Abnormalities in 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.

The K_m and V_max values for hepatic microsomal coumarin 3,4-epoxidation differ significantly between species (Born et al., 2000). CE formation, measured via the formation of o-HPA from CE, was greatest in mouse > rat > human, with the K_m for CE formation in human liver microsomes being 30- to 180-fold greater than that observed in rodent liver (Born et al., 2000). Furthermore, Eadie-Hofstee analysis of the data indicated that at least two P450 forms catalyzed CE formation in rodent liver, whereas a single form probably produced CE in human liver. Species differences in CE formation were also observed in the lung, with CE formation in whole mouse lung microsomes exceeding that in rat lung by 20-fold; no CE production was detected in whole human lung microsomal incubations (Caudill et al., 2000). The divergent rates of CE production in liver and lung suggested that different P450s, or at least forms with different affinity constants, catalyzed coumarin epoxidation in mouse, rat, and human target tissues. Using chemical induction studies in rats (Peters et al., 1991) or recombinant human P450s (Zhuo et al., 1999), other investigators have identified rat CYP2B1 and CYP1A1 and human P450 forms from the 1A, 2E, and 3A subfamilies as enzymes that catalyze CE production. The current work builds upon these initial studies through the use of recombinant enzymes and antibody- and chemical-mediated inhibition of o-HPA formation, defining the role of individual P450s in CE formation in mouse, rat, and human liver microsomes and in mouse lung microsomes.

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,

1 Abbreviations used are: CE, coumarin 3,4-epoxide; 3-HC, 3-hydroxycoumarin; o-HPA, o-hydroxyphenylacetaldelyde; P450, cytochrome P450; CHZ, chlorzoxazone; 5-PP, 5-phenyl-1-pentyne; EROD, ethoxyresorufin O-dealkylase.
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 Supersomes 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 (Güngerich, 1989). The microsomal protein was determined by the method of Bradford (1976) with bovine serum albumin as standard.

Coumarin Metabolism by Recombinant P450s. The recombiant enzymes (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. 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. δ-Dealkylation of 7-ethoxyresorufin, used to assess CYP1A activity, was determined according to the fluorometric method described by Burke et al. (1985). 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 CYP2E 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 × 150 mm) under isocratic conditions on a Waters Alliance 2690 chromatographic system. Samples were quantitated against a standard curve prepared with 6-OH chlorozoxone (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 CYP1A and CYP3A in 3-hydroxylation. Antibodies directed against rat CYP1A 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>
<thead>
<tr>
<th>CYP450</th>
<th>3-Hydroxycoumarin Formation</th>
<th>o-Hydroxyphenylacetalddehyde Formation</th>
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<tbody>
<tr>
<td>Human</td>
<td></td>
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<tr>
<td>CYP1A1</td>
<td>0.27*</td>
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<td>CYP1A2</td>
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<td>CYP2E1</td>
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</tr>
<tr>
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</tr>
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<td>CYP1A2</td>
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*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 did 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 CE 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 1.62 to 4.91 nmol/min/mg, respectively (Born et al., 2000). Incubations contained 500 μM coumarin were 1.6 to 3.6 nmol/min/mg 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 the 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), trichloroethylene (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 CYP2F 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-NC 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

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Table 3

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

<table>
<thead>
<tr>
<th></th>
<th>Anti-CYP1A1/2</th>
<th>Anti-CYP2E1</th>
<th>Anti-CYP1A1/2</th>
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<tr>
<td>% of Control</td>
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<tr>
<td>Mouse Lung Microsomes</td>
<td>91 ± 1</td>
<td>33 ± 0.4</td>
<td>31 ± 0.6</td>
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