The role of the *Ah* locus in hexachlorobenzene-induced porphyria

Studies in congenic C57BL/6J mice

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The role of the *Ah* locus in hexachlorobenzene (HCB)-induced porphyria and the possible involvement of *P*-450 cytochromes *P*₁450 and *P*₃450 in the pathogenesis of this disease were investigated in two congenic strains of C57BL/6J mice that differ only at this locus. Female B6-*Ah*^b mice (*Ah* receptor: ~ 30–70 fmol/mg of cytosolic protein) and B6-*Ah*^a mice (*Ah* receptor: undetectable) were pretreated with iron (500 mg/kg) and then fed a diet containing 0 or 200 p.p.m. of HCB for up to 17 weeks. Mice from the two strains consumed similar amounts of HCB. Urinary excretion of porphyrins was increased after 7 weeks of HCB treatment in B6-*Ah*^b mice, and after 15 weeks was over 200 times greater than that of mice given iron only. In B6-*Ah*^a mice, porphyrin excretion did not begin to increase until after 13 weeks, and after 15 weeks was only six times greater than that of controls. Similar differences were seen in the 15-week hepatic porphyrin concentrations (B6-*Ah*^b: 1110 ± 393; B6-*Ah*^a: 17.6 ± 14.5; controls: ~ 0.20 nmol/g). Uroporphyrinogen decarboxylase (EC 4.1.1.37) activity was diminished by 70 and 20 % in B6-*Ah*^b and B6-*Ah*^a mice respectively after 15 weeks of treatment with HCB. Cytochromes *P*₁450 and *P*₃450 were measured in hepatic microsomes (microsomal fractions) by radioimmunoassay and immunoblotting, using antisera raised against the orthologous rat isoenzymes *P*450c and *P*450d. HCB induced small amounts of a protein recognized by anti-*P*450c (*P*₁450) in B6-*Ah*^b mice, but not in B6-*Ah*^a mice. Relatively large amounts of a protein recognized by anti-*P*450d (*P*₃450) were induced in both strains, but to a somewhat greater extent in the B6-*Ah*^b mice. The hepatic accumulation of HCB at 15 weeks was greater in B6-*Ah*^b than in B6-*Ah*^a mice, in association with elevated hepatic lipid levels in the former strain. The results of this experiment indicate that the *Ah* locus influences the susceptibility of C57BL/6J mice to HCB-induced porphyria and are consistent with the suggestion that the sustained induction of *P*₃450 and/or *P*₁450 may be a causative factor in the development of this disease.

INTRODUCTION

Several halogenated aromatic hydrocarbons (HAH), including hexachlorobenzene (HCB), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and certain halogenated biphenyls, produce in humans and experimental animals a hepatic porphyria that closely resembles the human disease porphyria cutanea tarda [1–3]. HAH-induced porphyria is characterized by the hepatic accumulation and increased urinary excretion of highly carboxylated porphyrins [mainly uroporphyrin (URO) and heptacarboxyporphyrin]. This pattern of porphyrin overproduction is associated with a decrease in the hepatic activity of the soluble enzyme uroporphyrinogen decarboxylase (UD; EC 4.1.1.37) [1,3].

The exact mechanism by which UD activity is diminished in HAH-induced porphyria has not yet been elucidated. An alteration in the synthesis or degradation of the enzyme is possible, although there is some evidence that the amount of enzyme protein is unchanged after treatment with HCB or TCDD [4]. An alternative

hypothesis is that a reactive metabolite of HCB or TCDD may inactivate UD, perhaps by binding covalently to the enzyme [2,5]. However, no covalent binding of [¹⁴C]HCB-derived radioactivity to UD has been detected [6], and the porphyrogenic potency of TCDD [7,8], coupled with its resistance to metabolism [9], makes this hypothesis unlikely. It has also been suggested that the induction of one or more isoenzymes of hepatic cytochrome *P*-450 may be involved in the pathogenesis of HAH-induced porphyria, via the production of reactive species of oxygen or in association with lipid peroxidation [3,6,10–13]. Two *P*450 isoenzymes in particular are induced by all of the porphyrogenic HAHs (see below).

Most of the toxic effects of TCDD and structurally related compounds appear to be mediated by a specific interaction with the *Ah* receptor, the regulatory gene product of the *Ah* locus [14,15]. {The *Ah* locus was originally defined as the locus (loci) controlling the induction of cytochrome *P*-450-mediated multisubstrate mono-oxygenase activities (e.g. AHH) by polycyclic

Abbreviations used: AHH, aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase; B6-*Ah*^b, the *Ah*-responsive C57BL/6 mouse strain; B6-*Ah*^a, the *Ah*-non-responsive B6.D2-*Ah*^a strain consisting of the *Ah*^a allele of DBA/2 mice on a C57BL/6J genetic background; HAH, halogenated aromatic hydrocarbon; HCB, hexachlorobenzene; 3MC, 3-methylcholanthrene; *P*450, cytochrome *P*-450; r.i.a., radioimmunoassay; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran; UD, uroporphyrinogen decarboxylase (EC 4.1.1.37); URO, uroporphyrin; URO'gen, uroporphyrinogen.

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aromatic hydrocarbons such as 3MC, and was said to include structural, regulatory, and possibly temporal, genes [15]. In current usage, 'Ah locus' (or 'Ah gene') refers to the gene or genes encoding the Ah receptor protein. In crosses between C57BL/6 and DBA/2 mice, Ah-responsiveness is inherited in a dominant fashion; the pattern of inheritance is more complex in other strains [15-17].} Included among these receptor-mediated effects of TCDD are the aforementioned hepatic porphyria [7] and the induction in rats and mice of two hepatic cytochrome P-450 isoenzymes, P450c (P₁450) and P450d (P₃450) [18,19]. {The nomenclature used to describe the cytochrome P-450 isoenzymes is that of Thomas *et al.* [18] for rats and that of Kimura *et al.* [19] for mice. P450c and P450d of rats are orthologous to mouse P₁450 and P₃450 respectively [19]; these proteins are encoded by the rodent P450IA1 and P450IA2 genes respectively [20].} HCB produces an identical hepatic porphyria [1,2,21] and induces P450c and P450d in rats (although, in contrast with TCDD, HCB induces P450d preferentially) [22]. The similarity of the effects of HCB to those of TCDD is somewhat surprising, since HCB does not appear to obey the structural requirements thought to govern the binding of compounds to the Ah receptor [14,23]. Although HCB inhibits the specific binding of [³H]TCDD to the Ah receptor *in vitro* [22], the concentrations required (10⁻⁶-10⁻⁵ M-HCB) are quite high in comparison with those of known ligands for the Ah receptor and may therefore reflect non-specific effects of HCB on the solubility of TCDD. Alternatively, HCB may possess weak binding activity.

To examine the role of the Ah locus in TCDD- or HCB-induced porphyria, previous investigators have taken advantage of allelic differences at this locus, and the resulting differences in the properties of the Ah receptor, that exist among inbred strains of mice. C57BL/6 mice ('Ah-responsive') possess a high-affinity hepatic Ah receptor, whereas DBA/2 mice ('Ah-non-responsive') possess fewer receptors and/or receptors with a diminished affinity for TCDD and related compounds [14,15]. Several effects of TCDD have been shown to segregate with the Ah-responsive phenotype in crosses and back-crosses of these two strains [14]. Similarly, both TCDD and HCB produce porphyria in C57BL/6 (B6) mice, but not in DBA/2 (D2) mice [7,24]. Studies in other strains, however, have shown an incomplete correlation between the Ah phenotype and susceptibility to TCDD- or HCB-induced porphyria [8,21]. Interpretation of these results is complicated by the many other genetic differences that exist between these strains of mice [9,25,26]. In order to eliminate these differences, we utilized congenic strains of C57BL/6J mice that differ *only* at the Ah locus and a limited number of loci closely linked to the Ah locus (see [27] for a review of the genetics of congenic mice). Congenic C57BL/6J mice have also been used recently by other investigators to study the pharmacokinetics [25] and immunotoxicity [28] of TCDD, the characteristics of the Ah receptor [29], and the induction of cytochrome P-450 isoenzymes by HCB [30] and isosafrole [31]. The present study shows that B6-Ah^b (Ah-responsive) mice are more susceptible to the porphyrogenic effect of HCB than are congenic B6-Ah^d (Ah-non-responsive) mice and that this difference in susceptibility is associated with differences in the inducibility of two cytochrome P-450 isoenzymes.

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MATERIALS AND METHODS

Materials

HCB (99.8% purity), obtained from BDH (Poole, Dorset, U.K.), contained no detectable (< 0.5 p.p.m.) chlorinated dibenzo-*p*-dioxins or dibenzofurans [32]. The HCB-containing diet was prepared by mixing powdered HCB with powdered rodent chow (NIH-07; Zeigler Bros, Gardners, PA) to a level of 200 p.p.m. of HCB. Analysis of samples from eight batches of dosed feed demonstrated homogeneity of mixing and confirmed the dose level (197 ± 7 p.p.m.) [1,6-³H]TCDD (34.6 Ci/mmol) and TCDF were obtained and purified as previously described [33]. Uroporphyrin III octamethyl ester and porphyrin acid chromatographic marker kits were obtained from Porphyrin Products (Logan, UT, U.S.A.). H.p.l.c.-grade methanol and chloroform were from the J.T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.) and EM Science (Gibbstown, NJ, U.S.A.) respectively. Iron dextran injection USP (U.S. Pharmacopœia) (Imferon; Fisons Ltd., Loughborough, Leics., U.K.) was obtained from Merrill-Dow Pharmaceuticals (Cincinnati, OH, U.S.A.). Dowex 1X8-100 and 1X8-400 (both Cl⁻ form) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Animals

Female C57BL/6J (B6-Ah^b) mice were purchased from Jackson Laboratories (Bar Harbor, ME, U.S.A.). Female congenic B6-Ah^d mice were generously provided by Dr. Linda S. Birnbaum (National Institute of Environmental Health Sciences). These mice were originally bred in the laboratory of Dr. Daniel W. Nebert (National Institutes of Health, Bethesda, MD, U.S.A.) [34] using the cross-intercross method [27], whereby the Ah-non-responsive phenotype (Ah^d allele) of DBA/2N mice was bred on to a C57BL/6N background. These congenic mice were B6N.D2N-Ah^d(NE13) when obtained from Dr. Nebert by Dr. Alan Poland (McArdle Laboratory, University of Wisconsin, Madison, WI, U.S.A.), who bred these mice on to the C57BL/6J background for five cross-intercross cycles. Three additional cycles have occurred since these mice were obtained by Dr. Birnbaum. Because of a recent report of contamination of other congenic strains [35], these mice have been subjected to genetic monitoring. The congenic B6-Ah^d mice do not differ from the normal C57BL/6J (B6-Ah^b) mice in any of 32 biochemical markers examined to date [25,31].

All mice were received at the University of Rochester several weeks before treatment. They were housed in the Environmental Health Sciences Center's Hazardous Substances Facility for at least one week before treatment, and throughout the experiment, in stainless-steel cages (three to five mice per cage). They were maintained on a 12 h-light/12 h-dark cycle and allowed free access to food and water.

Treatment

Mice were 27-29 weeks old at the start of the experiment. After collection of pre-treatment urine

samples (see below), each mouse was injected subcutaneously with a single dose of iron dextran (500 mg of Fe/kg body wt.), which has been shown to potentiate HCB-induced porphyria [21]. After 1 day, half of the mice of each strain were placed on the HCB-containing diet, while the other half were maintained on the control diet. Treatment continued until the mice died or were killed. The diets were fed to the mice using a four-compartment powder feeder (Hazelton Systems, Aberdeen, MD, U.S.A.), which reduced spillage. Food consumption (corrected for spillage) was measured daily. Body weights were measured twice each week. One HCB-treated B6-*Ah^b* mouse and three HCB-treated B6-*Ah^d* mice died or were killed moribund during the experiment. Death was preceded by a rapid loss of body weight resulting from an inability to feed; this appeared to be due to severe neurological manifestations (tremor). The remaining mice were killed (by cervical dislocation under CO₂ anaesthesia) after 9 or 15 weeks (B6-*Ah^b*) or 15 or 17 weeks (B6-*Ah^d*) of treatment.

Preparation of liver

Livers were perfused *in situ* through the inferior vena cava with KCl (11.5 g/l, 4 °C). Two sections were taken from the left lobe; one was frozen in liquid N₂ and subsequently stored at -80 °C; the other was fixed in phosphate-buffered (37 mM-sodium phosphate, pH 7.0) 10% (v/v) formalin. The remaining hepatic tissue was homogenized in 0.25 M-sucrose (5 ml/g); this and all subsequent procedures were performed at 4 °C, unless otherwise stated. Portions of the homogenate were frozen (-80 °C) for later measurement of hepatic porphyrin, HCB and lipid concentrations. The remaining homogenate was centrifuged at 10000 *g* for 20 min. The resulting supernatant fraction was centrifuged at 105000 *g* for 75 min. Portions of the high-speed supernatant (cytosol) were frozen (-80 °C) until assayed for UD activity. The remaining cytosol was used immediately for the determination of the specific binding of [³H]TCDD (*Ah*-receptor assay). The 105000 *g* pellet (microsomal fraction) was suspended in 1.0 mM-EDTA/10% (v/v) glycerol/0.1 M-potassium phosphate buffer, pH 7.4, and stored at -80 °C before analysis for cytochrome *P*-450 isoenzymes.

Microscopy

Formalin-fixed hepatic tissue was embedded in paraffin, and 5 µm-thick sections were stained with haematoxylin/eosin or Prussian Blue/Nuclear Fast Red (iron stain). Frozen tissue was sectioned at 8 µm and stained with Oil Red O/haematoxylin (lipid). Appropriately stained sections were blindly scored for necrosis, iron and lipid content. Frozen sections were also used for visualization of porphyrins with an Olympus BH-2 fluorescence microscope, mercury 100 source and broad band-pass u.v. excitation and barrier filters.

Urine collection and porphyrin analysis

Urine samples (24 h) were collected each week from groups of three to five mice in plastic metabolism cages. Urine samples were preserved during collection with EDTA (~4 mg/ml of urine) and sodium carbonate (~6 mg/ml) and were analysed on the same day or frozen for later analysis.

Urinary porphyrins were separated and quantified by reversed-phase h.p.l.c. after 'clean-up' of the urine

samples by ion-exchange chromatography. Urine samples were applied to a 0.7 cm × 7.0 cm column of Dowex 1X8-100 resin that had been previously equilibrated with deionized water containing NaN₃ (0.1 g/l). The column was then washed with 20 ml of sodium citrate buffer (0.1 M, pH 4.0) and the porphyrins eluted with two 5 ml aliquots of 3 M-HCl.

A 20 µl aliquot of each HCl fraction was analysed by h.p.l.c. [36] using a Micropak SP-C18-5 column (15 cm × 4.6 mm int. diam.; Varian Instruments, Palo Alto, CA, U.S.A.) and a Perkin-Elmer LC-10 fluorescence detector (Perkin-Elmer, Norwalk, CN, U.S.A.) with excitation and emission filters of 360 nm and 500–700 nm respectively. The columns were maintained at 20 °C. Peak areas were quantified by using a Perkin-Elmer LCI-100 laboratory computing integrator. H.p.l.c. solvents were as described by Ford *et al.* [36] and were degassed with helium immediately before use. By using this method, recovery of porphyrins added to control urine samples was 94 ± 4%.

Hepatic porphyrins

The method of Kennedy *et al.* [37] was used to extract porphyrins from hepatic homogenates. The extracted porphyrins were then analysed by h.p.l.c. as described above. Recovery of porphyrin standards added to hepatic homogenates from untreated mice averaged 95 ± 3% for the five porphyrins.

Hepatic HCB concentrations

Aliquots of the hepatic homogenate were suspended in hexane and analysed for HCB by gas chromatography using a Perkin-Elmer Sigma 2000 gas chromatograph with a flame-ionization detector heated to 270 °C. The column (20% SP-2100/0.1% Carbowax 1500) was operated at 190 °C with nitrogen (30 ml/min) as the carrier gas. The mean recovery of HCB added at 100, 500 and 1000 µg/g was 97 ± 17%.

Hepatic lipids

Hepatic lipid concentrations were determined gravimetrically after extraction of ~1 ml of homogenate (equivalent to ~167 mg of tissue) with 9 ml of chloroform/methanol (2:1, v/v) as described by Folch *et al.* [38].

Ah-Receptor assay

The specific binding of [³H]TCDD to the *Ah* receptor was determined by the hydroxyapatite-adsorption method of Gasiewicz & Neal [33]. Hepatic cytosol was diluted in 1.5 mM-EDTA/1 mM-dithiothreitol/10% (v/v) glycerol/25 mM-Hepes buffer, pH 7.6, to 1.6–2.9 mg of protein/ml and then incubated with [³H]TCDD (2 nM), in the presence or absence of 200 nM-TCDF, for 18 h at 4 °C. Specific binding was calculated as the difference between total binding (in the absence of TCDF) and non-specific binding (in the presence of TCDF).

Preparation of URO'gen III and measurement of UD activity

UD activity was determined with URO'gen III as substrate by a modification of the method of Francis & Smith [39]. URO III octamethyl ester was hydrolysed in 5 M-HCl (~1.6 mg of URO ester/ml of HCl) for 24 h in the dark at room temperature. After evaporation of the acid *in vacuo* over KOH, the URO was dissolved

in 100 μ l of NH_4OH (1.0 M) and diluted to a final concentration of 1–2 mM-URO in 0.05 M- NH_4OH . This stock solution was stable for several months in the dark at 4 °C. Just before assay, the URO was reduced to URO'gen with NaBH_4 . To 150 μ l of the URO stock were added five or six drops of 1.0 M- NaBH_4 , followed by two or three drops of 0.5 M-HCl. After 30–60 min (and several colour changes) at 4 °C under helium and red light, the porphyrin was completely reduced (95 % minimum; routinely > 98 %), as indicated by its lack of fluorescence. Cold 1.0 mM-EDTA/3 mM-dithiothreitol/0.1 M-potassium phosphate buffer, pH 6.8, was then added to a final volume of 1.0 ml. This URO'gen solution was kept under helium until used (usually within 30 min).

In order to avoid interference with the assay and possible inhibition of UD by the elevated concentrations of endogenous porphyrins in livers from HCB-treated mice [40], porphyrins were removed from the cytosol by adsorption on to an anion-exchange resin [41]. A 2 ml portion of a ~ 50 % (v/v) suspension of Dowex 1X8-400 in 0.25 M-sucrose was added to a clean test tube. The resin was pelleted (1000 g for 10 min) and the excess sucrose drawn off. A 1 ml portion of cytosol was added, mixed with the resin, and incubated at 4 °C for 15 min with occasional mixing. After centrifuging for 10 min at 1000 g, the cytosol was removed for use in the assay. This procedure removed more than 95 % of the endogenous porphyrins from the cytosol of porphyric mice.

For the UD assay, 750 μ l of 0.1 M-potassium phosphate buffer, pH 6.8, 200 μ l of cytosol (1.1–1.3 mg of protein), and 50 μ l of URO'gen (final concn. 7.5–9.1 μ M) were added to amber-glass vials. After addition of substrate, the reaction was started by transferring the vials to a 37 °C shaking water bath. After a 30 min incubation under helium and red light, the reaction was stopped by placing the vials on ice and immediately (< 15 s) adding 500 μ l of cold 3 M-HCl. After being transferred to clear test tubes, the reaction mixtures were left overnight at 4 °C and then centrifuged at 700 g for 5 min. The porphyrins in the supernatant were separated and quantified by h.p.l.c. as described above. The major product formed under these conditions was heptacarboxyporphyrin. UD activity is expressed as the sum of all of the porphyrins formed, i.e. the decarboxylation of URO'gen. Product formation was linearly related to protein concentration (0.5–1.5 mg/ml) and incubation time (5–30 min) over the specified ranges.

Cytochrome P-450 isoenzymes

Antisera to the immunochemically similar rat cytochrome P-450 isoenzymes P450c and P450d were raised in rabbits and immunoadsorbed with the heterologous antigen to remove cross-reactivity of anti-P450c to P450d and of anti-P450d to P450c [42,43]. SDS/polyacrylamide-gel electrophoresis (7.5 % acrylamide) of mouse hepatic microsomal protein was performed as described by Laemmli [44]. Proteins were then electrophoretically transferred to nitrocellulose sheets by the method of Towbin *et al.* [45] and immunostained with immunospecific anti-P450c or anti-P450d [42]. In hepatic microsomes from 3MC-induced B6-*Ah^b* mice, anti-P450c recognized primarily a single band whose mobility was similar to that of P450c (Fig. 3; [22]), whereas anti-P450d recognized a polypeptide with a mobility equivalent to that of P450d [22]. The mouse hepatic microsomal

proteins recognized by anti-P450c and anti-P450d are presumably identical with P₁450 and P₃450 respectively, since there is 93 % amino acid sequence similarity between P₁450 and P450c and between P₃450 and P450d [19].

The relative amounts of P₁450 and P₃450 in hepatic microsomes were measured in radioimmunoassays (r.i.a.) by competition with ¹²⁵I-P450c and ¹²⁵I-P450d respectively [43]. Purified P450d was used to generate the standard curve for the r.i.a. of P₃450. Since preliminary studies indicated that microsomal P₁450 competed poorly in the r.i.a. compared with rat hepatic microsomal P450c, solubilized microsomes from 3MC-induced mice were used for the P₁450 standard curve rather than P450c. Solubilized microsomes from these mice inhibited the binding of both ¹²⁵I-P450c and ¹²⁵I-P450d by their respective antibodies, and the inhibition curves were parallel with those generated with pure P450c and pure P450d, indicating that the assays of P₁450 and P₃450 were linear and valid. All results are presented as relative amounts of P₁450 or P₃450, with the mean of all control values (for each isoenzyme) set equal to 1.0.

Protein

Microsomal protein was measured by the method of Lowry *et al.* [46]. The spectrophotometric method of Waddell [47] was used to measure cytosolic protein, bovine serum albumin being used as standard.

Data analysis

Statistical analyses were performed to compare HCB-treated groups with their controls or to compare the 15-week HCB-treated B6-*Ah^b* and B6-*Ah^d* groups [48]. An *F* test was used to test the homogeneity of variance, after which the appropriate *t* test was utilized to determine the probability that the observed difference in the means could have occurred by chance. Data are reported as means $\pm$ S.D.

RESULTS AND DISCUSSION

Food consumption and body weights

The total amounts of food consumed during the study by HCB-treated B6-*Ah^b* and B6-*Ah^d* mice were similar, and represented a total dose of approx. 2.9 and 3.0 g of HCB/kg body wt. respectively after 15 weeks of treatment (results not shown). Since one of the characteristic toxic effects of TCDD is a loss of body weight and mobilization of adipose tissue ('wasting syndrome') [14], mice were weighed twice each week to determine if HCB had a similar effect. No differences in body weight were seen between control and HCB-treated mice (Table 1).

Urinary porphyrins

HCB-induced porphyria developed sooner and was much more severe in B6-*Ah^b* mice than in B6-*Ah^d* mice. Urinary porphyrin excretion began to increase after 7 weeks of HCB treatment in B6-*Ah^b* mice and was elevated 200-fold after 15 weeks (Fig. 1c). In addition, URO replaced coproporphyrin as the major urinary porphyrin from 7 weeks onward (Fig. 1a). In B6-*Ah^d* mice, urinary porphyrin excretion did not begin to increase until after 13 weeks of HCB treatment and was elevated only 6-fold after 15 weeks. URO did not become the dominant porphyrin until after 14 weeks in these mice, and even after 17 weeks represented only 50 % of

Table 1. Body weights, relative liver weights and hepatic porphyrin concentrations in congenic C57BL/6J mice

Mice were treated with iron only (CON) or iron + HCB (HCB) as described in the Materials and methods section. Results are expressed as means $\pm$ s.d. for *n* mice per group. Results bearing a superscript 'a' indicate that samples within each group were pooled. Statistical significance: ***P* < 0.01 versus CON; †*P* < 0.05 versus B6-*Ah*^b (15 weeks).

Strain	Treatment	Period (weeks)	<i>n</i>	Body weight (g)	Liver weight (% of body weight)	Hepatic porphyrins (nmol/g wet wt.)
B6- <i>Ah</i> ^b	CON	9	4	23.9 $\pm$ 0.6	7.0 $\pm$ 0.5	0.26 ^a
	HCB	9	4	24.7 $\pm$ 1.1	13.0 $\pm$ 0.6**	75.4 $\pm$ 57.6
	CON	15	5	25.2 $\pm$ 1.3	7.2 $\pm$ 0.5	0.20 ^a
	HCB	15	4	25.4 $\pm$ 0.7	16.6 $\pm$ 1.5**	1110 $\pm$ 393
B6- <i>Ah</i> ^a	CON	15	4	23.3 $\pm$ 1.5	6.0 $\pm$ 0.4†	0.24 ^a
	HCB	15	3	23.6 $\pm$ 1.5	13.2 $\pm$ 0.9**†	17.6 $\pm$ 14.5†
	CON	17	5	23.8 $\pm$ 1.7	6.3 $\pm$ 0.4	0.18 ^a
	HCB	17	3	23.2 $\pm$ 1.2	13.3 $\pm$ 0.6**	48.5 $\pm$ 31.6

the total urinary porphyrin excretion (compared with 80 % in B6-*Ah*^b mice after 15 weeks; Figs. 1a and 1b).

Hepatic porphyrins and UD activity

The differences seen in urinary porphyrin excretion were confirmed by measuring hepatic porphyrin concentrations and UD activity. After 15 weeks of treatment, the relative increases in hepatic porphyrins in B6-*Ah*^b and B6-*Ah*^a mice were 5550-fold versus 73-fold respectively (Table 1). URO and heptacarboxyporphyrin comprised most (> 97 %) of the porphyrins (Fig. 2). The increased content of hepatic porphyrins was also observed by fluorescence microscopy (results not shown). After 9 weeks of HCB treatment, sections from B6-*Ah*^b mice exhibited focal areas of red fluorescence surrounded by non-fluorescent tissue. After 15 weeks, intense red fluorescence was seen throughout. Sections from B6-*Ah*^a mice given HCB for 15 or 17 weeks displayed focal fluorescence similar to that seen in the B6-*Ah*^b-9-week mice. No red fluorescence was observed in control mice of either strain. After 15 weeks, the hepatic UD activity of HCB-treated B6-*Ah*^b mice was diminished by 70 % (*P* = 0.0001) compared with that of control mice, whereas for B6-*Ah*^a mice the observed decrease was only 20 % (*P* = 0.030) (Table 2).

Ah Receptor

To confirm the *Ah* phenotype of the mice used in the present study, we measured the specific binding of [³H]TCDD in hepatic cytosol. No specific binding could be detected in B6-*Ah*^a mice, whereas B6-*Ah*^b mice contained significant amounts (mean values 30–70 fmol/mg of cytosolic protein). These data agree with receptor measurements made in other B6-*Ah*^b and B6-*Ah*^a mice [29,31], and are consistent with the absence of detectable *Ah* receptor in hepatic cytosol of DBA/2 mice [33,49]. HCB treatment had no effect on the amounts of [³H]TCDD specific binding detected in the B6-*Ah*^b mice.

Although we detected no cytosolic specific binding of [³H]TCDD in the B6-*Ah*^a mice, these mice are probably not completely lacking an *Ah* receptor. Specific binding has been detected in nuclei of DBA/2 mice treated with [³H]TCDD *in vivo* [50], consistent with the ability of relatively high doses of TCDD to induce P₁450 and P₃450 in these mice. It is likely that the B6-*Ah*^a mice used in the present study possess the same 'defective' receptor

found in DBA/2 mice. This might explain the modest porphyria that we observed in these mice. Although Smith & Francis [21] reported that DBA/2 mice did not become porphyric after treatment with iron and HCB, they maintained treatment for only 12 weeks. The B6-*Ah*^a mice used in our study were exposed for up to 17 weeks, and elevated porphyrin levels were not seen until after 13 weeks (Fig. 1). Moreover, DBA/2 mice may be resistant to HAH-induced porphyria as compared with B6-*Ah*^a mice, perhaps because of their greater adipose-tissue content [9,25] or resistance to peroxidative events [6,51].

Hepatic HCB concentrations

As shown in Table 3, hepatic HCB levels, when expressed per g of liver, were almost three times greater in B6-*Ah*^b mice than in B6-*Ah*^a mice after 15 weeks, despite the similar food consumption of the two strains. However, livers from HCB-treated B6-*Ah*^b mice contained more lipid than those of B6-*Ah*^a mice, as assessed biochemically (Table 3) and histologically (Oil Red O stain; results not shown). Similar elevations in hepatic lipids have been seen in C57BL/10 mice treated with HCB [21] or TCDD [8]. In the present study, hepatic HCB levels in the two strains were not significantly different (*P* = 0.12) when expressed relative to hepatic lipid content (Table 3), suggesting that the higher HCB levels seen in B6-*Ah*^b mice as compared with B6-*Ah*^a mice could be due in part to the greater accumulation of hepatic lipids in the former strain.

Induction of cytochrome *P*-450 isoenzymes

Because of the previously noted association between the induction of certain isoenzymes of hepatic cytochrome *P*-450 and HAH-induced porphyria, we measured the amounts of P₁450 and P₃450 by immunoblotting and radioimmunoassay (Figs. 3 and 4). No polypeptide was recognized by anti-P450c in control B6-*Ah*^b or B6-*Ah*^a microsomes. HCB induced a polypeptide recognized by anti-P450c (P₁450) in hepatic microsomes of B6-*Ah*^b mice, but to a lesser extent than acute treatment with 3MC (Fig. 3a). However, P₁450 was not induced in hepatic microsomes of HCB-treated B6-*Ah*^a mice.

The immunoblotting results were confirmed by radioimmunoassay (Fig. 4a), P₁450 was induced 16–36-fold by HCB in B6-*Ah*^b mice, whereas no significant induction

Fig. 1. Urinary porphyrin excretion of HCB-treated congenic C57BL/6J mice

Urinary porphyrins were analysed as described in the Materials and methods section. (a and b) Excretion of individual porphyrins as a percentage of total porphyrins; (c) total porphyrin excretion. The ranges of total porphyrin excretion of control mice were: B6-Ahb, 0.62–1.59; B6-Ahd, 0.68–1.83 nmol/day per mouse. Data were obtained from *n* mice at each time point as follows (where different from previous week). B6-Ahb: week 0, 9; week 10, 5; week 15, 4; B6-Ahd: week 0, 9; week 11, 8; week 15, 7; week 16, 4; week 17, 3. Key to numbers against curves: 8, URO; 7, heptacarboxyporphyrin; 6, hexacarboxyporphyrin; 5, pentacarboxyporphyrin; 4, coproporphyrin. For (c): ▨, B6-Ahb; ■, B6-Ahd.

was observed in B6-Ahd mice. The magnitude of the increase measured by r.i.a. in the B6-Ahb mice reflects the very low constitutive levels of this isoenzyme. The level of P₄₅₀ induction produced in B6-Ahb mice after 9 or 15 weeks of treatment with 200 p.p.m. of HCB is much lower than the 70-fold increase seen after administration of 3MC for 3 days [22,30]. However, the increase is greater than that seen after short-term treatment (7 days) with 1000 p.p.m. of HCB (5-fold) [22,30]. This suggests that the induction of P₄₅₀ may require hepatic levels of HCB higher than those attained during the 7-

Fig. 2. H.p.l.c. profiles of hepatic porphyrins from control or HCB-treated mice

Porphyrins were extracted from homogenate equivalent to 1.33–1.67 g (controls), 0.33 g (HCB-treated B6-Ahb) or 0.67 g (HCB-treated B6-Ahd) of liver, as described in the Materials and methods section. (a) Porphyrin standards (10 pmol of each porphyrin); (b) control (pooled samples from 4 or 5 control mice per group); (c) samples from individual HCB-treated mice (3 or 4 per group) were analysed; the samples shown are those whose total porphyrin concentrations were closest to the mean value for their group. The treatment period was 15 weeks in each case. Vertical bars in the ordinate position represent equivalent relative fluorescence. Key to numbers: 8, URO; 7, heptacarboxyporphyrin; 6, hexacarboxyporphyrin; 5, pentacarboxyporphyrin; 4, coproporphyrin; 2, mesoporphyrin.

day protocol, or that the increase in P₄₅₀ produced by long-term exposure to HCB might reflect secondary changes in the regulation of this isoenzyme.

Equivalent amounts of a polypeptide recognized by anti-P₄₅₀d (P₄₅₀) were present in control B6-Ahb and B6-Ahd mice (Figs. 3b and 4b). HCB induced relatively large amounts of this polypeptide in both strains; however, induction of P₄₅₀ was greater in B6-Ahb mice than in B6-Ahd mice (12-fold versus 4-fold after 15 weeks). This difference in the inducibility of P₄₅₀ in these strains was somewhat unexpected, since earlier studies had shown equivalent induction in the two strains

Table 2. UD activity in congenic C57BL/6J mice

Mice were treated with iron only (CON) or iron + HCB (HCB), as described in the Materials and methods section. Results are expressed as means $\pm$ S.D. for *n* mice per group. The control UD activities of the two strains are not directly comparable, owing to differences in the substrate concentrations. The treatment period was 15 weeks in each case. Statistical significance: **P* < 0.05 versus CON; ***P* < 0.01 versus CON.

Strain	Treatment	<i>n</i>	[URO'gen] (μ M)	UD activity	
				(pmol $\cdot$ min ⁻¹ $\cdot$ mg ⁻¹)	(% of CON)
B6- <i>Ah</i> ^b	CON	4	9.1	61.5 $\pm$ 2.5	100
	HCB	4	9.1	18.7 $\pm$ 1.4**	30
B6- <i>Ah</i> ^a	CON	4	7.5	47.2 $\pm$ 3.4	100
	HCB	3	7.5	37.8 $\pm$ 4.9*	80

Table 3. Hepatic HCB concentrations in HCB-treated congenic C57BL/6J mice

Mice were treated with iron + HCB as described in the Materials and methods section. The concentrations of HCB in hepatic homogenates were determined as described in the Materials and methods section and expressed relative to hepatic wet weight or hepatic lipid content. Results are expressed as means $\pm$ S.D. for *n* mice per group. Lipid concentrations in pooled hepatic homogenates from control mice were 38.7, 34.5, 33.2, and 33.0 mg of lipid/g of liver wet wt. for the four groups respectively. Statistical significance: ††*P* < 0.01 versus B6-*Ah*^b (15 weeks).

Strain	Length of treatment (weeks)	<i>n</i>	Lipid (mg/g of liver)	[HCB]	
				(μ g/g of liver)	(μ g/g of lipid)
B6- <i>Ah</i> ^b	9	4	58.6 $\pm$ 9.0	291 $\pm$ 74	4940 $\pm$ 770
	15	4	66.7 $\pm$ 3.7	635 $\pm$ 155	9490 $\pm$ 2120
B6- <i>Ah</i> ^a	15	3	36.6 $\pm$ 4.7††	220 $\pm$ 90††	6140 $\pm$ 2640
	17	3	42.0 $\pm$ 3.0	440 $\pm$ 68	10450 $\pm$ 1150

after short-term exposure to 1000 p.p.m. of HCB [30]. The observed difference could reflect the higher HCB concentrations present in B6-*Ah*^b mice after 15 weeks of treatment (Table 3). Alternatively, the *Ah* locus may influence the magnitude of the induction response during subchronic exposure. It has been suggested previously, however, that the induction of P₃450 by HCB, 3MC or isosafrole may not depend completely on the *Ah*^b allele [30,31,52]. Moreover, in primary cultures of rat hepatocytes, isosafrole and 2',3,4,4',5,5'-hexachlorobiphenyl retard the rate of degradation of P450d (but not P450c) [53]. Thus P₃450/P450d levels may be regulated by both the *Ah* locus and alternative mechanisms, such as protein stabilization.

Role of the *Ah* locus in HCB-induced porphyria

The data presented herein argue for the involvement of the *Ah* locus in HCB-induced porphyria in mice. The obvious suggestion is that HCB interacts with the *Ah* receptor in a manner similar to that proposed for TCDD. We have recently found that HCB is able to inhibit competitively the specific binding *in vitro* of [³H]TCDD in hepatic cytosol from mice or rats ([24,54; M. Hahn & T. Gasiewicz, unpublished work). These results could reflect non-specific effects of HCB on the solubility of TCDD, or weak binding of HCB to the receptor; further work is needed before firm conclusions can be drawn.

Previous investigators, using several inbred strains of mice, have found an incomplete correlation between the

Ah phenotype and susceptibility to HCB- or TCDD-induced porphyria [8,21]. The authors concluded, and we would agree, that genes other than the *Ah* gene must influence the porphyrogenicity of these compounds [8,21,24]. The susceptibility of the AKR strain to HCB-induced porphyria [21] is not inconsistent with our data. This strain is classified as *Ah*-non-responsive on the basis of the inability of 3MC to induce AHH activity (P₁450) and the absence of detectable *Ah* receptor [15]; induction of P₃450 has not been investigated in these mice. Genetic studies suggest, however, that the AKR and DBA/2 strains do not carry the same *Ah* allele [15,16]. Therefore it may be inappropriate to expect AKR and DBA/2 mice to respond similarly to HCB treatment. Moreover, DBA/2 mice might be resistant to HAH-induced porphyria for other reasons in addition to their *Ah*-non-responsive phenotype, as discussed above. Through the use of *congenic* strains of mice we have been able to circumvent these complicating factors and to show that the *Ah* locus does influence the susceptibility of mice to HCB-induced porphyria.

The *Ah* locus might be involved in the development of HCB-induced porphyria in several ways. Since a decrease in UD activity may be ultimately responsible for this porphyria, it is logical to suppose that the *Ah* locus influences the mechanism by which this occurs. Indeed, our results show a clear difference in the severity of this biochemical lesion between B6-*Ah*^b and B6-*Ah*^a mice (Table 2). However, the exact mechanism of the decreased UD activity is not yet known.

Fig. 3. Identification of hepatic microsomal cytochrome *P*-450 isoenzymes by immunoblotting

Immunoblots were prepared as described previously [22,42], using antisera raised against rat isoenzymes P450c (a) and P450d (b) to identify mouse cytochromes P₁450 and P₃450 respectively in microsomes from control (Con) or HCB-treated (HCB) mice after 9, 15, or 17 weeks (w) of treatment. Samples contained 40 µg of microsomal protein, except as noted below. P450c, purified rat hepatic P450c (2 pmol); 3MC, microsomal protein (10 µg) from B6-*Ah*^b mice treated with 80 mg of 3MC·day⁻¹·kg⁻¹ for 3 days; P450d, purified rat hepatic P450d (2 pmol).

Fig. 4. Relative amounts of hepatic microsomal cytochrome *P*-450 isoenzymes

Isoenzymes P₁450 (a) and P₃450 (b) were measured by r.i.a. as described in the Materials and methods section. All concentrations of P₁450 and P₃450 are expressed relative to the mean of the 16 control values (normalized to microsomal protein) for each isoenzyme. Control values for P₁450 or P₃450 did not differ significantly between the two strains. Open bars represent the mean relative values ± s.d. (*n* = 4) for mice in each concurrent control group. Hatched bars and solid bars represent values for HCB-treated mice (*n* = 4 for each group of B6-*Ah*^b mice; *n* = 3 for each group of B6-*Ah*^d mice).

It has been suggested that the pathogenesis of HAH-induced porphyria might involve the cytochrome *P*-450 system. According to one hypothesis, reactive oxygen species, such as superoxide and H₂O₂, are produced by cytochrome *P*-450 and either inactivate UD or oxidize its porphyrinogen substrates to the non-metabolizable porphyrins [6,10,11,55,56]. This hypothesis provides a possible explanation for the obligatory role of iron in HAH-induced porphyria [12,21], in that iron may catalyse the formation of hydroxyl radicals from H₂O₂ and superoxide (the Haber-Weiss reaction) [57]. The release of superoxide and H₂O₂ has been observed during the autoxidation of cytochrome *P*-450 isoenzymes and may

be increased in the presence of a substrate for the cytochrome *P*-450 [58] or after covalent modification of a specific isoenzyme [59].

The role of the *Ah* locus, then, might be to control the induction of an isoenzyme of cytochrome *P*-450 that can participate in such reactions. Two specific isoenzymes, P₁450 and P₃450, are induced in common by HCB, TCDD and the porphyrogenic halogenated biphenyls. The regulation of both P₁450 induction and porphyria by the *Ah* locus might seem to implicate this isoenzyme in the mechanism of porphyria. It is difficult, however, to reconcile the (modest) elevation of porphyrin excretion seen in the B6-*Ah*^d mice in the present study with the lack

of any detectable P₁450 induction in this strain. Moreover, the induction of P₁450 in the B6-*Ah*^b mice was small compared with that seen with 3MC. In contrast, the induction of P₃450 by HCB was quite striking in B6-*Ah*^b mice, suggesting that this isoenzyme could be involved in the porphyrogenic effect of HCB. The finding that this isoenzyme was induced to a lesser extent in B6-*Ah*^d mice is consistent with the attenuated porphyric response in this strain (cf. Table 1 and Fig. 4). Sustained high-level induction of P₃450 might be necessary for the porphyria to develop.

Conclusions

The results of the present experiments clearly indicate that the *Ah* locus influences the susceptibility of C57BL/6J mice to the porphyrogenic effects of HCB. Our study also shows that, in B6-*Ah*^b mice, HCB induces P₁450 and P₃450, which are known to be regulated by the *Ah* locus [50,60]. However, unlike most compounds that interact with the *Ah* receptor, HCB induces P₃450 preferentially. In addition, P₃450 is induced to some extent in *Ah*-non-responsive B6-*Ah*^d mice, unlike P₁450, which is only induced in B6-*Ah*^b mice. Thus P₃450 may be regulated by both the *Ah* locus and alternative mechanisms. The mechanism by which the *Ah* locus modulates the porphyrogenic effect of HCB is not yet clear. A decrease in UD activity has been demonstrated in porphyric mice, but it is not certain that this is the primary defect in the porphyria. Our data are consistent with the possibility that the porphyrogenic effect of HCB may be secondary to the sustained induction of P₁450 and/or P₃450, possibly via the production of reactive oxygen species that alter haem-biosynthetic enzymes or intermediates. Although the exact mechanism of HCB-induced porphyria remains to be elucidated, the demonstration of the involvement of the *Ah* locus provides evidence that all of the porphyrogenic HAHs may act by a similar mechanism. The recent demonstration that polycyclic aromatic hydrocarbon inducers of P₁450 and P₃450 can cause porphyria in iron-loaded C57BL/10 mice [56] provides further support for the role of the *Ah* locus and these cytochrome P-450 isoenzymes in this process.

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