Uroporphyrin produced in mice by iron and 5-aminolaevulinic acid does not occur in *Cyp1a2*(–/–) null mutant mice

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In the present study we have investigated the putative requirement for the cytochrome *P*-450 isoform CYP1A2 in murine uroporphyrin, by comparing *Cyp1a2*(–/–) knockout mice with *Cyp1a2*(+/+) wild-type mice. Uroporphyrin was produced by injecting animals with iron-dextran and giving the porphyrin precursor 5-aminolaevulinic acid in the drinking water. Some animals also received 3-methylcholanthrene (MC) to induce hepatic CYP1A2. In both protocols, uroporphyrin was elicited by these treatments in the *Cyp1a2*(+/+) wild-type mice, but not in the null mutant mice. Uroporphyrinogen oxidation activity in

hepatic microsomes from untreated *Cyp1a2*(+/+) mice was 2.5-fold higher than in *Cyp1a2*(–/–) mice. Treatment with MC increased hepatic CYP1A1 in both mouse lines and hepatic CYP1A2 only in the *Cyp1a2*(+/+) line, as determined by Western immunoblotting. MC increased hepatic ethoxy- and methoxy-resorufin O-dealkylase activities in both mouse lines, but increased uroporphyrinogen oxidation activity in the *Cyp1a2*(+/+) wild-type mice only. These results indicate the absolute requirement for hepatic CYP1A2 in causing experimental uroporphyrin under the conditions used.

INTRODUCTION

Porphyria cutanea tarda (PCT) is a human disease in which there is massive hepatic accumulation and urinary excretion of uroporphyrin (URO) and an inactivation of the haem-synthetic enzyme uroporphyrinogen decarboxylase [1,2]. Both sporadic and familial forms of PCT occur. In the inherited form there is a partial deficiency of uroporphyrinogen decarboxylase. Both forms of PCT are precipitated by the same etiological agents, which include alcoholic beverages and contraceptive steroids [1,2]. The disease is usually associated with some degree of hepatic siderosis and mild increases in body iron stores. Phlebotomy is a successful treatment and is presumed to remove excess iron stores. The role of iron in the disease, however, remains elusive. Recently it was shown that many PCT patients carry a genetic mutation found in hereditary haemochromatosis [3].

Experimental models of uroporphyrin in laboratory animals were developed as the result of an accidental poisoning in Turkey in the 1950s with hexachlorobenzene, a polyhalogenated aromatic hydrocarbon, which caused hepatic URO accumulation and excretion [4]. This type of human porphyria could be reproduced in rodents treated with hexachlorobenzene and other polyhalogenated compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [4].

The hepatic cytochrome *P*-450 isoform CYP1A2 has been postulated to play a major role in the URO accumulation. This hypothesis is based on the *in vitro* observation that microsomes containing induced CYP1A enzymes catalyse uroporphyrinogen oxidation to URO (UROX activity) [5,6] and that the UROX activity is inhibited by specific antibodies against CYP1A2 but

not by specific antibodies against CYP1A1 [7]. UROX activity is also catalysed by reconstituted purified mouse CYP1A2 [8] and its chicken equivalent, CYP1A5 [9]. Rodent CYP1A1 and its chick equivalent, CYP1A4, are much less effective in catalysing UROX [8,9]. The inactivation of uroporphyrinogen decarboxylase, observed in the human and animal uroporphyrins as well as *in vitro*, is thought to be due to production of an as yet unidentified inhibitor during the CYP1A2-catalysed UROX reaction [10,11].

Iron-dextran administration decreases the time required for the development of uroporphyrin in rats [12,13] and mice [14,15], whereas depletion of iron protects mice against TCDD-induced uroporphyrin [16]. In some mouse strains, even a single injection of iron-dextran can eventually cause uroporphyrin in the absence of any cytochrome *P*-450 inducers [17,18]. The development of this porphyria can be accelerated by administration of 5-aminolaevulinic acid (ALA), the precursor of porphyrins and haem [19,20]. In fact, uroporphyrin can be produced in the SWR mouse strain by administration of ALA without any other treatment [21]. The question arises, therefore, of whether all these causes of experimental uroporphyrin are dependent on CYP1A2.

In the present study we took advantage of the recently developed viable and fertile *Cyp1a2*(–/–) knockout mouse line [22] to determine the requirement for CYP1A2 in a mouse uroporphyrin model. In this model, animals are treated with the combination of iron-dextran, MC as inducer of CYP1A1 and CYP1A2, and ALA to cause hepatic porphyrin accumulation. We found that *Cyp1a2*(–/–) mice did not develop uroporphyrin after treatment with MC and ALA, either in the presence or in

Abbreviations used: AHR, Ah receptor; ALA, 5-aminolaevulinic acid; EROD, 7-ethoxyresorufin O-de-ethylase; MC, 3-methylcholanthrene; MROD, 7-methoxyresorufin O-demethylase; PCT, porphyria cutanea tarda; PHA, polyhalogenated aromatic compounds; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; URO, uroporphyrin; UROgen, uroporphyrinogen; UROX, uroporphyrinogen oxidation.

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the absence of iron overload. In addition, even in the absence of any CYP1A2 inducer, the uroporphyrin caused by iron loading and prolonged ALA feeding did not occur in the *Cyp1a2*(-/-) mouse line.

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EXPERIMENTAL

Animals

Cyp1a2(+/+) and *Cyp1a2*(-/-) mice were generated from the Nebert mouse colony. Development of the *Cyp1a2*(-/-) knockout mouse line [22] resulted initially in offspring with a mixed genetic background [approx. 50% 129/Ola (agouti) and 50% C57BL/6J (nonagouti)]. The problem of the lack of genetic homogeneity in a freshly generated knockout mouse line has recently been discussed [23]. Because the CYP1A enzymes cannot be induced by MC in the 129/Ola mouse (owing to the poor-affinity Ah receptor), it was important to generate the CYP1A2-deficient phenotype in the C57BL/6J mouse (having the high-affinity Ah receptor) (cf. [24]). Mice sent to the laboratory of P. R. S. contained approx. 87.5% of C57/BL6J genetic background and were genotyped for the C57BL/6J versus the 129/Ola allele of the Ah receptor (AHR) gene by the oligo PCR test, as described [25]. Further confirmation of the C57BL/6J *Ahr*^{b1} allele in this study was carried out by demonstrating via Western immunoblotting that CYP1A1 was inducible by MC.

Heterozygote *Cyp1a2*(+/-) × *Cyp1a2*(+/-) intercrosses resulted in the expected *Cyp1a2*(+/+)/*Cyp1a2*(+/-)/*Cyp1a2*(-/-) ratio of 1:2:1. The homozygotes were used for the experimental porphyria studies shown here, and the heterozygotes were used for further breeding or killed.

Treatment of animals and preparation of liver fractions

MC (Sigma), at a concentration of 6 mg/ml of corn oil, was injected intraperitoneally at 75 mg/kg body weight as indicated in the Figure legends. ALA hydrochloride (Sigma) was prepared in the drinking water at 2 mg/ml. Iron-dextran solution (100 mg of Fe/ml; Iron-Gard100TM; Fermenta, Kansas City, MO, U.S.A.) was diluted 1:1 with 0.9% NaCl in water and injected intraperitoneally at 375 mg of Fe/kg body weight at the times indicated.

C57BL/6J mice were also injected with MC, as described above, for preparation of 20% liver homogenates as CYP1A1 and CYP1A2 standards for the immunoblots. Microsomes from F344 male rats treated with 5.6% ethanol in a liquid diet for 7 days [26] or injected with 100 mg of dexamethasone/kg each day for 4 days [27] were also used as standards for the immunoblots. Liver homogenates and microsomes were prepared as described previously [11]. All animal protocols were approved by the VA Hospital Animal Use Committee.

Antibodies

A polyclonal antibody raised against rat CYP1A2 that detects both mouse CYP1A2 and CYP1A1, and the polyclonal antibody against human CYP3A, were generously given by Steve Wrighton (Eli Lilly Research Laboratories, Indianapolis, IN, U.S.A.). An antibody against rat CYP2E1 was generously given by Paul E. Thomas (Environmental and Occupational Health Sciences Institute, Piscataway, NJ, U.S.A.). The details of timing for each experiment are described in the Figure legends.

Assays

Porphyrin composition in liver homogenates was determined spectrophotofluorimetrically [28]. Protein concentrations were determined by the method of Lowry et al. [29], using BSA as a standard. Microsomal ethoxyresorufin O-de-ethylase (EROD), methoxyresorufin O-demethylase (MROD) and uroporphyrinogen oxidation (UROX) were measured as described previously [9]. The presence of CYP1A1 and CYP1A2 protein in liver homogenates was determined by immunoblotting after electrophoretic separation of proteins in a mixed-detergent system [30].

Statistical analysis

Results are presented as means ± S.D. Significance was determined by one-way analysis of variance, where *P* < 0.05 was considered significant.

RESULTS

CYP1A2 is necessary to elicit uroporphyrin in mice treated with iron plus ALA

Some strains of mice become highly uroporphyrin when treated with iron plus ALA, or even with ALA alone [18,20,21]. Here we examined whether such uroporphyrin is dependent on the constitutive expression of CYP1A2. Figure 1 shows that the livers from *Cyp1a2*(+/+) wild-type animals contained immunodetectable CYP1A2 protein, whereas livers from *Cyp1a2*(-/-) null mutant animals did not, as expected. CYP1A1 is not detected in these microsomes, as these animals were not treated with any Ah-receptor ligands to induce this cytochrome *P*-450 isoform. Using antibodies against CYP3A and CYP2E1, two other hepatic cytochromes *P*-450, we found that there was no preferential expression of constitutive CYP3A or CYP2E1 when

Figure 1 Immunoblots of liver homogenates from *Cyp1a2*(+/+) wild-type and *Cyp1a2*(-/-) knockout mice treated with iron plus ALA

Male 4-week-old mice were treated with iron-dextran (375 mg/kg) and, after 7 days, were given ALA in the drinking water (2 mg/ml) for 46 days. The mice were killed when urinary URO levels from the *Cyp1a2*(+/+) mice exceeded coproporphyrin levels by 2-fold. In *Cyp1a2*(-/-) mice, urinary URO always remained less than 20% of coproporphyrin. We applied 10 µg of liver homogenate protein per lane when analysing CYP1A1/1A2, and 5 µg when analysing CYP2E1 or CYP3A. Antibodies against CYP1A1/1A2, CYP2E1 and CYP3A were used. The positive control for CYP1A1 and 1A2 was from C57BL/6J mice treated for 48 h with MC (1 µg of liver homogenate protein) and for CYP2E1 and CYP3A were from rats treated with ethanol or dexamethasone respectively (1 µg of liver microsomal protein).

Figure 2 Hepatic uroporphyrin (URO) content in *Cyp1a2*($+/+$) and *Cyp1a2*($-/-$) mice treated with iron plus ALA

For the treatment regimen, see the legend to Figure 1. For the *Cyp1a2*($-/-$) mice, all URO values were less than 0.8 nmol/g of liver. The number of animals per group is indicated under each treatment.

Cyp1a2($+/+$) mice are compared with *Cyp1a2*($-/-$) mice (Figure 1).

Figure 2 shows that, following 46 days of ALA treatment, hepatic URO levels in the iron-loaded *Cyp1a2*($+/+$) mice became highly elevated (mean 162 ± 111 nmol/g of liver; range of 3–310), whereas URO levels were < 0.8 nmol/g liver in all ALA-treated iron-loaded *Cyp1a2*($-/-$) mice. These results indicate that the uroporphyrin is dependent on CYP1A2 expression. These data also suggest that the effect of iron loading appears to be CYP1A2-dependent.

Microsomal CYP1A1 and CYP1A2-catalysed activities induced by MC

Previous studies have established that EROD and MROD activities in rats and mice are catalysed by both CYP1A1 and 1A2; however, EROD and MROD are known to be predominantly carried out by CYP1A1 and CYP1A2 respectively [31,32]. UROX activity is principally associated with CYP1A2 [7,8]. Table 1 shows that, in microsomes from *Cyp1a2*($+/+$) and *Cyp1a2*($-/-$) mice treated with MC for 48 h, induction of hepatic EROD and MROD activities occurred in most of the mice in both lines; however, the ratios of MROD to EROD (i.e. CYP1A2 to CYP1A1) in hepatic microsomes from both untreated and induced animals were quite different, consistent with

Figure 3 Immunoblots of hepatic CYP1A1 and 1A2 in male *Cyp1a2*($+/+$) and *Cyp1a2*($-/-$) mice treated with MC for 48 h

A 5 μ g portion of liver homogenate was separated by 10%-PAGE using the special detergent mixture described in [30]. The antibody used was against rat CYP1A1/1A2. No sample was applied in lane 5.

the complete absence of CYP1A2 in the *Cyp1a2*($-/-$) null mouse (Table 1; Figure 3).

Microsomal UROX activity in untreated *Cyp1a2*($+/+$) mice was 2.5-fold greater than that in *Cyp1a2*($-/-$) mice (Table 1). The background UROX activity in microsomes from the *Cyp1a2*($-/-$) mice may be due to flavin oxidoreductase activity or to other cytochrome *P*-450 forms. UROX activities were induced in *Cyp1a2*($+/+$) wild-type mice by MC by about 2-fold, whereas no induction of UROX activity by MC was seen in the *Cyp1a2*($-/-$) knockout line. These results are consistent with previously published data from reconstitution and antibody inhibition experiments that had suggested UROX is catalysed primarily by CYP1A2 [7,8].

Effects of MC and iron loading on URO accumulation in *Cyp1a2*($+/+$) and *Cyp1a2*($-/-$) mice

It has previously been shown that C57BL/6 inbred strains of mice develop uroporphyrin 10–15 days following treatment with MC plus iron and ALA [19] and that iron overload stimulates the accumulation of hepatic URO in mice treated with hexachlorobenzene and TCDD in the absence of ALA [14,15]. In *Cyp1a2*($+/+$) mice treated with MC and ALA (Figure 4), we found that hepatic URO accumulated in both the presence and absence of iron overload; however, iron overload increased URO accumulation. In the *Cyp1a2*($-/-$) knockout mice treated with MC and ALA, hepatic URO was negligible in either the presence or absence of iron overload.

In the MC-treated *Cyp1a2*($-/-$) animals in which there was no URO accumulation, there was more protoporphyrin in the

Table 1 Microsomal CYP1A1 and CYP1A2-catalysed activities induced by MC

Mice were treated with a single dose of 75 mg of MC/kg in corn oil or with vehicle alone. After 48 h, animals were killed and hepatic microsomes prepared. EROD, MROD dealkylases and UROX activities were measured as described in the Experimental section. Values are means $\pm$ S.D. (*significantly different, $P < 0.005$).

Genotype	Treatment (no. of mice)	Dealkylase activity (pmol of resorufin/min per mg of protein)		Ratio MROD/EROD	UROX activity (pmol of URO/ min per mg of protein)
		EROD	MROD		
<i>Cyp1a2</i> ($+/+$)	Control (3)	39 ± 6	71 ± 7	1.9 ± 0.1	$8.1 \pm 0.9^*$
	MC (5)	370 ± 590	240 ± 210	1.3 ± 0.6	15 ± 6
<i>Cyp1a2</i> ($-/-$)	Control (2)	46 ± 12	18 ± 2	0.40 ± 0.02	$3.4 \pm 0^*$
	MC (3)	1500 ± 750	300 ± 150	0.20 ± 0.02	2.6 ± 0.7

Figure 4 Hepatic uroporphyrin (URO) accumulation in *Cyp1a2*(+/+) and *Cyp1a2*(-/-) mice treated with MC and ALA, in the presence ('+Fe') or absence ('no Fe') of iron overload

Male 4-week-old mice were first treated with iron-dextran (375 mg/kg); after 7 days, MC (75 mg/kg) was injected intraperitoneally, and ALA was given in the drinking water (2 mg/ml) each day thereafter. A second dose of MC (75 mg/kg) was given 11 days after the first dose to sustain high levels of CYP1A1/1A2 [33]. The animals were killed 11 days after the second MC dose. The number of animals per group is indicated under each treatment. For the *Cyp1a2*(-/-) animals, all URO values were less than 0.8 nmol/g of liver. *Iron overload significantly increased URO accumulation in *Cyp1a2*(+/+) mice ($P < 0.02$).

absence than in the presence of administered iron (12 ± 2 nmol/g of liver versus 0.60 ± 0.30 nmol/g liver respectively). These data suggest that, with excessive amounts of iron, protoporphyrin might be converted to haem by ferrochelatase or that the protoporphyrin might be degraded in an oxidative process. In MC-treated *Cyp1a2*(+/+) mice, in the absence of iron, hepatic protoporphyrin accumulation was 4 ± 2 nmol/g liver, suggesting that differences in URO accumulation in the two strains were not due to differences in ALA availability.

DISCUSSION

Cytochrome P-450 inducers that elicit experimental porphyria

There has been considerable controversy concerning the essential role of cytochromes P-450 in experimental uroporphyria induced by polyhalogenated and other compounds in laboratory animals. In the present study we have used *Cyp1a2*(-/-) knockout mice to determine the requirement for CYP1A2 in uroporphyria. We found that the expression of both constitutive and inducible CYP1A2, but not that of the CYP1A1, 2E1 or 3A proteins, was essential for development of uroporphyria. In MC-treated *Cyp1a2*(+/+) mice, uroporphyria occurred regardless of whether the mice were overloaded with iron. Further work is necessary to determine whether or not the experimental uroporphyria produced by other known CYP1A2 inducers, such as hexachlorobenzene, TCDD and polychlorinated biphenyls, might occur in the *Cyp1a2*(-/-) knockout line. Other cytochrome P-450 isoforms, induced by compounds such as hexachlorobenzene and ethanol (CYP3A) or ethanol and acetone (CYP2E1), have also been implicated in experimental uroporphyria [20,34,35]. It is also possible, but considered unlikely by us, that the deletion of CYP1A2 may cause metabolic effects other than suppressing the role of CYP1A2 in UROX.

Furthermore, the work described here does not indicate how CYP1A2-mediated UROX activity might lead to inactivation of uroporphyrinogen decarboxylase, which is an essential part of uroporphyria in animals and humans [1,2,10].

Experimental porphyria with iron plus ALA and no MC

Iron-loaded *Cyp1a2*(+/+) wild-type mice not treated with MC, having low constitutive uninduced levels of hepatic CYP1A2 and no detectable CYP1A1, became highly uroporphyrin when given prolonged ALA treatment, whereas *Cyp1a2*(-/-) mice did not (Figure 2). It had previously been shown that uroporphyria could be elicited without inducing CYP1A2, by iron plus ALA or simply by iron alone, in SWR and C57BL mice [17,18] or by ALA alone in SWR mice [21]. Among these various treatments, in the present study we used only the 'iron plus ALA' regimen, because of the more rapid onset of the uroporphyria, as compared with treatment with either iron alone or ALA alone. The results obtained herein indicate that uroporphyria, caused by iron overload in the absence of MC treatment, is still CYP1A2-dependent.

The SWR inbred mouse strain has been shown in independent studies to have higher hepatic non-haem iron than the C57BL/6 inbred strain (A. G. Smith, B. Clothier, N. Gorman and P. Sinclair, unpublished work) and also has twice as high hepatic constitutive levels of CYP1A2 and UROX activity [36]. The data in the present study support the view that higher constitutive levels of liver CYP1A2 in the SWR strain, in combination with its higher iron stores, might contribute to the observation that ALA alone can cause URO accumulation in the SWR but not in the C57BL/6J inbred mouse strain.

UROX, MROD and EROD activities

The low UROX activity in hepatic microsomes from *Cyp1a2*(-/-) knockout mice treated with MC (Table 1) indicated that most of the UROX activity is dependent on the presence of CYP1A2 and not CYP1A1. The result is in accord with the results of previous microsomal studies using specific inhibitory antibodies against either P-450 isoform [7] and with reconstitution studies using mouse purified CYP1A1 and CYP1A2 [8] or the equivalent chicken forms [9]. The results in Table 1 also suggest that UROX activity is a better measure of CYP1A2 than is MROD, since MROD is catalysed by both CYP1A1 and 1A2 [31,32].

Microsomes from *Cyp1a2*(+/+) mice that had not been treated with MC had about 2.5-fold greater UROX activity than those from *Cyp1a2*(-/-) mice (Table 1). This result, together with the *in vivo* experiment with mice treated with iron plus ALA (Figure 2), suggests that only a small amount of CYP1A2-dependent UROX activity might be sufficient to evoke uroporphyria. However, it is intriguing that microsomes from untreated *Cyp1a2*(-/-) mice had low, but measurable, UROX activity [40% of that from *Cyp1a2*(+/+) mice], but did not become uroporphyrin (Table 1; Figure 2). The background UROX activity in microsomes from the *Cyp1a2*(-/-) mice may be due to flavin oxidoreductase activity or to other cytochrome P-450 forms.

Comparison of mouse experimental porphyria with human PCT

The present study demonstrates the predominant role of CYP1A2 in mouse experimental uroporphyria under the conditions used and raises the question as to the role of this cytochrome P-450 isoform in the human disease PCT. There are no direct or indirect clinical data implicating any cytochrome P-450 form in PCT elicited by the most common etiologic agents, i.e. alcoholic beverages that might increase human CYP2E1 [37,38] or CYP3A5 [39], or oestrogenic steroids that might induce CYP3A isoforms. In a recent case-study of 13 PCT patients, 11 had very

low plasma ascorbic acid levels [40]. This finding is consistent with the high susceptibility for MC-induced uroporphyrin in ascorbate-deficient ODS mutant rats induced for CYP1A2 [41]. In addition, most of the PCT patients in the study quoted above [40] were smokers; tobacco smoking is a known inducer of CYP1A2 in humans [42]. The results shown in the present study also suggest that, if the mouse model is valid for PCT, and therefore CYP1A2 is involved in clinical PCT, then only small increases in this cytochrome *P*-450 isoform (or even no increases at all), i.e. just constitutive CYP1A2 levels, might be sufficient.

While the current data do suggest an interaction of iron with CYP1A2, they do not identify the role of iron in either the experimental uroporphyrin or in PCT [10]. Other factors that are not explained by the current experiments include (i) the effect of gender found in rats, and (ii) the presence of other apparently genetic factors reported in mice. For example, male rats are poorly responsive to the induction of uroporphyrin [43], even with iron-plus-ALA administration [44]. Moreover, iron-treated mice of the DBA/2 inbred strain exhibit hepatic inducible CYP1A2 following TCDD treatment, but remain resistant to the development of uroporphyrin [15].

Conclusions

In summary, the present study shows that, in the development of uroporphyrin in mice receiving the regimens of iron plus ALA or iron, ALA and MC, there is an essential requirement for CYP1A2. Whether CYP1A2 also plays a pivotal role in the mechanism by which other porphyrinogenic chemicals such as hexachlorobenzene and TCDD act, will be the subject of future work.

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