UROPORPHYRIN OXIDATION CATALYZED BY HUMAN CYTOCHROMES P450

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ABSTRACT:

Porphyria cutanea tarda is associated with excess hepatic production of uroporphyrin. Oxidation of uroporphyrinogen to uroporphyrin was previously demonstrated to be specifically catalyzed by cytochrome P450 (CYP) 1A2. Here, we investigated the ability of human CYP1A2 to catalyze uroporphyrinogen oxidation (UROX). UROX activity in human liver microsomes was maximally only 10% of the activity in microsomes from livers of untreated mice. There was a poor correlation of UROX activity with methoxresorufin demethylation, an activity catalyzed predominantly by CYP1A2 and strongly correlated with immunodetectable CYP1A2. With CYP forms expressed in HepG2 cells, the methoxresorufin demethylation and (ethoxyresorufin deethylation) activities of murine and human CYP1A2 forms were similar, but UROX activity catalyzed by human CYP1A2 was only 15–20% of the activity catalyzed by murine CYP1A2. Human CYP1A1, CYP1A2, and CYP3A4 expressed in lymphoblastoid cells all catalyzed UROX. In insect cells, CYP1A2 was more active in catalyzing UROX than was CYP1A1, CYP2E, CYP3A4, or CYP3A5. Human CYP1A2 expressed in Escherichia coli as a fusion protein with rat CYP oxidoreductase also catalyzed UROX. Reconstituted human CYP1A2 and CYP3A4 were active in catalyzing UROX, with reconstituted CYP1A2 having the highest specific activity obtained in this study. From inhibitor studies, it was concluded that some of the UROX activity in the insect cell microsomes was attributable to expressed CYP and some to an unidentified source. These results indicate that human CYP1A2 is active in catalyzing UROX but has lower activity than the murine orthologue. The results also indicate that most of the UROX activity found in human liver microsomes is not due to CYP1A2.

PCT1 is a human disease in which there is massive hepatic accumulation and urinary excretion of URO and inactivation of the heme synthetic enzyme URO-D (Elder, 1990; Anderson, 1996). Both sporadic and familial forms of PCT occur. Although patients with familial PCT have an inherited partial deficiency of URO-D, sporadic and familial forms of the disease are precipitated by the same etiological agents, such as alcoholic beverages and contraceptive steroids (Elder, 1990; Anderson, 1996).

Experimental models of this uroporphyrinaemia were developed as a result of an accidental poisoning with hexachlorobenzene (a polyhalogenated aromatic hydrocarbon) in Turkey in the 1950s, which caused hepatic URO accumulation and excretion in many people who were exposed to the agent present in treated wheat seed (Elder, 1978). Massive hepatic accumulation and urinary excretion of URO, together with decreased activity of hepatic URO-D, were also observed with rodents treated with hexachlorobenzene and other polyhalogenated compounds, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (Elder, 1978), which are all inducers of CYP1A forms (Whitlock, 1993). In cultured chick embryo hepatocytes, URO accumulation is coincident with induction of CYP1A and is prevented by inhibitors of CYP1A (Sinclair and Granick, 1974; Sinclair et al., 1984, 1986). In cultured mouse hepatocytes, URO accumulation is associated with increases in CYP1A2 but not CYP1A1 (Sinclair et al., 1990a). In vitro, hepatic microsomes containing induced CYP1A1 was found to catalyze UROX, resulting in the formation of URO (Sinclair et al., 1987; De Matteis et al., 1988). Rat microsomal UROX activity was inhibited by antibodies against CYP1A2 but not by antibodies against CYP1A1 (Jacobs et al., 1989a). UROX is also catalyzed by reconstituted purified mouse CYP1A2 and its chicken equivalent, CYP1A5 (Lambrecht et al., 1992; Sinclair et al., 1997b). In contrast, reconstituted rodent CYP1A1 and its chick equivalent, CYP1A4, are much less effective in catalyzing UROX. Recently, we showed that CYP1A2 knockout mice fail to become uroporphyrin when treated with iron and 5-aminolevulinic acid, with or without an inducer of CYP1A2 (Sinclair et al., 1998). Thus, evidence accumulated from experimental systems strongly implicates a role for CYP1A2 (or CYP1A5 in the chick system) in the uroporphyrinaemia caused by polyhalogenated compounds. This has led to the hypothesis that CYP1A2 also has a role in human PCT (Sinclair et al., 1997b). Although there is no direct evidence for such a hypothesis, we have noted that many patients with PCT are smokers (Sinclair et al., 1997b), and smoking has been considered to be an inducer of CYP1A2 (Sesardic et al., 1990b).

Here we have investigated whether human CYP1A2 catalyzes UROX. In a panel of human liver microsomes, we found that, al-

1 Abbreviations used are: PCT, porphyria cutanea tarda; CYP, cytochrome P450; CYP reductase, NADPH-cytochrome P450 oxidoreductase; EROD, ethoxyresorufin deethylation; MROD, methoxresorufin demethylation; URO, uroporphyrin; URO-D, uroporphyrinogen decarboxylase; UROX, uroporphyrinogen oxidation.
Materials and Methods

Materials. 7-Ethoxyresorufin, 7-methoxyresorufin, and resorufin were purchased from Molecular Probes (Eugene, OR). URO I was purchased from Porphyrin Products (Logan, UT). 5-Bromo-4-chloro-3-indole toluidine phosphate and p-nitroblue tetrazolium chloride were purchased from Bio-Rad Laboratories (Hercules, CA). Furafylline was prepared in the laboratory of Dr. W. Pfeifer (Fuhr and Pfeifer, 1995). Ketocozazole and α-naphthoflavone were purchased from Sigma Chemical Co. (St. Louis, MO). The primary antibodies used in the immunodetection of the different CYPs were as follows: for CYP1A2, a polyclonal antibody that detects both CYP1A1 and CYP2A in rats and mice (Sinclair et al., 1990a), kindly supplied by Dr. S. Wrighton (Eli Lilly Research Laboratories, Indianapolis, IN); for CYP3A, a polyclonal antibody kindly supplied by Dr. P. Guzelian (University of Colorado Medical School, Denver, CO) (Louis et al., 1994); for CYP2E, a monoclonal antibody (1–98-1) kindly supplied by Drs. H. Gelboin and S. Park (NCI, Bethesda, MD). Hernan Liver Microsomes. Liver microsomes were obtained either from the Department of Clinical Pharmacology, University Hospital of Frankfurt (Frankfurt, Germany) (Fuhr et al., 1992), from Human Biologics, University of Kansas (Kansas City, MO), or from a liver resection performed at the Veterans Administration Hospital (White River Junction, VT). These were obtained under protocols approved by the appropriate committees for the conduct of human research. The livers were stored at −80°C and the microsomes, prepared as described (Lambrecht et al., 1990), were also stored at −80°C.

Expression Systems. HepG2 cells were infected with vaccinia virus vector alone or containing cDNAs for human CYP1A2, human CYP3A4, or mouse CYP1A2, as described (Gonzalez et al., 1991; Tsydlov et al., 1993). Lysates were prepared by sonication to cell suspensions for 20 sec. Microsomes from human B lymphoblastoid lines expressing human CYP1A1, CYP1A2, or CYP1A4, from cells with the vector but without inserted cDNA, and from insect cells infected with baculovirus (Supersomes), with or without cDNA for human CYP1A1, CYP1A2, CYP3A4, CYP3A5, or CYP2E, were purchased from Gentest Corp. (Woburn, MA). Semipurified human CYP1A2-rat CYP reductase recombinant fusion protein expressed in Escherichia coli was kindly provided by Drs. C. Fisher and R. Estabrook (Southwestern Medical School, Dallas, TX) (Shet MS, Fisher CW, Estabrook RW, Expression, purification, and enzymatic properties of a recombinant fusion protein containing human P450 1A2 joined to NADPH-P450 reductase. Proceedings of the International Society for The Study of Xenobiotics 4, 1995). Reconstituted human CYP1A2 and CYP3A4 with human CYP reductase were purchased as reconstituted liposomes from Pan Vera Corp. (Madison, WI). Hepatic microsomes from untreated male C57BL/6 mice were prepared as described (Lambrecht et al., 1990).

The first four lanes contained microsomes from lymphoblastoid cells void of any CYP, or expressing CYP1A1, CYP1A2 or a mixture of these two as indicated. In each of the first four lanes, 1.7 μg of protein was applied; in the last five lanes (human liver microsomes), 3 μg of protein was applied.

Results

Comparison of UROX in Human and Mouse Liver Microsomes. A panel of eight human liver microsomal samples, derived from patients with histologically normal livers, were obtained from three different sources. The samples were chosen to represent widely different CYP1A2 contents and/or CYP1A2-specific activities, based on the values supplied by the sources. Caffeine N\textsuperscript{6}-demethylation activities, which are attributed mainly to CYP1A1 (Fuhr et al., 1996), ranged >6-fold in one group of samples that had been assayed together (samples 1–4) and >12-fold in the other group (samples 6–8). There was immunodetectable CYP1A2 in seven of the eight samples, and the amount varied by 8-fold (fig. 1, table 1). Sample 2 contained a protein that might be CYP1A1, because it migrated to the same position as human CYP1A1 expressed in lymphoblastoid cells (fig. 1). Detection of CYP1A1 depended on the distinct separation of CYP1A1 and CYP1A2 that was achieved here. It should be noted that sample 2 had the highest CYP1A2 level and some detectable

### TABLE 1

**Characteristics of human liver microsomes**

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Gender</th>
<th>Age (years)</th>
<th>CYP3A4 content (units)</th>
<th>CYP1A2 content (units)</th>
<th>Caffeine N\textsuperscript{6}-demethylation (pmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>F</td>
<td>64</td>
<td>54</td>
<td>48</td>
<td>123</td>
</tr>
<tr>
<td>02</td>
<td>F</td>
<td>54</td>
<td>48</td>
<td>41</td>
<td>290</td>
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<tr>
<td>03</td>
<td>M</td>
<td>80</td>
<td>51</td>
<td>45</td>
<td>15</td>
</tr>
<tr>
<td>04</td>
<td>M</td>
<td>51</td>
<td>45</td>
<td>40</td>
<td>68</td>
</tr>
<tr>
<td>05</td>
<td>M</td>
<td>38</td>
<td>38</td>
<td>&lt;5</td>
<td>275</td>
</tr>
<tr>
<td>06</td>
<td>M</td>
<td>22</td>
<td>22</td>
<td>15</td>
<td>ND</td>
</tr>
<tr>
<td>07</td>
<td>F</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>08</td>
<td>F</td>
<td>30</td>
<td>30</td>
<td>15</td>
<td>30</td>
</tr>
</tbody>
</table>

**Notes:**
- Patient number.
- F, female; M, male; NA, not available; ND, not determined.
- Arbitrary units, determined by densitometric analysis of immunoblots.
- Activities (expressed as picomoles of metabolites per minute per milligram of protein) were determined in two different laboratories, for samples 1–4 and 6–8.

Assays. Activities of caffeine N\textsuperscript{6}-demethylation (table 1), determined as

V\textsubscript{max} values for the high affinity site of caffeine N\textsuperscript{6}-demethylation, were determined in the laboratory of Dr. Fuhr (Fuhr et al., 1992) or were measured, at saturating caffeine concentrations, at Human Biologics, as described (Naline et al., 1987). EROD and MROD activities were measured spectrophotometrically, as previously described for EROD (Sinclair et al., 1997b). For MROD, the only difference was that methoxyresorufin (2 μM) was used as the substrate. UROX activity was determined spectrophotometrically from the formation of URO, as described, using 25–50 pmol of CYP and 5 μM uroporphyrinogen I as the substrate (Sinclair et al., 1997b). The activities obtained with the expression systems, on a per-milligram of protein basis, were corrected for the activity observed in lysates or microsomes from cells that were infected with vectors without CYP cDNA. For insect cell microsomes containing expressed CYP and CYP reductase, the control activity used was from microsomes from cells containing no expressed CYP or CYP reductase. Inhibition studies of the enzymatic activities were performed after preincubation of the reaction mixture (without the substrate) with the inhibitor in dimethylsulfoxide (1 μM, 0.25 ml of reaction mixture) for 5 min. This concentration of dimethylsulfoxide alone decreased the activity by <10%. CYP concentrations were determined spectrophotometrically as described (Omura and Sato, 1964).

Western Blots. Immunoblotting was performed as described previously (Louis et al., 1994; Gorman et al., 1998). The immunoblots were quantitated using a TRI 1000 scanning densitometer (Technology Resources, Nashville, TN).
CYP1A1. This sample also had the highest caffeine N\textsuperscript{4}-demethylation activity of the human microsomal samples studied in the Fuhr laboratory (Fuhr U, unpublished observations). Patient 2 was the only smoker in the group. As determined immunochemically, CYP3A content varied by 3-fold for all of the samples and CYP2E content varied by 2-fold (table 1).

MROD and EROD, in addition to UROX activities, were determined in these human microsomal samples, to investigate whether there was any correlation with UROX activities. MROD and EROD, in the absence of CYP1A1, are mainly catalyzed by CYP1A2 (Sesardic \textit{et al.}, 1990b; Tsyrlov \textit{et al.}, 1993). MROD and EROD activities were quite measurable and were similar to or higher than the activities of hepatic microsomes from untreated mouse liver, indicating that the CYP1A2 contents in mouse and human microsomes were of the same order (fig. 2). There was very good correlation of MROD and EROD activities (\(r = 0.99\)). There was also very good correlation of MROD and EROD with immunodetectable CYP1A2 content (\(r = 0.99\) and 0.92, respectively). These results are consistent with both activities being catalyzed by a single CYP in these preparations. We do not think the extra protein of 45 kDa detected in the immunoblot (fig. 1) was a proteolytic product of CYP1A2. Even if there was some degradation of CYP1A2, there was still considerable activity, as indicated by EROD and MROD measurements.

Fig. 2 shows that, whereas UROX activity was readily detected with untreated mouse microsomes, UROX activity catalyzed by the human liver microsomes was extremely low. The highest UROX activities in the human samples were only 10% of the activity determined in liver microsomes from uninduced C57BL/6 mice (fig. 2). Even with such low activities, an approximately 7-fold variation in the activities was catalyzed by human microsomes was detected, with only poor correlation with CYP1A2 content (\(r = 0.43\)). Sample 6 catalyzed UROX activity, even though there was no immunodetectable CYP1A2 and very low EROD and MROD activities.

There was only poor correlation of EROD or MROD activities with UROX activity (\(r = 0.12\) and 0.42, respectively) (fig. 2), suggesting that in these human hepatic microsomes, most of the UROX activity was not catalyzed by CYP1A2. These results indicate that the UROX activity in human liver microsomal samples was much lower than that in microsomes from untreated mice and could not be attributed to CYP1A2.

**Human CYP1A2 and UROX Activities in Expression Systems and in Reconstituted Liposomes.** We used four different recombinant model systems expressing individual CYP forms, as well as reconstituted human CYP1A2 and CYP3A4, to assess the abilities of particular forms to catalyze UROX, and to compare the efficacies of murine and human CYP1A2 to catalyze UROX.

**HepG2 Hepatoma Cells.** Mouse CYP1A2 and human CYP1A2 and CYP3A4 were expressed in HepG2 cells using vaccinia virus as the vector. When lysates of cells expressing CYP1A2 were analyzed for EROD and MROD, the activities of human CYP1A2 were greater than those of mouse CYP1A2 (table 2). Acetanilide 4-hydroxylase activities for human and mouse CYP1A2 were similar (3.1 and 4.6 pmol/min/pmol of CYP, respectively) (Tsyrlov IB, Gelboin HV, in \textit{Proceedings of the Xth International Symposium on Microsomes and Drug Oxidations}, 1994). The UROX activity of HepG2 cells expressing human CYP1A2 was only 20% of that of cells expressing murine CYP1A2. The UROX activity of human CYP1A2 was less than that of human CYP3A4 (table 2).

It is possible that some of the UROX activity in the HepG2 cell lysates was due to CYP reductase. Uninfected HepG2 cells and cells infected with wild-type vaccinia virus (devoid of detectable CYP) have reductase contents of approximately 4–5 pmol of reductase/mg of protein, based on a reductase activity of 5 \(\mu\)mol of cytochrome \(c\) reduced/min/nmol of reductase (Tsyrlov IB, Gelboin HV, unpublished results). Lysates of these cells demonstrated UROX activity of 0.26 pmol of URO/min/mg of protein. In cells infected with virus to overexpress human CYP reductase, there was approximately 26 pmol of reductase/mg of protein and a UROX activity of 0.62 pmol/min/mg of protein. These results suggest that the reductase could be one of the contributors to the UROX activity observed in lysates from cells with expressed CYPs. UROX values for the HepG2 lysates shown in table 2 were corrected for the contribution of background UROX activity (endogenous reductase and other factors) using the value of 0.26 pmol of URO/min/mg of protein. This represents approximately 50% of the

![Fig. 2.](Image)

F I G . 2 . MROD (A), EROD (B), and UROX (C) activities in human liver microsomes compared with microsomes from untreated mouse liver (Ctrl). Assays were performed as described in the “Methods” section. Values are means and SDs of three to four determinations.
UROX activity in lysates containing human CYP1A2 or CYP3A4 but only 6% of the UROX activity in lysates containing mouse CYP1A2.

**Lymphoblastoid Cells.** EROD, MROD, and UROX activities were measured in microsomes containing human CYP1A1, CYP1A2, or CYP3A4 (table 2). Both CYP1A1 and CYP1A2 catalyzed EROD and MROD, with preferential catalysis of EROD by CYP1A1 and of MROD by CYP1A2. CYP3A4 did not catalyze either activity. CYP1A1 was more active in catalyzing UROX than was CYP1A2, and both were more active than was CYP3A4. The UROX values obtained with microsomes from lymphoblastoid cells were very low, compared with the other expression systems (table 2). The non-CYP background activity in these microsomes was approximately 60% for microsomes containing CYP1A1 or CYP1A2 and approximately 90% for microsomes containing CYP3A4.

**Insect Cells.** High enzymatic activities were obtained with microsomes prepared from baculovirus-infected insect cells expressing both human CYP reductase and CYP1A1, CYP1A2, or CYP3A4 (table 2). UROX activity catalyzed by CYP1A2 in these microsomes was sevenfold higher than the activity catalyzed by CYP1A1, 1.4–3-fold higher than the activity of the one preparation of CYP3A4 tested, and 2–4-fold higher than the activity of either CYP3A5 or CYP2E1. CYP reductase expression levels in the microsomes containing CYP1A1 and CYP1A2 were similar, i.e., 1.5–2 nmol/mg of protein (based on information from the supplier of these microsomes). The reductase/CYP molar ratios were different for each preparation of insect microsomes; they were greater for the preparations containing CYP1A1 than for those with CYP1A2, and ratios for both of these preparations were greater than those for preparations with CYP3A4 (table 2). The UROX/MROD activity ratios for CYP1A2 were similar in the insect cell microsomes, HepG2 cell sonicates, and lymphoblastoid cells (table 2) but were only 10% of the UROX/MROD ratio for mouse CYP1A2 expressed in HepG2 cells. The UROX/MROD ratios for insect cell microsomes containing human CYP1A2 were approximately sevenfold higher than those for microsomes containing expressed human CYP1A1.

Microsomes containing CYP3A4 were more active in catalyzing UROX than were those containing CYP1A1, CYP3A5, or CYP2E1. The contributions of non-CYP activity in microsomes containing CYP1A1, CYP1A2, CYP3A4, CYP3A5, and CYP2E2 were 77–93, 37–50, 36–42, 45, and 43%, respectively.

Several inhibitors were used to characterize UROX, EROD, and MROD activities catalyzed by microsomes from insect cells expressing human CYP1A1 or CYP1A2. Table 3 shows the effect of these inhibitors on EROD, MROD, and UROX activities catalyzed by microsomes containing either of the two CYP1A forms. The values in table 3 were not corrected for the background activity of microsomes without expressed CYPs. Table 3 shows that furafylline, a selective inhibitor of human CYP1A2 (Sesardic et al., 1990a), inhibited both O-dealkylation activities catalyzed by CYP1A2. EROD and MROD activities catalyzed by CYP1A1 in this system were also inhibited. Furafylline inhibited microsomal UROX catalyzed by microsomes containing CYP1A2 by 50% but caused no inhibition of UROX catalyzed by microsomes containing CYP1A1. This result suggests that less than one half of the UROX activity catalyzed by microsomes containing CYP1A2 was due to CYP1A2 and that none of the UROX catalyzed by microsomes containing CYP1A1 was actually the result of catalysis by CYP1A1. The α-naphthoflavone inhibition of EROD, MROD, and UROX activities catalyzed by microsomes containing CYP1A2 was similar to the effects of furafylline on these reactions. However, whereas α-naphthoflavone totally inhibited EROD and MROD catalyzed by microsomes containing CYP1A1, it stimulated UROX by 50%. Ketoconazole (50 μM) completely inhibited EROD and MROD catalyzed by microsomes containing CYP1A1 but inhibited only 10–20% of these activities catalyzed by microsomes containing CYP1A2. The selective inhibition of human CYP1A1 by ketoconazole was also observed with CYP1A2 forms expressed in lymphoblastoid cells (data not shown). However, ketoconazole only partially inhibited the UROX activity catalyzed by microsomes containing CYP1A1, again indicating that UROX activity in these microsomes was mostly catalyzed by CYP1A2.

### Table 2: CYP contents and activities of EROD, MROD, and UROX catalyzed by mouse and human CYPs in various expression systems or purified CYPs

<table>
<thead>
<tr>
<th>Sample</th>
<th>CYP Content</th>
<th>EROD</th>
<th>MROD</th>
<th>UROX</th>
<th>UROX/MROD Ratio ((&lt;10^{-2}))</th>
<th>Reductase/CYP Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2 cells</td>
<td>Mouse 1A2</td>
<td>23</td>
<td>0.7 ± 0</td>
<td>0.1 ± 1</td>
<td>66 ± 4</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Human 1A2</td>
<td>17</td>
<td>1.2 ± 0</td>
<td>2.1 ± 1</td>
<td>12 ± 2</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>Human 3A4</td>
<td>32</td>
<td>0</td>
<td>0</td>
<td>23 ± 3</td>
<td>0.1</td>
</tr>
<tr>
<td>Lymphoblasts</td>
<td>Human 1A1</td>
<td>67</td>
<td>4.7 ± 0.1</td>
<td>0.8 ± 0</td>
<td>2.8 ± 0.4</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>Human 1A2</td>
<td>127</td>
<td>0.2 ± 0</td>
<td>0.7 ± 0</td>
<td>1.5 ± 0.2</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Human 3A4</td>
<td>49</td>
<td>0</td>
<td>0</td>
<td>0.6 ± 0.2</td>
<td>1.8</td>
</tr>
<tr>
<td>Insect cells</td>
<td>Human 1A1 (3)</td>
<td>121 ± 19</td>
<td>14.8 ± 6</td>
<td>6.8 ± 2</td>
<td>4 ± 2.5</td>
<td>0.9 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Human 1A2 (3)</td>
<td>181 ± 17</td>
<td>1.7 ± 0.3</td>
<td>3.6 ± 0.6</td>
<td>24 ± 8</td>
<td>6.6 ± 1</td>
</tr>
<tr>
<td></td>
<td>Human 3A4 (2)</td>
<td>560 ± 135</td>
<td>0</td>
<td>ND</td>
<td>12 ± 0.3</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Human 2E1 (1)</td>
<td>463</td>
<td>ND</td>
<td>ND</td>
<td>8 ± 0</td>
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</tr>
<tr>
<td></td>
<td>Human 3A5 (1)</td>
<td>500</td>
<td>ND</td>
<td>ND</td>
<td>7 ± 0.4</td>
<td>0.6</td>
</tr>
</tbody>
</table>

**ET AL.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>CYP Content</th>
<th>EROD</th>
<th>MROD</th>
<th>UROX</th>
<th>UROX/MROD Ratio ((&lt;10^{-2}))</th>
<th>Reductase/CYP Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>Human CYP1A2-rat CYP reductase fusion protein</td>
<td>NA</td>
<td>0.3 ± 0</td>
<td>0.3 ± 0</td>
<td>12 ± 0.2</td>
<td>40</td>
</tr>
<tr>
<td>Reconstituted CYP</td>
<td>Human 1A2</td>
<td>NA</td>
<td>0.4 ± 0</td>
<td>0.6 ± 0</td>
<td>36 ± 4</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Human 3A4</td>
<td>NA</td>
<td>ND</td>
<td>ND</td>
<td>12 ± 0.5</td>
<td>2</td>
</tr>
</tbody>
</table>


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**Notes:**
- HepG2 samples were cell lysates; reconstituted CYPs were mixtures of CYP, CYP reductase, and liposomes; lymphoblasts and insect cells were microsomal suspensions; and the fusion protein was partially purified (see Materials and Methods).
- UROX values were corrected for values for cells without vector, except for the fusion protein and reconstituted CYPs. The insect cells without vector did not contain CYP reductase, which was expressed in these cells with various CYPs. The UROX values for cells without vector were 0.26, 0.28, and 3.2 pmol of URO/min/mg of protein for HepG2 cell lysates, lymphoblast microsomes, and insect cell microsomes, respectively.
- Values in parentheses refer to the number of different preparations of insect cell microsomes used.
- ND, not determined; NA, not available.
Effects of CYP inhibitors on EROD, MROD, and UROX activities catalyzed by microsomes from insect cells expressing human CYP1A1 and CYP1A2

<table>
<thead>
<tr>
<th>CYP1A2</th>
<th>EROD&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>MROD&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>UROX&lt;sup&gt;a&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 ± 1</td>
<td>100 ± 2</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>+Furafylline</td>
<td>8 ± 1</td>
<td>15 ± 2</td>
<td>54 ± 3</td>
</tr>
<tr>
<td>+α-NF</td>
<td>0 ± 2</td>
<td>0 ± 2</td>
<td>50 ± 4</td>
</tr>
<tr>
<td>+Ketoconazole</td>
<td>85 ± 0</td>
<td>86 ± 3</td>
<td>70 ± 14</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>100 ± 2</td>
<td>100 ± 1</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>+Furafylline</td>
<td>45 ± 0</td>
<td>18 ± 1</td>
<td>99 ± 6</td>
</tr>
<tr>
<td>+α-NF</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>153 ± 7</td>
</tr>
<tr>
<td>+Ketoconazole</td>
<td>4 ± 4</td>
<td>1 ± 1</td>
<td>81 ± 8</td>
</tr>
</tbody>
</table>

Values represent means and ranges of two to four determinations. Inhibitor concentrations were as follows: furafylline, 50 μM; α-naphthoflavone (α-NF), 30 μM; ketoconazole, 50 μM.

Discussion

Previous work, mainly using animal models and reconstituted CYP1A2, implicated CYP1A2 as the key enzyme catalyzing UROX in the process leading to experimental uroporphyria (Jacobs et al., 1989a; Lambrecht et al., 1992; Sinclair et al., 1997b, 1998). In this report, we have investigated two questions, i.e., 1) whether human CYP1A2, compared with other human CYPs, catalyzes UROX at rates similar to that of the murine orthologue and 2) whether UROX activity in human liver microsomes can be attributed to CYP1A2.

Assessment of the ability of human CYP1A2 to catalyze UROX is important in the evaluation of the potential role of CYP1A2 in the human uroporphyria known as PCT.

The experiments with HepG2 cells expressing both human and murine CYP1A2 allowed a comparison of UROX activities catalyzed by each orthologue in the same cell line. The severalfold difference in the UROX activities of mouse and human CYP1A2 (table 2) supported the finding of low human UROX values obtained in the comparison of mouse and human liver microsomes (fig. 2). These results clearly indicated that even expressed human CYP1A2 is much less active in the catalysis of UROX than is the mouse orthologue. This was an unexpected finding because, as shown here, the two orthologues were quite comparable in catalyzing EROD and MROD.

The relationship is seen clearly in table 2, where the UROX/MROD ratios for human CYP1A2 expressed in several systems were maximally only one tenth of that for mouse CYP1A2 expressed in HepG2 cells. The only expressed human CYP1A2 forms with a UROX/MROD ratio approximating that of the mouse form were the E. coli CYP1A2-CYP reductase fusion protein and the reconstituted CYP1A2. The fusion protein of covalently linked CYP reductase and CYP1A2 was not inhibited by typical CYP inhibitors. Thus, catalysis of UROX by the fusion protein was anomalously low in comparison with the other expressed CYP1A2s, which are not covalently bound to reductase. Reconstituted CYP1A2 had a higher activity than the expressed forms but was still less active than mouse CYP1A2 expressed in HepG2 cells (table 2). Similarly, UROX activity catalyzed by the reconstituted human CYP1A2 was one third of the value obtained previously with reconstituted mouse CYP1A2 (Lambrecht et al., 1992). Mouse and human CYP1A2 bear a high degree of amino acid identity (77%). Nucleotide identity is approximately 80% for exons 2–6 and 58% for exons 1 and 7 (Ikeya et al., 1989). Presumably, differences in amino acid residues in the vicinity of the active center of the mouse orthologue are responsible for the higher UROX specific activity of the mouse form. It appears that during evolution the specific activity of CYP1A2 for oxidizing uroporphyrinogen, an intermediate of the heme biosynthetic pathway, has decreased. This does not present an obvious evolutionary advantage, other than a decreased susceptibility to porphyria.

CYP forms other than CYP1A2 were also active in catalyzing UROX. CYP1A1 and CYP2E were less active than human CYP1A2 and much less active than mouse CYP1A2. However, the UROX activity of CYP3A4 (either the purified reconstituted form or that expressed in HepG2 cells and lymphoblasts) was closest to the activity of CYP1A2.

The data obtained with microsomes from the insect cells indicated considerable non-CYP background activity. Only approximately one half of the UROX activity of these microsomes containing CYP1A2, CYP3A4, CYP3A5, or CYP2E was catalyzed by the CYPs, and very little of the UROX activity of microsomes containing CYP1A1 was catalyzed by this form. The high CYP reductase/CYP ratio in those
microsomes (Crespi and Miller, 1997) suggests that the reductase may contribute to UROX activity. We demonstrated previously, with mouse hepatic microsomes, that iron-EDTA increases a catalase-sensitive UROX activity through reactive oxygen species generated at the reductase level, rather than by iron stimulating CYP-catalyzed UROX (Jacobs et al., 1989b). Catalase only partially inhibited UROX catalyzed by both CYP1A1 and CYP1A2 expressed in the insect cells, supporting the view that the excess reductase contributes some but not all of the UROX activity. The problem of the effect on drug metabolism of high CYP reductase/CYP ratios in insect cells has recently been encountered by others (Crespi and Miller, 1997). One clear advantage of the HepG2 vaccinia and lymphoblast expression systems is their relatively low reductase/CYP ratios (table 2), producing a minimal contribution of reductase to measured UROX. This ratio is in the range (1:10 to 1:20) found in this laboratory for mammalian liver microsomes from humans, mice, and rats (Gorman N and Sinclair P, unpublished observations).

We observed some interesting results with ketoconazole when it was used as a nonspecific CYP inhibitor of activities in the expression systems (Newton et al., 1995). Ketoconazole, at 50 μM (a concentration where it acts nonspecifically) (Newton et al., 1995), inhibited human CYP1A1-catalyzed EROD and MROD activities almost completely but had little effect (<20%) on CYP1A2-catalyzed EROD and MROD activities. However, ketoconazole did not inhibit UROX catalyzed by CYP1A1 in the insect cell microsomes, which suggests, as stated previously, that this UROX activity was not catalyzed by CYP1A1. Interestingly, whereas ketoconazole had little effect on human CYP1A2-catalyzed UROX, it inhibited UROX catalyzed by reconstituted mouse CYP1A2 (Lambrecht et al., 1992) and by chick CYP1A1 (Sinclair et al., 1997b).

Based on findings with experimental animal systems, it has been suggested that human CYP1A2 may have a role in human PCT (Elder, 1990; Sinclair et al., 1997a). The finding that human CYP1A2 is much less active in catalyzing UROX activity than is murine CYP1A2 has implications for this hypothesis. There is as yet no direct evidence linking CYP1A2 to PCT, other than the recently noted common association with smoking (Sinclair et al., 1997a), which is a known CYP1A2 inducer (Sesaric et al., 1990b). Uroporphyrin can be caused in mice by 5-aminolevulinic acid and iron overload in the presence or absence of treatment with 3-methylcholanthrene, a CYP1A2 inducer (Urquhart et al., 1988; Dean and Elder, 1991; Smith and Francis, 1993). Using Cyp1a2-knockout mice, this uroporphyrinemia was recently shown to be absolutely dependent on CYP1A2 expression (Sinclair et al., 1998). Because human CYP1A2 is shown here to be less active than the mouse orthologue in catalyzing UROX, a role for CYP1A2 in human PCT may depend on induction, such as occurs in smokers. Although the current study does not support a major role for CYP1A2 in UROX in humans, it is still possible that a small increase in CYP1A2 might, over a long period, be sufficient to produce the hepatic UROX accumulation observed in PCT.

The difference in the abilities of human and murine CYP1A2 orthologues to catalyze UROX raises the additional possibility that in PCT there is a mutation in the human form, making it more active in catalyzing UROX than the normal human form that was used in the current studies. No genetic polymorphism in human CYP1A2 has yet been found (Eaton et al., 1995). Another possibility raised here is that other CYP isoforms, such as CYP3A4 or CYP2E, may be active in UROX. Both CYP2E and CYP3A4 are inducible by the alcohols in alcoholic beverages (Koop and Coon, 1984; Louis et al., 1994; Roberts et al., 1995). Consumption of alcoholic beverages is a known precipitant of PCT (Elder, 1990; Anderson, 1996). CYP2E is known to cause the production of more reactive oxygen species, such as superoxide and hydrogen peroxide, than do other forms (Ekstrom et al., 1986). CYP2E was active in catalyzing UROX but to no greater extent than CYP3A4. We previously concluded that superoxide has no role in UROX, because there was no correlation of UROX activities with production of superoxide (as measured by lucigenin chemiluminescence) by mouse microsomes induced for different CYP forms (Sinclair et al., 1990b). Superoxide production was proportional to the total CYP content and not to any particular CYP form.

In summary, UROX activity in human liver microsomes was not correlated with CYP1A2 content. Experiments with different expression systems confirmed that CYP1A2 catalyzes UROX, but with a lower specific activity than that of the mouse orthologue. Whether there is a role for this CYP in the development of human PCT and whether reductases or other CYP forms have roles in the development of PCT remain to be determined.

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References


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