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UROPORPHYRIA PRODUCED IN MICE BY IRON AND 5-AMINOLEVULINIC ACID

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Abstract—Porphyria cutanea tarda and the analogous hepatic uroporphyria produced in rodents by aromatic hydrocarbons result from inactivation of hepatic uroporphyrinogen decarboxylase (UROD). Inactivation appears to be iron-dependent and may require induction of cytochromes of the P450IA subfamily. To investigate the hypothesis that the mechanism of inactivation involves an intermediate of haem biosynthesis, we administered iron and the haem precursor, 5-aminolevulinate (ALA), to mice. Iron-overloaded male mice of the *Ah*-responsive C57BL/6 strain, given ALA solution as their only drink, developed severe uroporphyria after 49 days. ALA did not produce uroporphyria in iron-overloaded male mice of the *Ah*-nonresponsive DBA/2 strain. Iron or ALA alone did not produce porphyria in either strain. Hepatic iron concentrations and rates of ethoxyresorufin deethylation (an indicator of cytochrome P450IA-mediated activity) were similar in both strains. These experiments show that a haem precursor is involved in iron-dependent inactivation of UROD. They emphasize the importance of inherited factors in determining susceptibility to this type of porphyria, even in the absence of administration of compounds that act through the *Ah* locus to induce cytochromes of the P450IA subfamily.

The human disease, porphyria cutanea tarda (PCT), and the analogous chronic uroporphyria produced in rodents by polyhalogenated or polycyclic aromatic hydrocarbons result from inactivation of an enzyme of haem biosynthesis, uroporphyrinogen decarboxylase (UROD) (EC 4.1.1.37), in the liver [1–3]. This inactivation process appears to be iron-dependent. In PCT, hepatic siderosis is frequent [4]; depletion of hepatic iron stores leads to remission [5], with restoration of hepatic enzyme activity to normal in some patients [2]; and administration of iron provokes relapse [6]. In rodents, the porphyrogenic action of aromatic hydrocarbons is accelerated by iron overload [3], delayed by desferrioxamine [7] and prevented by iron deficiency [1]. The mechanism by which iron influences the development of this type of hepatic porphyria is unknown. Recent theories have focused on possible mechanisms involving iron-catalysed formation of reduced oxygen species [8–10].

In rodents, susceptibility to polyhalogenated aromatic hydrocarbon-induced uroporphyria varies markedly between species and strains. Production of this type of porphyria appears to require induction of arylhydrocarbon (*Ah*)-inducible microsomal cytochromes of the P450IA subfamily [1, 11, 12] and, in inbred strains of mice, susceptibility partially correlates with responsiveness to induction of these cytochromes [13]. Thus, hexachlorobenzene produces porphyria much more rapidly in iron-loaded mice of the *Ah*-responsive C57BL strains than in the non-responsive DBA/2 strain [14].

Here we show that administration of the haem precursor, 5-aminolevulinic acid (ALA), which is not an inducer of cytochromes P450IA, causes severe uroporphyria within 7 weeks in iron-overloaded C57BL/6 mice but does not produce porphyria in the DBA/2 strain.

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MATERIALS AND METHODS

Materials. Synthetic pentacarboxylic porphyrin III with the acetic acid substituent at position 5 was a gift from the late Professor A. H. Jackson, Department of Chemistry, University of Wales College of Cardiff, Cardiff, U.K. 7-Ethoxyresorufin was from Pierce (Rockford, IL, U.S.A.). ALA dihydrochloride was from the Sigma Chemical Co. (Poole, U.K.). Imferon (iron dextran injection B.P.) was from Fisons Ltd (Loughborough, U.K.).

Animals. Male C57BL/6 and DBA/2 mice (22–28 g body wt) (Harlan Orlac Ltd, Bicester, U.K.) housed under identical conditions were allowed free access to a standard diet and to either tap water or to a solution of ALA in tap water (2 mg/mL) which was prepared fresh every 4 days. Iron overload was produced by a single intraperitoneal injection of Imferon (0.25 mL, 12.5 mg iron). Administration of ALA was started 3 days after the injection of iron and stopped 24 hr before the mice were killed in order to decrease the concentration in the liver of those porphyrins (mainly protoporphyrin) that had been produced by recent metabolism of ALA.

Mice were killed by cervical dislocation. Livers were removed, rinsed in saline, diced, washed with saline to remove blood, and homogenized in 0.25 M sucrose containing 20 mM Tris–HCl buffer (pH 7.4). Supernatant fractions were prepared from homogenates (20%, w/v) by centrifugation for 3 min at 15,000 *g*.

Table 1. Effect of iron and ALA on hepatic uroporphyrinogen decarboxylase activities of CB7BL/6 and DBA/2 mice

Treatment	Length of treatment (days)	Uroporphyrinogen decarboxylase (nmol/min/g)	
		C57BL/6 mice	DBA/2 mice
None	—	6.2 (5.4–7.1)	4.5 (4.4–5.6)
Iron	49	5.0 (4.5–5.5)	5.2 (4.4–5.9)
ALA	49	8.9 (7.3–10.9)	4.1 (3.1–5.2)
Iron and ALA	49	1.1 (0.8–1.2)*	5.7 (5.2–6.5)

Results are means and ranges for groups of three (C57BL/6 mice, no treatment) or four animals.

* Activity significantly decreased when compared with other treatment groups: $P < 0.01$.

Activities and concentrations are expressed per g wet weight of liver.

Methods. UROD was measured in liver homogenates (20%, w/v) using pentacarboxylate porphyrinogen III as substrate [15]. Ethoxyresorufin deethylase was assayed in supernatant fractions of liver in the presence of 0.01 mM dicoumarol [16]. Total porphyrin in liver homogenates was measured fluorometrically after extraction into methanol/1 M $HClO_4$ (1:1, v/v) using coproporphyrin I as standard and porphyrins were fractionated by TLC after methyl esterification [17]. Hepatic iron concentrations were determined as described by Richmond *et al.* [18] except that the iron released was measured by atomic absorption spectrophotometry.

Significance of differences between groups was assessed by the Mann–Whitney test.

RESULTS

Seven weeks after a single injection of iron dextran (500 mg iron/kg body wt), hepatic iron concentrations were increased 60-fold in male C57BL/6 mice but neither UROD activities (Table 1) nor hepatic porphyric concentrations (mean 1.0, range 0.4–1.5 nmol/g vs 0.3, 0.1–1.04 nmol/g) were significantly altered.

Addition of ALA to the drinking water of iron-loaded C57BL/6 mice produced severe uroporphyria; an 80% decrease in hepatic UROD activity (Table 1) being accompanied by a marked increase in porphyrin concentration (mean 153, range 140–174 nmol/g). Fractionation of hepatic porphyrins by TLC showed that the major components were uroporphyrin and heptacarboxylic porphyrin. In contrast, iron-overloaded male DBA/2 mice did not develop UROD deficiency (Table 1) or accumulate uroporphyrin after prolonged administration of ALA. Neither strain of mouse became porphyric when treated with ALA alone.

Table 2 shows that the difference in the susceptibility of these two strains of mice to the porphyrogenic effect of iron and ALA is unlikely to be explained by differences in hepatic iron concentrations or by inadvertent induction of cytochromes of the P450IA subfamily, as assessed by measurement of ethoxyresorufin deethylase activity. The small decrease in deethylase activity in iron-loaded mice (Table 1) has been noted previously [17] but remains unexplained.

DISCUSSION

Smith *et al.* [14] have recently shown that male C57BL/10 mice develop uroporphyria between 3 and 6 months after a single dose of iron dextran. Our results show that continuous oral administration of ALA greatly accelerates this response so that mice become markedly porphyric within 7 weeks. The time-course of onset of uroporphyria in response to ALA and iron has not been established but there is some evidence [17, 19] that hepatic UROD activity starts to decrease between 3 and 5 weeks.

These experiments suggest that inactivation of UROD in iron-loaded mice requires interaction between iron and either ALA or a subsequent intermediate of haem biosynthesis, since orally-administered ALA is converted in the liver to protoporphyrin and haem [20]. The same interaction may also be involved in the production of uroporphyria by polyhalogenated and polycyclic aromatic hydrocarbons and acetone because the porphyrogenic effect of these chemicals is both iron-dependent and potentiated by ALA [17, 19]. The nature of the interaction is uncertain but iron may be involved in the oxidation of uroporphyrinogen by cytochromes P450IA to an unidentified, catalytic site-specific inhibitor of UROD [8, 9, 21].

The striking difference between strains in the development of uroporphyria (Table 1) indicates that the response to iron and ALA is determined by inheritance. DBA/2 mice are also much more resistant to the porphyrogenic effect of aromatic hydrocarbons [3]. For these chemicals, there is much evidence, including experiments with congenic mice, that inherited differences at the *Ah* locus contribute to susceptibility [12]. Structural genes at this locus encode high-(Ah^b allele) or low-affinity (Ah^d allele) receptors for aromatic hydrocarbon inducers of microsomal cytochromes of the P450IA subfamily and a number of other proteins. C57BL/6 mice are homozygous for the Ah^b gene and respond to inducers whereas the non-responsive DBA/2 strain

Table 2. Hepatic iron concentrations and ethoxyresorufin deethylase (EROD) activities in C57BL/6 and DBA/2 mice

Strain	Treatment	Iron (μg/g wet wt)	EROD (pmol/min/mg protein)
CB7BL/6	None	32 (28–38)	41 (39–44)
	ALA and iron	1820 (1570–2080)*	25 (23–27)*
DBA/2	None	43 (20–53)	47 (41–51)
	ALA and iron	1760 (1440–2330)*	29 (27–31)*

Figures are means and ranges for 3–8 animals.
* Significance of difference from untreated group: $P < 0.01$.

is homozygous for the Ah^d gene [22]. Since chemicals that cause hepatic uroporphyria in rodents are both inducers of the cytochrome P450IA subfamily and ineffective in DBA/2 mice, induction of one or more of the cytochromes of this subfamily has been considered a prerequisite for the development of this type of porphyria [1, 3, 11–13].

Our results show that administration of an inducer of cytochromes P450IA is not required either to produce uroporphyria or to demonstrate the difference in susceptibility between these strains of mice. Inadvertent induction of cytochromes P450IA during our experiments cannot be completely excluded because we have not measured individual isoenzymes by a specific immunochemical method. However, the finding that rates of dealkylation of ethoxyresorufin, an activity that is catalysed to different extents by cytochromes P450IA1 and IA2 [23], were similar in both strains (Table 2) provides evidence against this possibility. The apparent lack of induction does not exclude involvement of cytochrome P450 in the mechanism whereby iron and ALA interact to cause porphyria. Provided excess iron and substrate (uroporphyrinogen) are present, constitutive concentrations of cytochrome P450 may be sufficient to catalyse formation of the putative inhibitor at a slow rate. This explanation is consistent with the observation that uroporphyria develops much more rapidly when iron-loaded C57BL/6 mice receive both ALA and an inducer of cytochromes P450IA [17].

There seem to be two possible explanations for the persistence of the strain difference in apparently uninduced animals. First, inherited differences between the strains that are not under the control of the *Ah* locus may be important. For example, although hepatic iron concentrations were similar (Table 2), more subtle differences in iron metabolism or mobilization in response to ALA are not excluded by our experiments. Similarly, although hepatic UROD activities are similar in the two strains, at present there is no evidence that susceptibility of the enzymes to inactivation is the same. Experiments using congenic mice [12] might provide information on these points. Secondly, the difference may be determined by qualitative or quantitative differences in the products of genes activated by the *Ah* receptor–ligand complex. Constitutive concentrations of cytochromes P450IA differ little between the two strains but there is a one amino acid difference in the structures of cytochrome P450IA2 [24], the isoenzyme that catalyses uroporphyrinogen oxidation in rats [10]. However, rates of oxidation of uroporphyrinogen by uninduced hepatic microsomes and by cytochrome P450IA2 are similar in the two strains [25, 26]. The rate of hepatic microsomal lipid peroxidation provoked by NADPH/ADP-iron is lower in DBA/2 mice and it has been suggested that this may be a factor in their resistance to uroporphyria [27].

Although the precise explanation of the strain difference remains to be determined, our results emphasize the importance of inherited factors in determining susceptibility to uroporphyria. Identification of these factors in mice should enable molecular genetic techniques to be used to search for similar inherited determinants of the apparent predisposition of certain individuals to develop PCT in response to various common forms of liver injury, notably alcoholic liver disease with siderosis.

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