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Uroporphyrin accumulation in hepatoma cells expressing human or mouse CYP1A2: relation to the role of CYP1A2 in human porphyria cutanea tarda

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Abstract

In experimental animals, CYP1A2 is absolutely required for the development of uroporphyria induced by treatment with polyhalogenated aromatic compounds or other compounds. Although the role of this CYP in clinical uroporphyria, porphyria cutanea tarda (PCT), is not clear, *Cyp1a2(−/−)* mice are resistant to the development of uroporphyria. Here, we compared the abilities of human and mouse CYP1A2 expressed in mouse hepatoma Hepa-1 cells to: (i) catalyze CYP1A2-dependent methoxyresorufin demethylase (MROD), and (ii) support uroporphyrin (URO) accumulation. Both CYP1A2 orthologs were expressed at similar levels as indicated by immunodetectable CYP1A2 proteins and MROD activities. URO accumulation was increased in cultures expressing either ortholog when supplemented with 5-aminolevulinic acid, the porphyrin precursor. Cells expressing mouse CYP1A2 produced more URO than cells expressing human CYP1A2. The results indicate that human CYP1A2 can support URO accumulation in hepatoma cells and thus may play a role in human PCT.

Keywords: CYP1A2; Porphyria cutanea tarda; Uroporphyrin; 5-Aminolevulinic acid; Uroporphyrinogen oxidation

1. Introduction

CYP1A2 is a cytochrome P450 of the CYP1 family that is principally and constitutively expressed in the liver of humans and animals [1]. CYP1A2 has been implicated in the development of experimental uroporphyria caused by AH receptor ligands, in which there is a massive hepatic accumulation and urinary excretion of URO and other highly carboxylated porphyrins [2]. In experimental animals, treatment with ALA alone, the precursor of porphyrins (Fig. 1), can lead to the development of uroporphyria. This is accelerated when animals are also treated with iron [3,4]. Recently, we showed that the uroporphyria caused by several different treatments, including ALA and iron, is prevented completely in *Cyp1a2(−/−)* mice [5–7], indicating the absolute requirement of CYP1A2 expression. Whether CYP1A2 also has a role in chronic clinical uroporphyria, known as PCT, has not been established, and some skepticism has been raised about this role [8,9]. PCT is associated with the consumption of alcoholic beverages, hepatic viral infection, or the use of estrogenic steroids [2], and is usually associated with some iron overload [2].

In this report, we investigated whether human CYP1A2 supports URO accumulation in intact liver-like cells. We

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Abbreviations: ALA, 5-aminolevulinic acid; CYP, cytochrome P450; MROD, methoxyresorufin demethylase; PCT, porphyria cutanea tarda; RT–PCR, reverse transcriptase–polymerase chain reaction; URO, uroporphyrin; and UROX, uroporphyrinogen oxidation.

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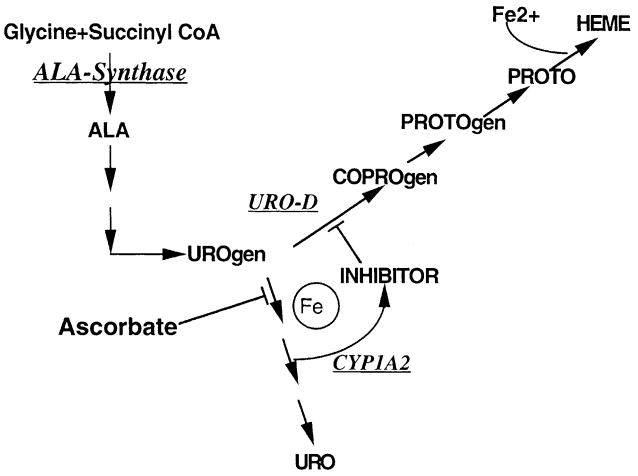

Fig. 1. Scheme of heme biosynthesis indicating that URO accumulation is due to the combination of the UROX reaction and inhibition of URO-D activity. Abbreviations used in the figure: ALA, 5-aminolevulinic acid; COPROgen, coproporphyrinogen; PROTO, protoporphyrin; PROTOgen, protoporphyrinogen; URO, uroporphyrin; URO-D, uroporphyrinogen decarboxylase; and UROgen, uroporphyrinogen.

present the results of studies with mouse hepatoma cells transiently transfected with mouse or human CYP1A2. When treated with ALA, the hepatoma cells transfected with either of the CYP1A2 orthologs accumulated URO. These results support the hypothesis of a role for CYP1A2 in human uroporphyria.

2. Materials and methods

2.1. Materials

Lymphoblasts expressing human CYP1A2 were obtained from Gentest. Male C57BL/6N mice were purchased from the NCI.

2.2. Cell culture

Hepa-1 cells, provided by Dr. O. Hankinson, Department of Pathology, UCLA Medical School, were maintained in Williams' E medium (prepared from powder; GIBCO) supplemented with 5% fetal bovine serum (GIBCO), penicillin/streptomycin (GIBCO), and glutamine (2 mM) under 5% CO_2. Cells were passed prior to becoming 100% confluent.

2.3. Vector construction

The mouse CYP1A2 eukaryotic expression vector ("pm1A2") was constructed as follows. To produce a source of RNA enriched in mouse CYP1A2 mRNA, CYP1A2 expression was induced in C57BL/6N male mice by treatment with 3-methylcholanthrene (Sigma) for 24 hr (100 mg/kg body weight, injected i.p. in corn oil, 6 mg/mL). This animal protocol has been approved by the Animal Use Committees of Dartmouth College and the VA Medical Center. Total RNA was purified using a commercial kit (RNeasy, Qiagen) from whole liver following the instructions of the manufacturer, and cDNA was produced by reverse transcription, using an oligo-dT primer. Sense (5′-ACGTGGTACCATGGCGTTCTCCCATGACAT-3′) and antisense (5′-CGTCACTAGTATGCCTCGACAATC-TTCACT-3′) oligonucleotides were designed that introduce: (i) a Kozak translation recognition sequence upstream of the ATG start site [10,11], and (ii) *Kpn*I and *Spe*I sites into a PCR product encoding the full-length mouse CYP1A2 protein. Liver cDNA was PCR-amplified using standard procedures, and the *Kpn*I/*Spe*I-digested PCR product was ligated into *Kpn*I and *Spe*I sites of the parent vector, pANloxPY (see below). Following transformation of competent DH5α bacteria, clones were isolated, and the integrity of the plasmid DNA was verified by restriction enzyme analysis and sequencing of both DNA strands. The sequence was identical to the mouse CYP1A2 sequence in the database (GenBank Access No. #XØØ479). An expression clone for human CYP1A2 was generated by the amplification of human CYP1A2 from a previously described cDNA clone [12]. The primers designed for this cloning operation were similar to that for RT–PCR of the mouse CYP1A2 cDNA clone. Both mouse and human CYP1A2s were cloned into the mammalian expression vector, pANloxPY. This vector was prepared in our laboratory and uses the CMV early promoter and enhancer to drive high-level expression. It also contains an SV40 intron and a poly A signal.[1]

2.4. Transfection

For transfection experiments, $1–4 \times 10^6$ trypsinized Hepa-1 cells were plated in 100-mm diameter tissue culture plates and grown to 40% confluence. Culture medium was removed immediately prior to transfection. For each plate, 5.6 μg of plasmid DNA was complexed to Lipofectamine Plus according to the instructions of the manufacturer (GIBCO) and added to OptiMEM medium (GIBCO) at transfection. Three hours after transfection, the medium was supplemented with fetal bovine serum, penicillin/streptomycin, and glutamine to give the same final concentrations used for growing cells. Twenty-four hours after transfection ALA was added to the cell medium to give a final concentration of 150 μM. Up to 500 μM ALA had no effect on cell growth (data not shown). Transfection efficiency was monitored in some experiments by co-transfection with the luciferase reporter, pGL3 (Promega). Luciferase activity varied less than 5% between triplicates. Porphyrin accumulation was assayed as described below, 24 hr after adding ALA. Cells

[1] For restriction map and complete sequence of this clone, contact Dr. T. Dalton (tim.dalton@uc.edu).

transfected with empty pANloxPY vector constituted the negative controls for CYP1A2 expression.

2.5. Preparation of microsomes

Cells were rinsed with 0.15 M NaCl and scraped into 25 mM Tris/0.25 M sucrose/1 mM EDTA, pH 7.4. The suspension was sonicated for 6 sec using a Branson Sonifier, model W140D at setting "3." The sonicate was centrifuged at 10,000 *g* for 10 min at 4°, and the supernatant at 100,000 *g* for 1 hr at 4°. The pellet was overlayed with 50 mM HEPES/0.25 M sucrose/1 mM EDTA, pH 7.4, and was stored at −80° for 1–2 weeks prior to assay.

2.6. Antibodies

A polyclonal antibody raised against rat CYP1A2, which detects CYP1A1 and CYP1A2 proteins from both humans and mice, was a gift from Dr. Steven Wrighton (Lilly Research Laboratories).

2.7. Assay of porphyrin composition

Porphyrin composition in cells plus medium was determined by reverse-phase HPLC after extraction with an equal volume of acetone–conc. HCl (97.5:2.5, v/v), as described previously [13]. During the extraction procedure, uroporphyrinogen is oxidized to URO. Protein concentrations were determined by the method of Lowry *et al.* [14], using bovine serum albumin as a standard.

2.8. Assay of microsomal MROD and UROX, and immunoblotting

Microsomal MROD and UROX activities were measured as described previously [15]. The presence of CYP1A1 and CYP1A2 proteins in cell homogenates or microsomes was determined by immunoblotting, following electrophoretic separation of proteins, as described [16].

2.9. Data analysis

Results are presented as means ± SD. Significance was determined by one-way ANOVA, where $P < 0.05$ was considered significant.

3. Results

3.1. CYP1A2 expression as detected by immunoblots and MROD activity in mouse hepatoma Hepa-1 cells transfected with vectors expressing mouse or human CYP1A2

Immunoblotting was used to determine whether the CYP1A2 proteins were produced in mouse hepatoma cells transfected with vectors expressing CYP1A2. Figure 2 shows an immunoblot of sonicates from two experiments with hepatoma cells expressing either human or mouse CYP1A2. As indicated, the antibody prepared against rat CYP1A2 detected both CYP1A1 and CYP1A2 in liver microsomes from 3-methylcholanthrene-treated mice (lane 9). Hepa-1 cells transfected with empty vector expressed no protein of similar size to either mouse or human CYP1A2 (lanes 1 and 2), whereas cells transfected with the mouse CYP1A2 vector expressed a protein of the same apparent molecular weight as mouse CYP1A2 protein (lanes 3, 4, and 7). The cells transfected with human CYP1A2 vector expressed a protein of higher apparent molecular weight (lanes 5, 6, and 8) than mouse CYP1A2. Expressed human CYP1A2 was of similar size to that detected in lymphoblasts expressing human CYP1A2 and in human liver microsomes (data not shown).

To measure the activities of each expressed CYP1A2 in these cultures, MROD, a microsomal activity highly specific for hepatic CYP1A2, especially in the absence of concurrent CYP1A1 expression [17], was used. This activity indicates whether the expressed CYP1A2 protein identified by immunoblotting is present as apo- or holocytochrome, since only the heme-containing holoprotein catalyzes MROD activity. There was no MROD activity detected in microsomes prepared from cells transfected with empty vector only (Fig. 3). The MROD activities of microsomes from cells transfected with vectors carrying either mouse or human CYP1A2 were similar, in accord with previous literature [18].

3.2. Accumulation of URO in ALA-treated cells transfected with mouse CYP1A2 or human CYP1A2

Table 1 shows the URO accumulation in cells transfected either with empty vector or with vectors for mouse

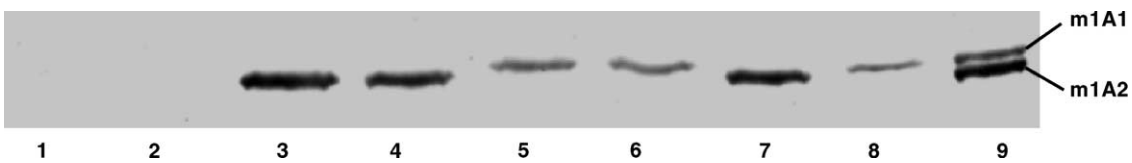

Fig. 2. Immunodetectable mouse and human CYP1A2 expressed in transfected hepatoma cells. Hepatoma cells were transfected and treated with 150 μM ALA for 24 hr before harvesting. Three micrograms of sonicate protein was loaded in each well. Lanes 1 and 2: hepatoma cells transfected with empty vector; lanes 3, 4, and 7: cells transfected with mouse CYP1A2 vector; lanes 5, 6, and 8: cells transfected with human CYP1A2 vector; lane 9: liver microsomes from a 3-methylcholanthrene-treated mouse showing induced levels of CYP1A1 and CYP1A2. Samples in wells 1–6 and 7, 8 were from two separate experiments.

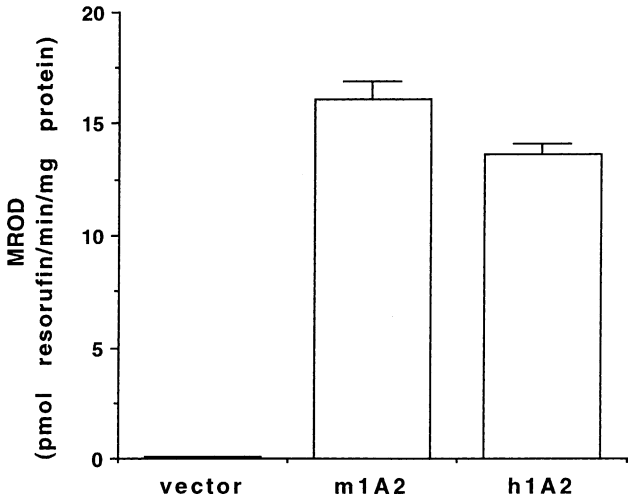

Fig. 3. Microsomal MROD activity in hepatoma cells transfected with vectors expressing mouse or human CYP1A2. Cells were treated and microsomes prepared as described in "Section 2." The values are means ± SD from two separate experiments with two dishes per experiment. The activities for mouse and human CYP1A2 were significantly different from the activity of cells transfected with empty vector ($P < 0.05$).

or human CYP1A2, and then incubated with ALA for 24 hr. Cells transfected with mouse CYP1A2 vector accumulated 2- to 4-fold more URO than those treated with empty vector alone. Similarly, cells transfected with human CYP1A2 vector accumulated 1.5- to 3.5-fold more URO compared to cells transfected with empty vector alone. In two of the three experiments shown in Table 1, the increases in URO were significantly different ($P < 0.05$) in cells expressing mouse CYP1A2 compared to those expressing human CYP1A2.

The major porphyrin accumulating from ALA in cells transfected with empty vector was protoporphyrin, followed by coproporphyrin (data not shown), as previously observed with ALA-treated mouse or chick primary hepatocyte cultures [16,19].

Table 1
Effect of expression of mouse or human CYP1A2 on URO accumulation by hepatoma cells

Experiment	URO[a] (pmol/dish)		
	Vector alone	m1A2	h1A2
1[b]	48 ± 5	190 ± 6	66 ± 5
2[b]	76 ± 9	162 ± 9	119 ± 8
3[c]	68 ± 4	222 ± 23	240 ± 14

Mouse cells were transfected as described in "Section 2." After 24 hr, 150 μM ALA was added and, after a further 24 hr, cells and/or medium were assayed for URO accumulation, as indicated below.

[a] URO was measured as 8 + 7-carboxyl porphyrins by HPLC. In each experiment, the values for URO in cells transfected with either mouse (m1A2) or human (h1A2) CYP1A2 were significantly different from those transfected with vector alone ($P < 0.05$).

[b] Expt. 1 and 2: Values represent means ± SD of porphyrins in cells plus medium from three separate dishes.

[c] Expt. 3: Values represent means ± SD of porphyrins in medium only from three separate dishes.

Since iron has been shown to stimulate hepatic URO accumulation *in vivo* [4,20], we tested the effect of treatment with 20 μM iron nitrilotriacetate (FeNTA) on URO accumulation in transfected cells treated with ALA. FeNTA treatment did not increase URO accumulation in cells transfected with vector alone. There was a small (25–30%) increase in URO caused by FeNTA in cells transfected with mouse or human CYP1A2 vectors (data not shown). In all cultures treated with FeNTA, there was a decrease in protoporphyrin of about 50% (data not shown).

UROX activity was measured in microsomes from transfected cells. In this reaction, uroporphyrinogen, the first reduced tetrapyrrole intermediate of heme biosynthesis, is metabolized through a six electron oxidation to URO, a highly conjugated red fluorescent porphyrin [21–23]. This reaction is considered to be an important contributor to the development of experimental uroporphyria [21–23]. The UROX activity of microsomes isolated from cells transfected with mouse CYP1A2 was readily measurable, being 7-fold greater than the activity of microsomes from cells transfected with empty vector alone (1.4 ± 0.1 vs 0.2 ± 0.1 pmol URO/min/mg protein, respectively). However, UROX activity in microsomes from cells transfected with human CYP1A2 vector was not significantly greater than that in microsomes from cells transfected with empty vector alone (data not shown). This result is in accord with our previous finding using a different expression system [18] that human CYP1A2 has a much lower UROX activity than the mouse ortholog.

4. Discussion

Previous studies using *Cyp1a2(−/−)* mice have indicated that CYP1A2 is a major contributor to hepatic URO accumulation in experimental animals [5–7]. However, it is not yet clear whether CYP1A2 has a role in the development of PCT, the most common form of human uroporphyria. The main question addressed here was whether CYP1A2, which has been shown to be required for experimental uroporphyria [5–7], is also required in human PCT. To investigate this, we examined whether hepatoma cells expressing human CYP1A2 would support URO accumulation. Hepatoma cells expressing mouse CYP1A2 were used as a positive control.

Using transient transfection, high levels of human and mouse CYP1A2 expression were achieved, as indicated by both immunodetected proteins and MROD enzyme activities (Figs. 2 and 3). We calculated whether the levels of expression of CYP1A2s were similar to those in liver microsomes. The MROD activities of hepatic microsomes from the transfected cells expressing mouse CYP1A2 were about 25% of the activity of microsomes isolated from untreated C57BL/6 mice [18]. The microsomal MROD activities in cells expressing human CYP1A2 (Fig. 3) were within the range of activities measured in microsomes

isolated from seven human livers (mean 50, range 1–105 pmol resorufin/min/mg protein; [18]). Since only 25–50% of the cells would have been transfected, it is likely that CYP1A2 expression in the transfected hepatoma cells probably was similar or higher than the range of basal expression in human liver. Furthermore, the readily measurable values of MROD activities catalyzed by both expressed CYP1A2s indicate that endogenous CYP reductase activities in the mouse hepatoma cells were not rate-limiting for MROD.

Human CYP1A2 expressed in the hepatoma cells supported URO accumulation from added ALA (Table 1). This result is consistent with the hypothesis that CYP1A2 has a role in human PCT. Support for the role of CYP1A2 induction in PCT is the apparent association of PCT with tobacco smoking [24], a recognized inducer of CYP1A2 in humans [25].

Our hypothesis that increases in CYP1A2 in PCT patients might be a factor in susceptibility to the disease has not been supported by recent studies examining whether hepatic CYP1A2 induction is selectively elevated in PCT patients [8,9]. These patients at the time of diagnosis did not have increased levels of CYP1A2 compared to the control group. However, the sensitivity of assessment of CYP1A2 *in vivo* is limited, and elevations in CYP1A2 levels may have occurred earlier in the long time course of the development of PCT. It is important to note that although expression of CYP1A2 is essential in experimental uroporphyria [5–7], increases above the basal level of CYP1A2 are not required, but shorten the time to appearance of uroporphyria. For example, acetone treatment of iron-loaded mice increases URO accumulation without increasing CYP1A2 [26], yet the same treatment does not cause uroporphyria in *Cyp1a2(−/−)* mice [26]. Thus, increased levels of CYP1A2 may not be required for the development of this disease.

These findings also indicate that cells other than primary hepatocytes have the capacity to accumulate URO, provided they also express sufficient CYP1A2. Previously, only primary chick and mouse hepatocytes were shown to accumulate URO in a CYP1A-dependent manner [16,19,27,28].

In addition to showing that transfected cells expressing either mouse or human CYP1A2 accumulated URO when incubated with ALA, we also measured the UROX activity of microsomes from these cells. UROX activity catalyzed by CYP1A2 has been postulated, mainly on the basis of experiments from rodents [5–7], to be important in the development of uroporphyria. In a previous study with vaccinia-infected hepatoma cells, we observed that UROX activity catalyzed by the mouse form was much greater than that catalyzed by the human ortholog [18]. This was confirmed in the present study. In the hepatoma cells expressing human CYP1A2 it was not possible to detect UROX activity above background, probably due to the reduced efficiency of transient transfection as compared to the vaccinia system. Thus, from the data of the current study, we cannot conclude whether it is the UROX activity of human CYP1A2 that is responsible for the URO accumulation in the hepatoma cells. We are currently pursuing stable expression of human CYP1A2 in mice that lack expression of mouse CYP1A2. Microsomes from these mice are expected to have much higher levels of human CYP1A2 than were present in the transfected hepatoma cells used here. These transgenic mice should allow determination of the relationship between URO accumulation and human CYP1A2-catalyzed UROX.

In vivo, hepatic URO accumulation in experimental porphyria is associated with some degree of hepatic iron loading [4,29], this factor being one of the key elements common to clinical uroporphyria [2]. In the current study, only minor increases in URO accumulation were produced by adding iron to hepatoma cultures transfected with vectors for either mouse or human CYP1A2, but not with empty vector. This finding is interesting since treatment with iron chelators has no measurable effect on CYP1A5-mediated URO accumulation in primary chick hepatocyte cultures [28]. Accumulation of URO in primary cultures of mouse hepatocytes is dependent upon induction of CYP1A2, with no stimulation by iron [27]. Thus, the striking effects of iron *in vivo* have not yet been reproduced in cell cultures.

In summary, mouse hepatoma cells transfected to express mouse or human CYP1A2 proteins can accumulate URO when incubated with ALA. These results are compatible with a role for CYP1A2 in the human disease PCT.

Acknowledgments

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