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Role of Small Differences in CYP1A2 in the Development of Uroporphyria Produced by Iron and 5-Aminolevulinate in C57BL/6 and SWR Strains of Mice*

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ABSTRACT. Previous work has implicated CYP1A2 in experimental uroporphyria caused by polyhalogenated aromatic compounds, and in uroporphyria caused by iron and 5-aminolevulinate (ALA) in the absence of inducers of CYP1A2. Here we examined whether the different susceptibilities of SWR and C57BL/6 strains of mice to uroporphyria in the absence of inducers of CYP1A2 are related to different levels of CYP1A2. Enzymological assays (ethoxy- and methoxyresorufin dealkylases, and uroporphyrinogen oxidation) and immunoblots indicated that there was about twice the amount of hepatic CYP1A2 in SWR mice compared with C57BL/6 mice. Immunohistochemistry revealed that CYP1A2 was located centrilobularly in the liver, and the staining was more intense in SWR mice than in C57BL/6 mice. Hepatic non-heme iron was about double in SWR compared with C57BL/6 mice. In SWR mice given iron dextran, hepatic iron was 1.7-fold that of C57BL/6 mice given iron dextran. SWR mice administered ALA in the drinking water accumulated much less hepatic protoporphyrin than did C57BL/6 mice. To confirm the importance of small increases in CYP1A2, C57BL/6 mice were given a low dose of 3-methylcholanthrene (MC) (15 mg/kg), as well as iron and ALA. There was about a 5- to 6-fold increase in hepatic uroporphyrin accumulation after 32 days on ALA compared with animals not given MC. In these animals, CYP1A2 was increased by 10-fold at 2 days, but returned to basal levels by 14 days. We conclude that small and transient differences in CYP1A2 may be important in the development of uroporphyria. BIOCHEM PHARMACOL **58**;2:375–382, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. CYP; uroporphyrin; C57BL/6; SWR; iron; uroporphyrin accumulation caused by iron and 5-aminolevulinate

PCT** is a human disease in which there is massive hepatic accumulation and urinary excretion of URO accompanied by inactivation of the heme synthetic enzyme URO-D [1, 2]. Inherited (familial) and non-inherited forms of PCT occur; both of these forms are precipitated by the same etiologic agents, such as alcoholic beverages and contraceptive steroids [1, 2].

Hepatic accumulation and urinary excretion of URO, together with decreased activity of hepatic URO-D, can be produced in rodents treated with hexachlorobenzene and other polyhalogenated compounds such as TCDD [3–5], which are all inducers of CYPs of the CYP1A subfamily [6]. Several experimental results from this laboratory [7–10] and others [11] have implicated CYP1A in the development of uroporphyria. The oxidation of uroporphyrinogen, a reduced intermediate in the biosynthesis of heme, is catalyzed by reconstituted purified mouse CYP1A2, whereas reconstituted mouse CYP1A1 is much less effective in catalyzing UROX [12]. Recently, we demonstrated that chemically induced uroporphyria is prevented completely in CYP1A2 knockout mice [13].

Excess hepatic Fe is another factor implicated in the development of uroporphyria in both animal models and human PCT [1, 2]. Some PCT patients carry a common mutation (C282Y) in the gene responsible for the iron-overload disease hemochromatosis [14]. However, the exact role of Fe in uroporphyria is not understood. In addition to roles for CYP1A2 and Fe, other susceptibility and resistance factors may regulate the occurrence of uroporphyria in mice

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** *Abbreviations*: ALA, 5-aminolevulinic acid; CYP, cytochrome P450; EROD, ethoxyresorufin-*O*-deethylase; Fe, iron, regardless of form; MC, 3-methylcholanthrene; MROD, methoxyresorufin-*O*-demethylase; PCT, porphyria cutanea tarda; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; URO, uroporphyrin; URO-D, uroporphyrinogen decarboxylase; and UROX, uroporphyrinogen oxidation.

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[15–17]. Overall, uroporphyria appears to have a multifactorial etiology in which the interaction of the various factors is not understood completely. There are also protection factors, such as ascorbic acid [18].

There is controversy whether variations in the levels of constitutive CYP1A2 contribute to the development of uroporphyria. The requirement for constitutive levels of CYP1A2 in uroporphyria was demonstrated recently in CYP1A2 knockout mice [13]. SWR, C57BL/6, and C57BL/10 strains of mice treated with Fe and ALA, or SWR mice treated with ALA alone, can become uroporphyric in the total absence of inducers of CYP1A2 [19–21]. In uroporphyria caused by Fe plus ALA, SWR mice become uroporphyric much faster than C57BL/6 mice [20, 21]. SWR mice have higher endogenous levels of non-heme hepatic Fe than C57BL/6 and C57BL/10 mouse strains, are not inducible for CYP1As by low affinity Ah receptor ligands [20, 21, *], and may also have higher basal CYP1A2 levels [22]. However, Smith and Francis [20] found no differences in constitutive CYP1A2 hepatic content in four different mouse strains, including C57BL/6 and SWR, and concluded that differences in CYP1A2 content could not account for the different susceptibilities of the strains to uroporphyria produced in the absence of CYP1A inducers.

Here, we present data to show that SWR mice have about twice the hepatic CYP1A2 content of C57BL/6 mice, as determined enzymatically and immunochemically. These and other data presented here suggest that small increases in CYP1A2 above the basal level found in C57BL/6 mice may be important in producing uroporphyria.

MATERIALS AND METHODS

Chemicals

MC and ALA were from the Sigma Chemical Co. Iron-dextran solution (100 mg Fe/mL; Iron-Gard100™) was from Fermenta. All other chemicals were of the highest grade available.

Animals

SWR and C57BL/6 male mice (20–25 g) were purchased from Jackson Laboratories and Charles River Laboratories, respectively. Some mice were treated with a single i.p. injection of MC (15, 50, or 100 mg/kg, 1–6 mg MC/mL of corn oil) as indicated in the figure legends. The ALA concentration in the drinking water was 2 mg/mL. Iron dextran solution was injected i.p. at 500 mg Fe/kg as a 50 mg Fe/mL solution. At the times indicated in the figure legends, the mice were euthanized, and liver homogenates (20%, w/v) and liver microsomes were prepared as previously described [23]. All animal protocols were approved by the VA Hospital Animal Use Committee and the Dartmouth College Institutional Animal Care and Use Committee.

Assays

Porphyrin composition in liver homogenates was determined spectrofluorimetrically as described previously [8]. Some of the porphyrin analyses were confirmed by reverse-phase HPLC [10]. Protein concentrations were determined as described [24] using BSA as standard. Microsomal EROD and UROX were measured as described previously [25]. MROD was assayed as described for EROD using 2 μM methoxyresorufin as substrate.

Liver homogenates were analyzed for CYP1A1 and CYP1A2 after electrophoretic separation of CYP1A1 and CYP1A2 in a mixed-detergent system [10] and transfer to nitrocellulose. The goat antibody against rat CYP1A2, which detects both mouse CYP1A2 and CYP1A1, was a gift from Dr. Steve Wrighton (Eli Lilly Research Laboratories).

Non-heme iron was determined essentially by the method of Torrance and Bothwell [26], except that ascorbate was used instead of thioglycollic acid.

For immunohistochemical detection of CYP1A2, sections of liver were fixed in 10% neutral buffered formalin, and after embedding in paraffin, 4- to 5-μm sections were cut and mounted on sialinized slides. The dried slides were deparaffinized with xylene and then rinsed in ethanol. After blocking with buffer containing normal serum from the species in which the secondary antibody was made, the slides were incubated with the primary and secondary antibodies. The primary antibody was the same as that used for immunoblots. Binding of this antibody was detected using a Vectastain ABC-AP kit and a phosphatase substrate kit (Vector Red, Vector Laboratories) according to the manufacturer's instructions. Samples lacking primary or secondary antibodies were used as controls.

Statistical Analysis

Results are presented as means ± SD, as indicated. Statistical significance was determined by one-way analysis of variance, and $P < 0.05$ was considered significant.

RESULTS

Uroporphyria in SWR and C57BL/6 Mice Treated with ALA with and without Fe

As previously reported [20], in Fe dextran-treated mice administered ALA in the drinking water for 28 days, SWR mice were much more sensitive to uroporphyria than were C57BL/6 mice (Fig. 1A). In this time period, URO accumulation in both strains was dependent upon treatment with Fe (Fig. 1A). With ALA treatment, C57BL/6 mice accumulated significantly more protoporphyrin than did

* Smith AG, personal communication. Cited with permission.

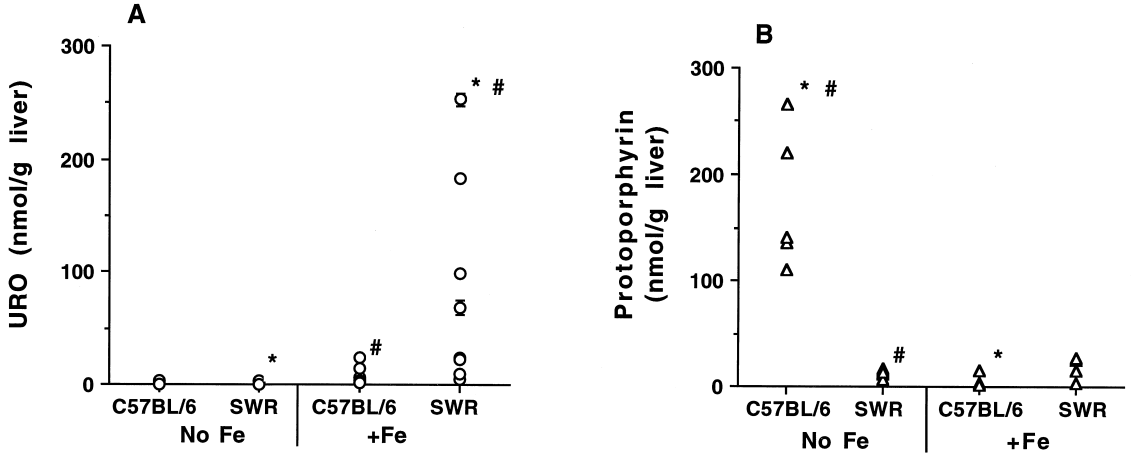

FIG. 1. Effects of Fe and ALA on URO (A) and protoporphyrin (B) accumulations in livers of SWR and C57BL/6 mice after 28 days. Male mice (22–25 g) were injected with Fe dextran (500 mg Fe/kg) as indicated, and after 5 days, ALA (2 mg/mL) was added to the drinking water. Animals were killed after 28 days on ALA. Porphyrins were determined spectrofluorimetrically, where URO represents mainly 8- and 7-carboxyl porphyrins. Values represent means of duplicate measurements, with the vertical lines indicating the range. There were 6–8 animals per group. Key: (*#) values for groups with the same symbol are significantly different from each other ($P < 0.05$).

SWR mice (Fig. 1B). With Fe treatment, protoporphyrin levels were low in both strains (Fig. 1B).

Table 1 shows that the hepatic non-heme Fe concentrations in SWR mice not treated with Fe were more than 2-fold those of C57BL/6 mice, as noted previously [20]. Interestingly, even after Fe dextran treatment, there was still a significantly higher Fe content in SWR livers, regardless of treatment with ALA (Table 1).

Prolonged feeding of ALA alone has been shown to cause URO accumulation in SWR mice [21]. When C57BL/6 and SWR mice were administered ALA alone for 56 days, SWR mice accumulated hepatic URO, whereas none of the C57BL/6 mice accumulated hepatic URO (<1 nmol/g liver) (Fig. 2A). Similar to the findings with 28 days of treatment with ALA, the livers of C57BL/6 mice had higher protoporphyrin contents than did the livers of SWR mice (Fig. 2B).

Comparison of Hepatic Levels of CYP1A2 Activities in SWR and C57BL/6 Mice

Since administration of Fe to C57BL/6 mice did not make them as sensitive as SWR mice to uroporphyria, we next examined whether CYP1A2, the catalyst for UROX, was present at a higher level in SWR mice. Three enzymatic activities were used to measure CYP1A2: EROD, MROD, and UROX. EROD and MROD activities mainly represent CYP1A2, since in untreated mice there is very low expression of CYP1A1 mRNA and no immunodetectable protein [13, 27]. All three activities were significantly higher (1.5- to 2-fold) in microsomes from SWR compared with C57BL/6 mice (Fig. 3). The 1.5- to 2-fold difference between SWR and C57BL/6 values remained, regardless of the Fe dextran treatment (data not shown).

Figure 4 shows a representative immunoblot indicating the absence of CYP1A1 and higher levels of CYP1A2 in liver homogenates from SWR mice. Similar differences were observed in liver homogenates from ALA-treated animals (data not shown).

In animals treated with Fe dextran, there was a 35–55% decrease in enzyme activities (data not shown), as reported by Smith's group [20, 28]. Treatment with Fe decreased immunodetectable CYP1A2 by 50–70% in C57BL/6 mice and by 30–70% in SWR mice, as determined densitometrically.

Immunohistochemical Detection of Hepatic CYP1A2 in C57BL/6 and SWR Mice

In this study, we used the same antibody as for immunoblotting CYP1As. Since there was no detectable CYP1A1 in these livers (Fig. 4), the red staining was specific for CYP1A2. CYP1A2 was detected readily in the centrilobular region of the SWR liver lobule (Fig. 5A), whereas there was much less CYP1A2 in the liver sections from untreated C57BL/6 mice (Fig. 5B). Centrilobular localization of many CYP forms has been observed previously [29]. Liver sections fixed with ethanol rather than formalin, in order to retain URO [30], had intense red fluorescence in centrilobular regions when viewed under UV light (data not shown).

TABLE 1. Hepatic Fe in C57BL/6 and SWR mice treated with Fe dextran and ALA

	Non-heme Fe (μg/g wet liver)	
Treatment	**C57BL/6**	**SWR***
Dextran	62 ± 3	143 ± 30
Fe dextran	969 ± 358	1630 ± 30
Dextran + ALA	83 ± 17	203 ± 24
Fe dextran + ALA	1350 ± 269	1842 ± 157

Values are means ± SD, 4 mice/group.

*All values for SWR mice are significantly different from similarly treated C57BL/6 mice ($P < 0.05$).

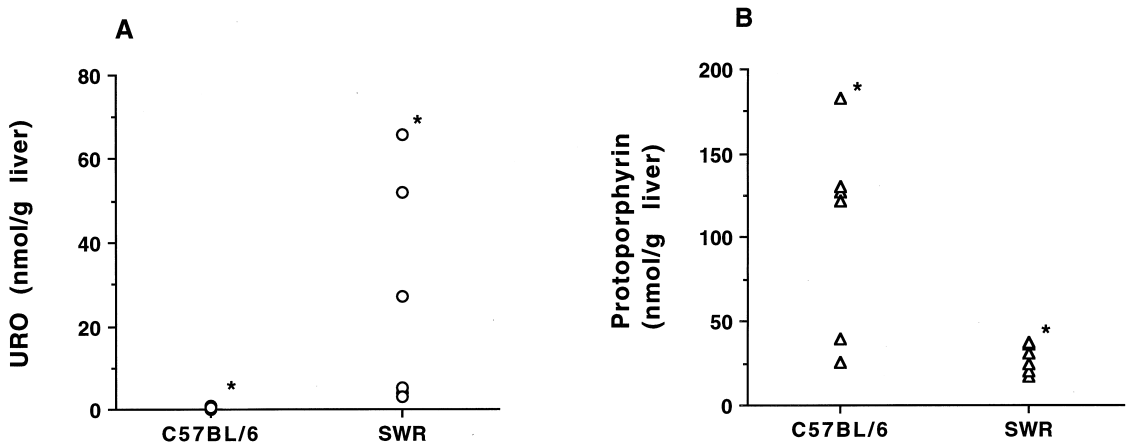

FIG. 2. Effect of 56 days of ALA administration on hepatic URO accumulation in SWR and C57BL/6 mice in the absence of Fe treatment. This experiment is essentially the same as that described in the legend of Fig. 1 except that ALA administration continued for 56 days and there was no treatment with Fe dextran. There were 6 mice per group. Key: (*) indicates a statistically significant difference from mice of the other strain given the same treatment ($P < 0.05$).

Effect of Transient Increase in CYP1A2 on URO Accumulation in C57BL/6 Mice

Previously, we had shown that high doses of MC (100 mg/kg), an inducer of CYP1A2, result in high levels of URO accumulation in Fe-treated C57BL/6 mice given ALA in the drinking water for 14–16 days [31]. Here, we determined the dose response of MC on the URO accumulation in C57BL/6 mice treated similarly. Table 2 shows that at 14 days there was a significant increase in hepatic URO accumulation at 15 mg MC/kg in Fe-treated mice. Increasing the dose of MC from 15 to 50 mg/kg caused further URO increases, but there was no further increase in URO at 100 mg MC/kg. At 32 days, in animals given 15 mg MC/kg, there was a considerable increase in URO accumulation, but only if the animals also had been treated with Fe. This result has been confirmed in a separate experiment (data not shown). The accumulation of URO at this MC dose was very similar to that observed in SWR mice treated with Fe and ALA for the same time (Fig. 1). Table 2 also shows that at 14 days, treatment with 50 and 100 mg MC/kg caused accumulation of URO without any Fe treatment. As previously observed [31], Fe appears to potentiate accumulation of URO at these MC doses in animals treated with ALA. The increases were not statistically significant. However, in other experiments using similar high doses of MC, the effect of Fe was significant [13].

We next investigated the time course of CYP1A2 protein following administration of the low doses of MC (Fig. 6). Densitometric analysis of the immunoblots shows that

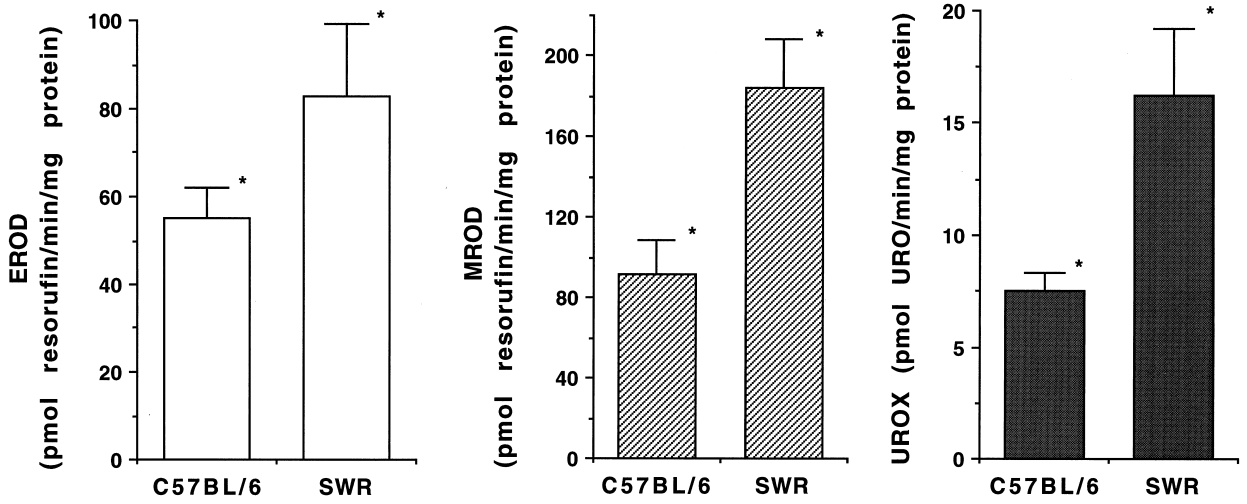

FIG. 3. EROD, MROD, and UROX activities in hepatic microsomes from C57BL/6 and SWR mice. Hepatic microsomes were prepared as described in Materials and Methods from animals that were either untreated or treated with ALA alone as in Fig. 1. Because the enzymatic values for microsomes from animals treated with ALA did not differ from those from untreated animals, we combined these groups. Values are means ± SD for a total of 6 animals per group. Key: (*) indicates compared groups that are significantly different ($P < 0.05$).

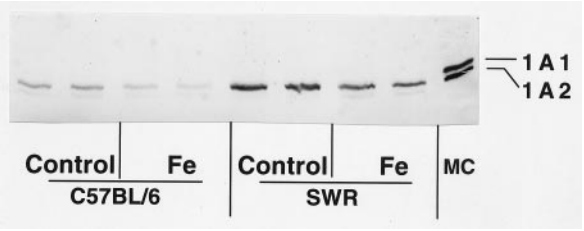

FIG. 4. Immunoblots of liver homogenates for detection of hepatic CYP1A2 expressed in C57BL/6 and SWR mice. Immunoblotting was performed as described in Materials and Methods using an antibody that detects both CYP1A1 and CYP1A2 [13]. One microgram of liver homogenate protein was applied to each well. Results from duplicate livers per treatment are shown. MC indicates 0.5 μg of liver homogenate protein from a C57BL/6 mouse treated with MC to show the resolution of CYP1A1 and CYP1A2. Control, untreated; Fe, Fe dextran.

hepatic CYP1A2 was increased 10-fold by 15 mg/kg, and 20-fold by 50 mg/kg at 2 days after treatment. Thereafter, the amount of CYP1A2 rapidly declined, reaching basal levels by 14 days. CYP1A1, which was readily detected after 2 days of treatment with MC, was essentially undetectable at 14 days. Following a dose of 100 mg MC/kg, CYP1A2 was still detectable at 14 days, but had decreased from the peak by about 80%, and CYP1A1 was not detectable (data not shown).

These results suggest that in Fe-treated mice only a transient increase in CYP1A2 may be needed to promote a later development of uroporphyria.

DISCUSSION

In this study, we compared the constitutive levels of hepatic CYP1A2 in C57BL/6 and SWR strains of mice, since the SWR strain has a faster rate of development of uroporphyria when treated with ALA, a precursor of heme, which accelerates the onset of uroporphyria [20, 21, 31]. Use of ALA also avoids any strain differences in the induction of ALA synthase, the rate-limiting enzyme of the heme biosynthetic pathway. The results clearly demonstrated that the SWR mouse has about twice the constitutive hepatic CYP1A2 content of the C57BL/6 mouse, and has a higher endogenous hepatic Fe content.

TABLE 2. Effect of MC dose and Fe on hepatic URO accumulation in C57BL/6 mice treated with ALA for 14 or 32 days

MC (mg/kg)	Fe dextran (500 mg Fe/kg)	Uroporphyrin (nmol/g liver)	
		At 14 days	At 32 days
0	−	ND*	2 ± 2†
	+	<1	26 ± 17†‡
15	−	<1§	<1‖
	+	6 ± 4§	126 ± 39‡‖
50	−	24 ± 12	ND
	+	63 ± 50	ND
100	−	29 ± 27	ND
	+	59 ± 58	ND

C57BL/6 mice were treated with MC and Fe dextran followed by ALA in the drinking water as described in the legend to Fig. 1. Animals were killed 14 or 32 days after ALA treatment began. Values are means ± SD for 5–11 mice/group.

*ND = not done.

†‡§‖Values with the same symbol are significantly different from each other ($P < 0.05$).

We suggest that these differences contribute to the different sensitivities for the development of uroporphyria in these two strains. Using low doses of MC to increase hepatic CYP1A2, we found that transient submaximal increases in CYP1A2 could considerably increase the uroporphyric response of the C57BL/6 mouse. Interestingly, this new finding was observed only if the mice were also treated with Fe. These results are in agreement with our previous conclusion, using CYP1A2 knockout mice [13], that CYP1A2 has a central role in uroporphyria and that interactions of Fe and CYP1A2 are important. Although previous work with CYP1A2 knockout mice demonstrated that C57BL/6 mice given Fe require CYP1A2 to become uroporphyric [13], the minimal amount of CYP1A2 required was not known.

The differences in uroporphyric responses between SWR and C57BL/6 mice reported in this paper are similar to those obtained previously [20, 21]. However, the finding that CYP1A2 in liver of SWR mice was about double that of C57BL/6 mice is different from the previous report of no difference [20]. In the latter study, which examined four different mouse strains including C57BL/6 and SWR, the differences in EROD, UROX, or immunodetectable CYP1A2 were much smaller than those reported here. The higher CYP1A2 level in SWR compared with C57BL/6 mice obtained in our study was established by five different assays: three enzyme assays, immunoblots, and immunohistochemistry. Although EROD and MROD can be catalyzed by both CYP1A1 and CYP1A2 [13], there is no CYP1A1 present in untreated mice [13]. Therefore, these activities mainly represent the activity of CYP1A2 in uninduced mice. This was confirmed by the immunoblots (Fig. 4). For untreated mice, the EROD activities obtained by Smith and Francis [20] for the SWR strain were only 20% greater than those of C57BL/6 mice, and this difference was observed only in the absence of Fe treatment. However, in a later study, consistent with our data (Fig. 3), this group observed hepatic MROD activities to be greater in SWR mice (approximately 2-fold) compared with C57BL/6 mice.* Casley *et al.* [22] recently reported that SWR mice had constitutive CYP1A2 values and associated enzyme activities that were among the higher values observed in six other strains, although they did not examine tissues from C57BL/6 mice. Smith *et al.* [15, 17, 32] have shown that there are additional genetic factors that are responsible for the different susceptibilities of mouse strains to the induction of uroporphyria. While these factors have yet to be identified, we suggest that the difference in CYP1A2 between the C57BL/6 and SWR mice, although small, may

* Scullion M and Smith AG, personal communication. Cited with permission.

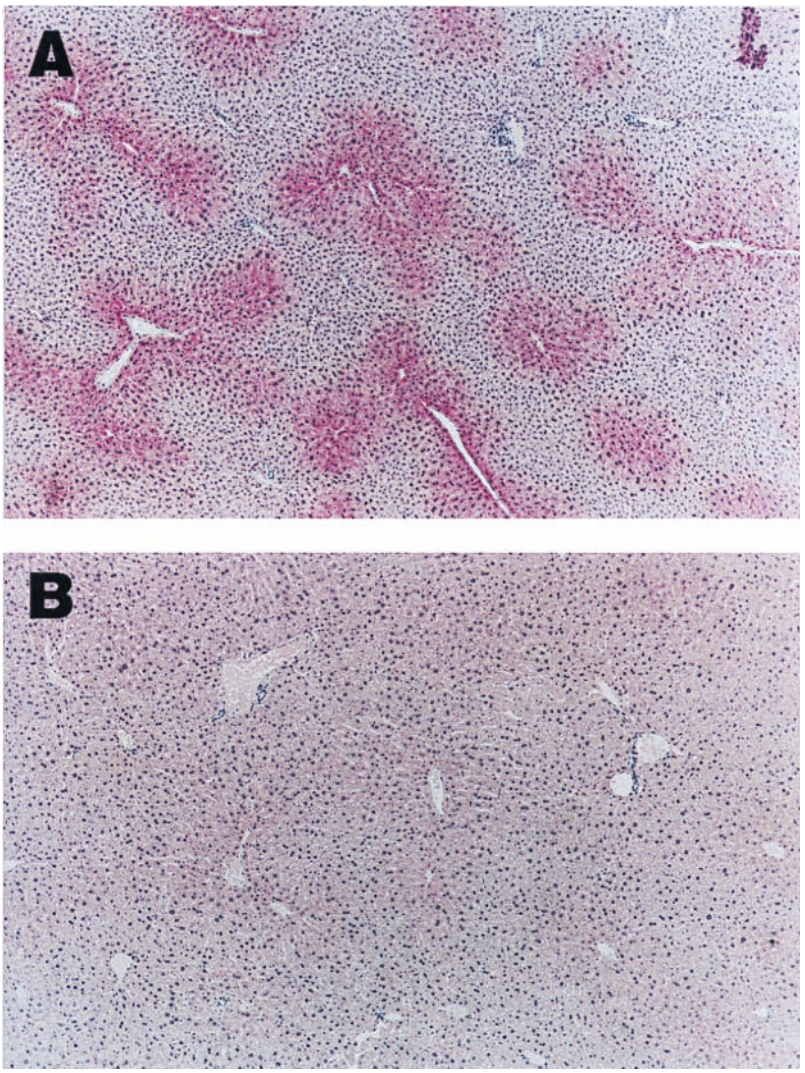

FIG. 5. Immunohistology of liver sections from untreated C57BL/6 and SWR mice using an antibody to CYP1As. Liver was fixed in formalin, and immunostaining was performed as described in Materials and Methods. The red stain indicates the presence of CYP1A2. Magnification was 100×. (A) SWR; and (B) C57BL/6.

be one factor contributing to the development of uroporphyria caused by ALA with or without Fe.

In contrast to the results obtained here for hepatic UROX activity, no detectable differences between C57BL/6 and SWR hepatic microsomes were observed by Smith and Francis [20]. The absolute UROX activities in that study [20] were 5–6 times those obtained in the current work and in previous studies [33, 34]. We do not know why the absolute UROX activities determined in the two laboratories were so different. It is possible that the high values obtained in Smith's laboratory may have obscured the 2-fold differences we have observed between the two strains.

We also found that endogenous liver non-heme Fe was higher in the SWR strain. Previously, SWR mice were found to have considerably higher endogenous hepatic Fe than C57BL/10 mice [20]. The increased Fe levels in SWR mice are considered to be genetically determined [35], but the biochemical mechanism responsible for these higher levels is not known. The low hepatic protoporphyrin in SWR mice may have been due to higher endogenous Fe levels in this strain compared with C57BL/6 mice, resulting in conversion of accumulating protoporphyrin to heme (Fig. 1B). Consistent with this interpretation was the

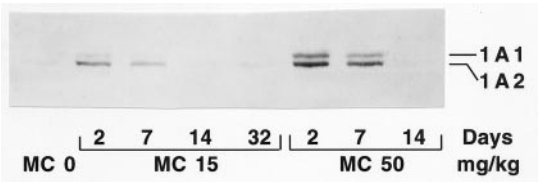

FIG. 6. Time course of CYP1A2 in liver homogenates from C57BL/6 mice treated with 15 or 50 mg MC/kg. C57BL/6 mice (3 per group) were treated with a single injection of MC as indicated, and then with ALA for the times indicated. Homogenates were immunoblotted as described in Materials and Methods, with 2 μg of protein applied per well. This is a representative gel. Similar results were obtained with the other samples of each group.

finding that in Fe-treated C57BL/6 mice, hepatic protoporphyrin values were decreased markedly compared with C57BL/6 mice not treated with Fe (Fig. 1B). Similar conclusions for a larger pool of available Fe in SWR mice than in C57BL/10 mice were made by Smith *et al.* [35].

We also report here that in Fe-treated C57BL/6 mice given ALA a transient increase in CYP1A2 caused by treatment with low doses of MC resulted in hepatic URO accumulation, which occurred even after CYP1A2 protein had returned to basal levels (Fig. 6, Table 2). This result suggests that in the presence of excess Fe even transient increases in CYP1A2 can influence subsequent URO accumulation. Previously, others had noted in much longer time–course studies that uroporphyria can be initiated and maintained by a single dose of hexachlorobenzene well after the peak of induction has occurred [36–38]. However, unlike our results, in some of those studies, CYP1A2, as indicated by EROD, remained well above basal levels during the extended uroporphyria [36, 38]. It is possible that initiation of uroporphyria by the increased UROX catalyzed by CYP1A2 may generate the as yet uncharacterized URO-D inhibitor, which has been detected in porphyric liver, and which seems to be derived from ALA, and, hence, uroporphyrinogen [39]. The low dose of MC used here may be effective in increasing uroporphyria, not only by transiently inducing CYP1A2, but also by some other gene product regulated by the Ah receptor, as has been suggested by genetic experiments [15–17, 32]. This may, for example, affect the amount of biochemically active Fe.

In summary, this paper demonstrates that SWR mice have higher basal levels of hepatic CYP1A2 and Fe compared with the C57BL/6 strain of mice. The data suggest that these higher CYP1A2 levels contribute to the higher susceptibility of the SWR mouse to development of uroporphyria. The results also suggest that only submaximal and transient increases in CYP1A2 are sufficient to accelerate the development of uroporphyria.

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