Synergism of iron and hexachlorobenzene inhibits hepatic uroporphyrinogen decarboxylase in inbred mice

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The combination of a single subcutaneous dose of iron (12.5 mg/mouse) and subsequent treatment with hexachlorobenzene (0.02% of the diet) caused a progressive inhibition of hepatic uroporphyrinogen decarboxylase in male C57BL/10 mice leading to the accumulation of uroporphyrin in 4–6 weeks. There was only a slight inhibition of the enzyme in the absence of iron and none without hexachlorobenzene. Females were less sensitive than males. In addition, comparisons between the C57BL/10, BALB/c, AKR and DBA/2 strains indicated that the susceptibilities of mice to induction of porphyria did not completely correlate with their classification as *Ah*-responsive or *Ah*-non-responsive.

Prolonged treatment of rats with HCB causes an inhibition of hepatic uroporphyrinogen decarboxylase (porphyrinogen carboxylase, EC 4.1.1.37) with resulting accumulation of uroporphyrin (Elder *et al.*, 1976). This is used as a model for HCB-induced and spontaneous porphyria cutanea tarda in humans (Smith & De Matteis, 1980). TCDD causes a similar porphyria in C57BL/6 or C57BL/10 mice after a single small dose (75–150 µg/kg body wt.) (Goldstein *et al.*, 1973; Smith *et al.*, 1981), whereas multiple dosing is apparently needed for rats (Cantoni *et al.*, 1981; Goldstein *et al.*, 1982).

Certain inbred strains of mice, e.g. C57BL/6, C57BL/10 and BALB/c, when treated with 3-methylcholanthrene, respond by induction of the hepatic microsomal polycyclic aryl hydrocarbon hydroxylase system of enzymes controlled by the *Ah*-locus and are termed *Ah*-responsive (Nebert *et al.*, 1982*a,b*). Other strains, such as DBA/2 and AKR, are much less sensitive and are termed *Ah*-non-responsive. TCDD is a much more potent inducer of this drug metabolizing system than 3-methylcholanthrene (Poland & Glover, 1975; Poland & Knutson, 1982) and it has been proposed that the susceptibility of C57BL/6 mice to TCDD-induced porphyria is due to their being *Ah*-responsive (Jones & Sweeney, 1980). However, HCB, unlike TCDD, induces a mixture of microsomal mono-oxygenases in mice (Holme & Dybing, 1982). Comparisons of the porphyrogenic effects of HCB and TCDD in

genetically defined mice would be a significant step in elucidating the mechanism of uroporphyrinogen decarboxylase inhibition. Unfortunately, mice are extremely sensitive to the neurotoxicity of HCB and no porphyrogenic effects have been reported (De Matteis *et al.*, 1961; Ippen & Aust, 1972). Iron appears to influence the severity of HCB-induced porphyria in rats (Taljaard *et al.*, 1972; Smith *et al.*, 1979) and the reduction of hepatic iron levels protects C57BL/6 mice from the porphyrogenicity of TCDD (Sweeney *et al.*, 1979). We have recently used iron to maximize the hepatic toxicity of TCDD in susceptible mouse strains; some only reacted after this pretreatment (J. B. Greig & A. G. Smith, unpublished work).

In the present paper we describe the inhibition of hepatic uroporphyrinogen decarboxylase by HCB in some mouse strains after pretreatment with iron and compare the sensitivities of *Ah*-responsive and *Ah*-non-responsive mice.

Experimental

Chemicals

HCB (Organic Analytical Grade) was purchased from BDH Chemicals Co. (Poole, Dorset, U.K.). No TCDD or related compounds could be detected by selected-ion monitoring g.l.c.–mass spectrometry at a detection limit of less than one part per 50 000 (Karasek & Onuska, 1982). Imferon (50 mg of Fe/ml) was obtained from Fisons (Loughborough,

Abbreviations used: HCB, hexachlorobenzene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

Leics., U.K.) and its carbohydrate precursor, dextran C, was a gift from Fisons.

Animals and treatment

Mice (7–10 weeks old) were either bred in these laboratories or obtained from OLAC (1976) Ltd., Bicester, Oxon, U.K., and were fed a powdered MRC diet 41B supplemented with 2% arachis oil. Animals receiving HCB (0.02% of diet) were kept in a negative-pressure isolator. Imferon or dextran C were given by subcutaneous injection. Mice were killed by cervical dislocation and the livers were rinsed in distilled water, weighed and if necessary frozen with liquid N₂ and stored at -70°C.

Analyses

Liver homogenates (1:4, w/v) were prepared in 0.1 M-Na₂HPO₄/NaH₂PO₄ buffer (pH 6.8)/0.1 mM-EDTA. Porphyrin concentrations were estimated by fluorescence spectroscopy (Granick *et al.*, 1975) using uroporphyrin III as a standard. Porphyrins that had been isolated from porphyric liver were chemically decarboxylated in 1 M-HCl (Edmondson & Schwartz, 1953). H.p.l.c. of porphyrins was carried out using LDC/Milton Roy instruments with a Spherisorb 5 μ ODS column (25 cm $\times$ 0.46 mm internal diam.). Gradient solvent systems were produced using an Apple II computer and were adaptations of published methods (Johansson & Nilsson, 1982; Wright *et al.*, 1983).

Uroporphyrinogen decarboxylase was estimated as previously described (Smith & Francis, 1979) using uroporphyrin (isomers I:III, 32:68) isolated from porphyric rats. Activity is expressed as nmol of coproporphyrinogen formed/h per g of liver after a 1 h incubation with liver homogenate or as pmol/min per mg of protein after a 30 min incubation with a 40000g supernatant. Protein was determined with bovine serum albumin as standard (Lowry *et al.*, 1951).

HCB content of liver was estimated by g.l.c., with electron-capture detection using a Varian Vista 6000 chromatograph fitted with a column (2 m $\times$ 2 mm internal diam.) of 3% OV-210 coated on 100–120 mesh Gas-Chrom Q (Rizzardini & Smith, 1982).

Incubations *in vitro* with iron

Livers from male C57BL/10 and DBA/2 mice were homogenized in 0.25 M-sucrose and centrifuged at 40000g for 30 min. Portions of the supernatants were incubated in 4 mM-(NH₄)₂SO₄-FeSO₄ for 10 min at 37°C. Uroporphyrinogen decarboxylase was then assayed as before except that 0.05 M-sodium cacodylate buffer adjusted to pH 6.8 with 0.1 M-HCl was employed (so that the incubations were phosphate-free) and no EDTA was included.

Results

Prolonged treatment with HCB

BALB/c mice treated with HCB for up to 25 weeks did not show any increases in hepatic porphyrin levels, although their livers became extremely fatty. Males were more affected by neurotoxicity, with a greater mortality than females. In further experiments, male and female C57BL/10, BALB/c and DBA/2 mice were fed HCB for 15 weeks. Small and highly variable increases in liver porphyrins were observed in surviving male C57BL/10 mice (seven of 19 animals). Liver porphyrin levels were only slightly elevated in the other groups. All of the DBA/2 mice survived.

Response after pretreatment with iron

Male C57BL/10, BALB/c and DBA/2 mice together with C57BL/10 females were given Imferon (12.5 mg of iron/mouse; approx. 500 mg/kg body wt.) by subcutaneous injection and then fed HCB for up to 6 weeks. After an initial lag phase C57BL/10 male mice, and to a lesser extent C57BL/10 females, developed a massive porphyria with no deaths (Fig. 1). In contrast, DBA/2 mice did not react to HCB, even after 12 weeks (results not shown). Although concentrations of porphyrins in the livers of the BALB/c strain were only slightly increased after 6 weeks they were significantly raised

Fig. 1. Accumulation of porphyrins in the livers of different strains of mice fed HCB in the diet (0.02%) after a single subcutaneous injection of Imferon (12.5 mg of iron)

Mice were treated as described in the Experimental section. ●, Male C57BL/10; ○, female C57BL/10; ■, male BALB/c; □, male DBA/2. Results are means $\pm$ S.E.M. of four to nine animals per time point. There were no increases in porphyrin levels in mice not fed HCB. Note the logarithmic scale for porphyrins.

Table 1. Comparison of the combined effects of iron and HCB on the liver size, hepatic porphyrin levels and uroporphyrinogen decarboxylase activity of some inbred mouse strains

Male mice were given either dextran C (0.25 ml; 200 mg/ml) or Imferon (0.25 ml; 12.5 mg of Fe) by subcutaneous injection and then fed a diet containing 2% arachis oil with or without 0.02% HCB for 7 weeks. They were killed and analysed as described in the Experimental section. Results are means $\pm$ S.E.M. for the numbers of animals shown in parentheses. Significance assessed by Student's *t*-test: **P* < 0.001; †*P* < 0.005; ‡*P* < 0.01; §*P* < 0.05, when compared with the corresponding dextran C-treated control mice.

Strain	Dextran C	Fe	HCB/dextran	HCB/Fe
Liver wt. (% body wt.)				
C57BL/10	4.5 $\pm$ 0.2 (5)	5.5 $\pm$ 0.3 (5)§	10.9 $\pm$ 0.8 (7)*	11.5 $\pm$ 0.7 (7)*
BALB/c	4.7 $\pm$ 0.1 (4)	5.8 $\pm$ 0.2 (4)†	6.1 $\pm$ 0.3 (6)†	7.2 $\pm$ 0.3 (6)*
AKR	4.6 $\pm$ 0.2 (3)	7.1 $\pm$ 0.2 (3)*	6.7 $\pm$ 0.2 (4)*	7.6 $\pm$ 0.2 (4)*
DBA/2	4.9 $\pm$ 0.1 (4)	6.4 $\pm$ 0.2 (4)*	5.6 $\pm$ 0.2 (6)‡	6.4 $\pm$ 0.2 (6)*
Porphyrins (nmol/g of liver)				
C57BL/10	0.40 $\pm$ 0.09	0.71 $\pm$ 0.02	3.14 $\pm$ 0.73†	1171 $\pm$ 135*
BALB/c	0.63 $\pm$ 0.07	0.67 $\pm$ 0.04	0.87 $\pm$ 0.05§	4.46 $\pm$ 0.88†
AKR	0.60 $\pm$ 0.07	0.46 $\pm$ 0.03	0.64 $\pm$ 0.04	82.6 $\pm$ 15.8†
DBA/2	0.76 $\pm$ 0.05	0.69 $\pm$ 0.04	0.82 $\pm$ 0.12	0.76 $\pm$ 0.05
Uroporphyrinogen decarboxylase (nmol of coproporphyrinogen/h per g of liver)				
C57BL/10	41.4 $\pm$ 1.4	38.1 $\pm$ 0.9	33.1 $\pm$ 1.8‡	6.2 $\pm$ 0.7*
BALB/c	40.9 $\pm$ 1.0	40.7 $\pm$ 1.7	43.1 $\pm$ 0.9	38.4 $\pm$ 1.4
AKR	43.5 $\pm$ 0.5	39.2 $\pm$ 0.9	40.7 $\pm$ 0.8	30.5 $\pm$ 1.3§
DBA/2	41.1 $\pm$ 1.1	43.7 $\pm$ 0.6	40.2 $\pm$ 0.9	42.6 $\pm$ 1.6

after 10 weeks (36.5 ± 7.0 nmol/g). This response was not greatly altered by a larger dose of iron (25 mg/animal). Giving iron to C57BL/10 males at 4 weeks was far less effective in producing porphyria after 6 weeks than when the mice were dosed before treatment with HCB. H.p.l.c. analysis of the porphyrins accumulated in C57BL/10 males showed uroporphyrin to be the major one with smaller amounts of heptacarboxyporphyrin (92:8). The uroporphyrin consisted of a mixture of the I and III isomers (isomer I 35–65% of total). When the heptacarboxyporphyrin was chemically decarboxylated to coproporphyrin only a trace of the I isomer was detected (I:III, 7:93). This distinction in isomer composition between uroporphyrin and heptacarboxyporphyrin is very similar to that previously described for HCB-induced porphyria in rats (Smith & Francis, 1981).

Inhibition of uroporphyrinogen decarboxylase

To demonstrate the individual and combined effects of iron and HCB on liver size, porphyrins and uroporphyrinogen decarboxylase activity, male C57BL/10, BALB/c, AKR and DBA/2 mice were fed HCB for 7 weeks. Animals not pretreated with Imferon received dextran C. There was a high correlation between increased liver size caused by HCB, inhibition of uroporphyrinogen decarboxylase and the degree of porphyria (Table 1). Iron caused no significant decreases in the activity of the enzyme but when combined with HCB far greater

inhibition occurred in C57BL/10 and AKR mice than with HCB alone.

Time course of uroporphyrinogen decarboxylase inhibition

Liver weight, HCB content, porphyrin levels and uroporphyrinogen decarboxylase were monitored over 5 weeks in C57BL/10 male mice fed HCB after iron treatment. Liver weight and HCB content rose continually (Fig. 2a). The upturn in HCB levels after 4 weeks was accounted for by association with increases in lipid accumulation. Control animals given iron also showed a small increase in liver weight (compare Table 1). Uroporphyrinogen decarboxylase activity (estimated in 40000g supernatants) appeared to fall from the beginning of the experiment but overt porphyria was not observed until 3–4 weeks later when enzyme activity was reduced by at least 50% (Fig. 2b).

Effects of iron in vitro on uroporphyrinogen decarboxylase

Liver supernatants from male C57BL/10 and DBA/2 mice were treated with 4 mM- $(\text{NH}_4)_2\text{FeSO}_4$ for 10 min at 37°C and then assayed in a 1 mM solution to determine any effects *in vitro* on uroporphyrinogen decarboxylase. Activity was considerably reduced but there were no differences between the strains: control C57BL/10, 6.1 ± 0.1 ; control DBA/2, 6.0 ± 0.2 ; iron-treated

Fig. 2. Effects of HCB in the diet (0.02%) on C57BL/10 male mice after a subcutaneous injection of Imferon (12.5 mg of Fe)

(a) Liver size (■) and HCB content (□); (b) liver porphyrins (●) and uroporphyrinogen decarboxylase activity (○). Results are means $\pm$ S.E.M. for three mice per time point. Animals given iron alone showed a small increase in liver size but no changes in porphyrins or enzyme activity after 5 weeks.

C57BL/10, 2.9 ± 0.6 ; iron-treated DBA/2, 2.6 ± 0.3 pmol of coproporphyrinogen formed/min per mg of protein ($n = 3$ per group). These results confirm that under some experimental conditions ferrous ions at relatively high concentrations can cause inhibition of uroporphyrinogen decarboxylase *in vitro* (Kushner *et al.*, 1975).

Discussion

Although there is some evidence for the influence of iron on HCB-induced porphyria in rats (Taljaard *et al.*, 1972; Louw *et al.*, 1977; Smith *et al.*, 1979), Hanstein *et al.* (1981), in contrast, were unable to demonstrate a stimulatory effect of iron on the development of porphyria. However, the results reported in the present paper clearly demonstrate that a dose of iron before HCB treatment caused porphyria in C57BL/10 male mice, whereas many

animals not given iron eventually died from other toxic effects of HCB without any large rises in liver porphyrin levels. The onset of porphyria was due to a much greater inhibition of uroporphyrinogen decarboxylase in the presence of high levels of hepatic iron, although iron alone was ineffective. Females were less sensitive than males. Inhibition of uroporphyrinogen decarboxylase seemed to occur a short time after the start of HCB treatment but a large rise in liver porphyrin levels did not begin until 3 to 4 weeks later. Although the resistance of DBA/2 mice might imply that the toxic effect is directly linked to the inducibility of aryl hydrocarbon hydroxylase (Poland & Glover, 1975; Poland & Knutson, 1982), the relatively low susceptibility of BALB/c mice (*Ah*-responsive), but greater sensitivity of the AKR strain (*Ah*-non-responsive), demonstrate that the sequence of toxicity is more complex. The relative sensitivities of the sexes and strains of mice to HCB-induced porphyria after iron treatment are very similar to findings with TCDD (J. B. Greig & A. G. Smith, unpublished work) and suggest that these two halogenated compounds act by a common mechanism. This may involve stimulation of lipid peroxidation (Jones *et al.*, 1981) and other manifestations of iron toxicity (De Matteis & Stonard, 1977), although it is unclear whether the effects *in vitro* of ferrous ions on decarboxylase activity have any significance *in vivo*. Alternatively, iron may stimulate metabolism of these compounds to toxic products as has been suggested for the action of chlorinated chemicals on cultured avian hepatocytes (Sinclair & Granick, 1974). By comparing the effects of HCB and TCDD on certain inbred mouse strains with known genetic differences, together with the synergism of iron, some of these hypotheses can be tested more rigorously than has hitherto been possible.

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