogenase, and 100 μM coumarin, as suggested by the enzyme supplier. The reaction was initiated by adding 1 mM NADP, and the samples were incubated for 30 min at 37°C. For 3-HC, the reaction was terminated with 0.5 ml of methanol, and the product was quantitated by liquid chromatography coupled with mass spectrometry. For CE, the reaction was terminated with 0.2 ml of 30% perchloric acid. Samples were extracted, and CE (detected as o-HPA) was quantitated by gas chromatography coupled with flame ionization detection (Born et al., 2000). Enzyme activities were determined in replicate samples. In the case of CYP3A4 and rat CYP1A2, different lots of enzyme were compared with regard to specificity and activity.

Immunoinhibition Studies. Anti-P450 antibodies (5–10 μg of IgG/mg of microsomal protein or 1–2 μl of IgG/μg of microsomal protein, as recommended by the manufacturer to inhibit >85% of P450 activity) were incubated with 0.25 to 0.5 mg of mouse, rat, and human liver microsomes or 0.25 mg of mouse lung microsomal protein. Under these conditions, >90% of CYP1A-mediated EROD activity was inhibited by anti-CYP1A IgG in both rodent and human liver microsomes (data not shown). Incubation with the monoclonal mouse anti-human 2E1 blocked >90% of chlorozoxazone 6-hydroxylation in human liver microsomes and >80% of p-nitrophenol 4-hydroxylation in rodent liver microsomes (data not shown). The antibodies were preincubated with microsomal protein for 15 min at room temperature before dilution into a 1-ml reaction mixture containing 100 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA, 2 mM MgCl2, 5 mM glucose 6-phosphate, 1 IU/ml glucose-6-phosphate dehydrogenase, 1 mM NADP, and coumarin. Samples were incubated at 37°C for 30 min and were then terminated with 0.2 ml of 30% perchloric acid. The kinetics of CE formation in rodent and human liver microsomes were previously determined using similar reaction conditions (Born et al., 2000). When 5-PP, a mechanism-based inhibitor (Chang et al., 1996; Carlson, 1997; Roberts et al., 1998), was used to block CYP2F2 activity in mouse liver (50 μM coumarin) and lung (500 μM coumarin) microsomes, the microsomes were first inhibited with antibodies and/or the microsomes were incubated with 5 μM 5-PP and 1 mM NADP in a 1-ml reaction mixture, largely as described above. After a 15-min incubation with 5-PP, the reaction mixture was diluted 100-fold into a secondary incubation containing coumarin and a NADPH-generating system, and a final 30-min incubation was used to monitor coumarin metabolism by the inhibited P450s. Samples were extracted, and coumarin 3,4-epoxidation was quantitated. 3-Hydroxycoumarin samples were quenched with methanol and did not require extraction before quantita-
tion. For each microsomal sample and combination of antibodies and chemical inhibitors, samples were run in duplicate. Rat and mouse liver and lung microsomal samples were run in triplicate.

Analysis of 3-Hydroxycoumarin Formation. Coumarin metabolites were separated using a Waters 2790 HT high-pressure chromatograph and a Waters Xterra RP18 (3.5 μm, 2.1 × 100 mm; Milford, MA) column. Metabolites were eluted from the column under isocratic conditions (0.1% formic acid in water/0.1% formic acid in acetonitrile, 86:14) and detected using a Micromass ZMD mass-selective detector (Manchester, UK) with selective ion monitoring at m/z 163. Quantification was based on an external calibration curve that ranged from 1 to 100 pmol on column. The limit of quantitation was 0.5 pmol of 3-HC on column.

Determination of CYP1A Activity. o-Dealkylation of 7-ethoxyresorufin, used to assess CYP1A activity, was determined according to the fluorometric method described by Burke et al. (1995). Microsomal protein (0.4 mg) was used in this assay, and the substrate concentration was 2 μM. Quadruplicate reactions were conducted at 37°C in fluorometric cuvettes using a PerkinElmer LS50-B luminescence spectrometer (Norwalk, CT).

Determination of CYP2E1 Activity. CHZ 6-hydroxylation was measured in 0.25-ml incubations containing 0.2 mg of microsomal protein and 200 μM CHZ, according to the method of Newton et al. (1995). Triplicate CHZ samples were analyzed using a Waters Symmetry C18 column (5 μm, 3.9 × 50 mm) under isocratic conditions on a Waters Alliance 2690 chromatographic system. Samples were quantitated against a standard curve prepared with 6-OH chlorozoxazone (GENTEST).

Results and Discussion

Initial studies used a panel of recombinant human P450 enzymes to rapidly screen for forms that catalyzed coumarin 3-hydroxylation and 3,4-epoxidation. Recombinant CYP3A4 was identified as the most active coumarin 3-hydroxylase (Table 1). Recombinant rat CYP1A1 and CYP1A2 formed 3-HC at rates 2-fold higher than the orthologous human forms, a result consistent with rat hepatic microsomes having a higher rate of 3-hydroxylation than human liver microsomes (Fentem and Fry, 1992). Immunoinhibition studies in rodent liver microsomes confirmed the involvement of CYP1A1 and CYP3A1 in 3-hydroxylation. Antibodies directed against rat CYP1A1 and CYP3A2 decreased 3-hydroxylation in rat liver microsomes by 72 and 64%, respectively, in incubations containing 500 μM coumarin; similar results were obtained in mouse liver microsomes (data not shown). Antibody inhibition studies with human liver microsomes and 500 μM coumarin were difficult to interpret due to low 3-hydroxylation rates (data not shown).

| TABLE 1  |
| Coumarin metabolism by recombinant P450 enzymes |
| Microsomal preparations of SF9 cells expressing human CYP1A1 (reductase/P450, 18:1), CYP1A2 (reductase/P450, 7:1), CYP2E1 (reductase/P450, 3:1, h/b/P450, 2:1), CYP3A4 (reductase/P450, 7:1, b/h/P450, 5:1), CYP2A6 (reductase/P450, 1:1), and rat CYP1A1 (reductase/P450, 2:1) and CYP1A2 (reductase/P450, 2:1) were incubated with 100 μM coumarin, and 3-hydroxycoumarin or 3-hydroxyphenylacetaldehyde formation was quantitated by liquid chromatography-mass spectrometry or gas chromatography coupled with flame ionization detection, respectively. |
| P450 | 3-Hydroxycoumarin Formation | o-Hydroxyphenylacetaldehyde Formation |
| nmol/nmol P450 |
| Human | CYP1A1 | 0.27* |
| | CYP1A2 | 0.10 |
| | CYP2E1 | 0.17 |
| | CYP3A4 | 0.68 |
| | CYP2A6 | N.D. |
| Rat | CYP1A1 | 0.44 |
| | CYP1A2 | 0.38 |

N.D., not detected; b, cytochrome b5.

* Values are the average of duplicate determinations.
CE formation was catalyzed by recombinant CYP2E and CYP1A forms (Table 1), confirming that CE and 3-HC are the products of different metabolic pathways (Fig. 1). Recombinant human CYP1A1 and CYP1A2 catalyzed o-HPA formation at rates 20 times those for coumarin 3-hydroxylation. Furthermore, recombinant rat CYP1A2 catalyzed o-HPA formation at a rate 12 times greater than the orthologous human form, which is consistent with the higher rate of CE formation in rat liver. o-HPA formation was not detected in incubations containing CYP2A6, CYP2C8, CYP2C9*1 (Arg144), CYP2C19, CYP2D6*1, or CYP3A5. Additionally, recombinant CYP1B1 and CYP4A11 do not catalyze o-HPA formation (Zhuo et al., 1999). In contrast to an earlier report that described the kinetics of o-HPA formation by recombinant CYP3A4 (Zhuo et al., 1999), the current studies indicate that CYP3A4 does not catalyze CE formation at 100 or 1000 μM coumarin.

The formation of o-HPA by human recombinant enzymes has previously been examined. Zhuo et al. (1999) identified CYP1A1, CYP1A2, and CYP2E1 as catalysts of o-HPA formation. The $K_m$ and $V_{max}$ for CYP1A1, CYP1A2, and CYP2E1 were 12, 19, and 51 μM and 2.0, 2.4, and 7.1 nmol/min/nmol, respectively. These data indicate that multiple P450 forms may contribute to o-HPA formation and that the affinities of each enzyme for coumarin are similar. However, the $K_m$ and $V_{max}$ for o-HPA formation in human liver microsomes ranges from 1320 to 7420 μM and 1.32 to 4.91 nmol/min/mg, respectively (Born et al., 2000). This disparity may be due to competition for substrate between multiple enzymes. Specifically, CYP2A6-mediated 7-hydroxylation greatly exceeds CE formation and is the predominant route of coumarin clearance in humans at low, toxicologically relevant concentrations, with $K_m$ and $V_{max}$ values ranging from 0.2 to 3.6 μM and 0.18 to 2.47 nmol/min/mg, respectively (Draper et al., 1997; Lake, 1999).

In immunoinhibition studies confirmed the importance of hepatic CYP1A and 2E forms in coumarin epoxidation (Table 2). Previous rodent liver microsomal studies indicated that o-HPA formation was biphasic, with the high-affinity $K_m$ being approximately 40 μM and the low-affinity $K_m$ being >500 μM (data not shown). To focus on identifying the high-affinity enzyme, a substrate concentration of 50 μM was selected for the current studies. Significant differences were not observed between anti-CYP1A- and CYP2E1-mediated inhibition in rodent liver microsomes; therefore, it was not apparent which P450 was the high affinity form.

Inhibition of CYP1A1/2 and 2E1 decreased o-HPA formation in rat liver microsomes by 84%. In contrast, o-HPA production was decreased by only 38% in mouse liver microsomes. Additional data obtained in mouse liver microsomes with anti-rat antibodies directed against CYP2B1/2B2, CYP2C11, CYP2D6, and CYP3A5/3A2 are consistent with results obtained with recombinant P450s and suggest that these forms are not involved in o-HPA formation in mouse liver microsomes.

The role of CYP1A1 and CYP2E1 in CE formation in human liver microsomes was also examined, using liver microsomes with high (4.1 nmol/min/mg; H0017), medium (0.16 nmol/min/mg; H0018), and low (0.10 nmol/min/mg; H0019) coumarin 7-hydroxylase (CYP2A6) activity, a pathway that is known to compete with the formation of CE. The $K_m$ and $V_{max}$ values for o-HPA formation in these microsomal preparations ranged from 2720 to 4970 μM and 1.62 to 4.91 nmol/min/mg (Born et al., 2000). Incubations contained 500 μM coumarin, a substrate level greatly exceeding toxicologically relevant concentrations but sufficient to support CE formation at a readily detectable level in each of the samples. Antibodies to CYP1A1/2 and CYP2E1 inhibited o-HPA formation by 67 to 92% (Table 2).

To determine the putative role of CYP1A and CYP2E in CE production in a larger number of samples, 7-EROD and CHZ activities were determined for 11 hepatic microsomal samples from individual human donors (data not shown), and these values were correlated with CE formation rates that had previously been determined (Born et al., 2000). In this small number of samples, the correlation between CE and 7-EROD was poor ($r^2 = 0.164$). CE and CHZ formation correlated to a greater degree ($r^2 = 0.544$), which may suggest that hepatic CYP2E1 activity is a more important determinant of CE formation in human liver than CYP1A activity (results not shown). However, the contributions CYP1A and CYP2E to coumarin epoxidation may vary according to the relative hepatic abundance of these enzymes and exposure to P450-inducing agents.

Although CE production is observed in hepatic microsomes from rodents and humans, and hepatic CYP1A and CYP2E forms contribute to this reaction in each species, it should also be noted that in vitro CE formation rates alone are not predictive of liver toxicity in vivo. CE formation is clearly a prerequisite for liver injury (Lake et al., 1989, 1994). However, CE formation is greatest in mouse liver microsomes, a species relatively resistant to coumarin-mediated hepatotoxicity (Zhuo et al., 1999; Born et al., 2000). These data suggest that detoxification mechanisms play an important role in determining...
susceptibility to injury (Zhuo et al., 1999), a conclusion that is supported by recent studies demonstrating that o-HPA is extensively detoxified by liver cytosolic enzymes in the mouse but not the rat (Born et al., 2000).

Species differences have also been observed in coumarin-mediated lung injury, with the mouse lung being uniquely susceptible to acute coumarin-mediated toxicity (Born et al., 1998). Although injury is localized to Clara cells of the distal-terminal bronchioles, the rate of o-HPA formation in pooled whole mouse lung microsomes is comparable to that observed in mouse liver microsomes, reaching 6 nmol/min/mg at 500 μM coumarin. These findings indicate that coumarin belongs to a select family of diverse chemicals that require metabolic activation and target the mouse lung Clara cell. Although differing in their mechanisms of metabolism and toxicity, naphthalene (Mahvi et al., 1977), styrene (Gadberry et al., 1996), 3-methylindole (Durham and Castleman, 1985), trichloethylen (O’Brien et al., 1990), and methylene chloride (Odum et al., 1992) injure the mouse lung. Of these chemicals, naphthalene (Ritter et al., 1991), styrene (Carlson, 1997), and 3-methylindole (Wang et al., 1998) are bioactivated to electrophilic intermediates by members of the CYP2F subfamily. Therefore, when immunoinhibition by 1A and 2E IgG failed to significantly block o-HPA formation in whole mouse lung microsomes (Table 3), the role of CYP2F2 in lung CE formation was examined using the mechanism-based CYP2F inhibitor 5-PP. Incubation of whole mouse lung microsomal protein with 5 μM 5-PP decreased CE formation rates by 67%, demonstrating the central role of CYP2F2 in coumarin epoxidation in the mouse lung. These data are consistent with the fact that the Clara cell is the sole site of CYP2F2 expression in the mouse lung (Ritter et al., 1991) and that coumarin selectively targets the Clara cell (Born et al., 1998). The differential effects of coumarin in the mouse and rat lung may result from species differences in the substrate specificity or affinity of the CYP2F enzymes expressed in the lung, although further studies will be required to address this issue. Incubation of mouse liver microsomes with 5 μM 5-PP decreased CE formation by 17%, suggesting a minor role for 2F2 in the liver. Together, CYP1A and CYP2E1 IgG with 5 μM 5-PP reduced CE production by 60% in the mouse liver, suggesting that the majority of hepatic CE formation is catalyzed by CYP1A1/2 and CYP2E1.

In summary, the current data demonstrate that cytochromes P450 from the 1A and 2E subfamilies are the major catalysts of CE formation in mouse, rat, and human liver microsomes, whereas 3-HC formation is catalyzed primarily by CYP3A and CYP1A1 forms. Furthermore, recent studies with recombinant mouse CYP2A4 indicate that this form also catalyzes CE production, albeit at low levels (Vmax ~0.2 nmol/min/mg) (von Weymarn and Murphy, 2001). The involvement of multiple P450s in CE formation in the mouse liver, some of which remain to be identified, may be an important factor in the high rate of CE production in mouse liver microsomes. Finally, the unique susceptibility of the mouse lung to coumarin-mediated toxicity seems linked to the high level of expression of CYP2F2 in this target organ, a result consistent with the selective lung toxicity of other CYP2F2 substrates in the mouse.

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References


### Table 3

Antibody- and chemical-mediated inhibition of coumarin 3,4-epoxidation in mouse whole lung microsomes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Anti-CYP1A1/2</th>
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<th>Anti-CYP1A1/2</th>
<th>Anti-CYP2E1</th>
<th>5-PP</th>
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<td>Mouse Lung Microsomes</td>
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