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SEX-LINKED HEPATIC UROPORPHYRIA AND THE INDUCTION OF CYTOCHROMES P450IA IN RATS CAUSED BY HEXACHLOROBENZENE AND POLYHALOGENATED BIPHENYLS*

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Abstract—A marked sex difference in the development of uroporphyria occurred after administration of polychlorinated and polybrominated biphenyls (PCBs and PBBs), as well as hexachlorobenzene (HCB), to F344 rats for 15 weeks. Thus the propensity of female rats to develop uroporphyria appears to be a general response to this class of halogenated chemicals. A heat-stable inhibitor(s) of liver uroporphyrinogen decarboxylase was extractable from uroporphyric livers. Although oxidation of uroporphyrinogen I to uroporphyrin I by hepatic microsomes from rats pretreated with porphyrogenic regimes of HCB and PCBs was induced, there was no correlation with the *in vivo* sex difference in porphyria development. Levels of total cytochrome P450 and pentoxyresorufin and benzyloxyresorufin dealkylase activities (associated with cytochrome P450IIB1) were greater in microsomes from control, HCB, PCB and PBB treated male rats than females. In contrast, ethoxyresorufin deethylase activity (associated with cytochrome P450IA1) was always significantly greater in females. These findings were confirmed by immunoblotting with polyclonal antibodies to cytochromes P450IA1, IA2 and IIB1. Immunocytochemical studies showed that, even after 30 weeks of HCB exposure, cytochromes P450IA1 and P450IA2 were still more highly induced in female liver, especially in the centrilobular region. The results are consistent with the association of cytochrome P450IA isoenzymes with uroporphyria development, although the sex difference in P450IA levels alone may not be marked enough to provide the complete explanation for the pronounced susceptibility of females to HCB.

A partial block in hepatic haem biosynthesis at uroporphyrinogen decarboxylase (EC 4.1.1.37) occurs in rodents and humans following exposure to a variety of polyhalogenated aromatic chemicals which interact chronically with the *Ah*-receptor [2–5]. The resulting uroporphyria is very similar to the non-familial hepatic porphyria that can occur in some patients with moderate liver damage caused by alcohol and some drugs [2, 5]. In rats, hexachlorobenzene (HCB) has been the most studied example of this class of chemicals. Female rats are considerably more sensitive than males to the induction of porphyria [6–9], with accompanying depression of uroporphyrinogen decarboxylase activity [10]. Treatment with oestrogenic drugs increases the susceptibility of male rats to HCB-induced inhibition of the enzyme [11]. Female rats are also markedly more susceptible to the hepatocarcinogenicity of HCB [9, 12] and there may be a link between uroporphyria and liver tumour development [9]. Human patients with non-familial uroporphyria (porphyria cutanea tarda) also have a high risk of developing liver carcinoma [13]. Other polyhalogenated aromatics such as polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), some hexachlorodibenzo-*p*-dioxins and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) also cause liver tumours predominantly in female rats (for references see [10]), but much less is known of any sex difference in uroporphyria development.

Mechanistically, the depression of uroporphyrinogen decarboxylase activity by polyhalogenated aromatic chemicals in rodents appears to be associated with chronic induction of members of the cytochrome P450 supergene family, especially those inducible by 3-methylcholanthrene, P450IA1 and P450IA2, and mediated through the *Ah*-receptor [2, 3, 14–17]. Recent studies indicate that in the rat, cytochrome P450IA2, but not P450IA1, might be involved by oxidizing uroporphyrinogen to uroporphyrin [17]; other more oxidized products could be inhibitors of the decarboxylase [18].

In the present work, we have shown that PCBs and PBBs, like HCB, also cause hepatic uroporphyria predominantly in female rats, but that, under *in vitro* conditions, there was no marked sexual dimorphism in the microsomal oxidation of uroporphyrinogen. However, both cytochromes P450IA1 and P450IA2 were persistently induced to a significantly greater degree in females than in males.

MATERIALS AND METHODS

Chemicals and polyclonal antibodies. HCB (Organic Analytical Standard) was purchased from BDH (Poole, U.K.). Aroclor 1254 was originally

* A preliminary account of part of this work has been reported [1].

a gift to Dr J. B. Greig from Monsanto, U.S.A. Firemaster BP-6 was a gift from Dr R. Kimbrough and Dr V. W. Burse (Center for Disease Control, Atlanta, U.S.A.). 3,3',4,4'-Tetrachlorobiphenyl and 3,3',4,4',5,5'-hexachlorobiphenyl were supplied by Prochem., Wessel, F.R.G. and British Greyhound, Birkenhead, U.K., respectively. TCDD was prepared as reported in Greig *et al.* [19]. Pentacarboxyporphyrin I, uroporphyrin I and iron-uroporphyrin I were purchased from Porphyrin Products (Logan, UT, U.S.A.). Ethoxyresorufin and pentoxyresorufin (more correctly the alkyl phenoxazones) were prepared as described previously [16]. Benzyloxyresorufin was obtained from Boehringer Corp. Ltd (Lewes, U.K.). Metyrapone was from Sigma Ltd, and 9-hydroxyellipticine was a gift from Prof. C. Paoletti. Purified rat liver cytochrome P450 isoenzymes and rabbit polyclonal antibodies were prepared as described by Wolf *et al.* [20].

Rats and protocols. Male and female F344/N rats (10 weeks old) bred on site, were fed powdered 41B diet (Christopher Hill Group, Poole, U.K.) containing 2% corn oil. HCB, Aroclor 1254 and Firemaster BP-6.were added to the diet at levels of 0.02 or 0.005% as indicated. Animals were maintained in plastic boxes with wire-mesh floors over trays in a plastic isolator under negative pressure. Rats were killed by decapitation and livers were homogenized in water (1:4 w/v) for analysis of porphyrins and to detect inhibitory activity towards uroporphyrinogen decarboxylase. For the preparation of microsomes, liver (1 g) was homogenized in 4 mL of 0.25 M sucrose/50 mM Tris–HCl buffer (pH 7.5), spun at 10,000 *g* for 15 min and then the supernatant centrifuged at 100,000 *g* for 60 min. The pellet was washed by resuspension in 75 mM KCl/25 mM Tris–HCl buffer (pH 7.5) and recentrifuged at 100,000 *g* for 60 min. The final microsomal pellet was suspended in 4 mL of the same 0.25 M sucrose/50 mM Tris–HCl buffer system before use.

Assays. Porphyrin contents of liver homogenates were determined by fluorescence spectroscopy and expressed in terms of uroporphyrin [21]. The inhibitory properties of heat-treated extracts of livers towards hepatic uroporphyrinogen decarboxylase were estimated with C57BL/10ScSn mouse liver cytosol as described previously [22]. Sigma kit 59-UV was used to assay plasma alanine aminotransferase activity (EC 2.6.1.2). Cytochrome P450 levels were estimated as in Stewart and Smith [23] and protein by the method of Lowry *et al.* [24] using bovine serum albumin as the standard.

Oxidation of uroporphyrinogen I by microsomes in the presence of NADPH was performed in a similar manner to that described by Jacobs *et al.* [17]. Incubation mixtures at 30°C consisted of 2.36 mL of 0.25 M sucrose/1 mM EDTA/0.05 M HEPES buffer (pH 7.6), 0.1 mL of microsomal suspension (about 10 mg protein) and 20 μL of an NADPH regenerating system (0.9 mg NADPH, 2.1 mg glucose 6-phosphate, 4.5 mg $MgCl_2 \cdot 6H_2O$ and 0.2 units of glucose 6-phosphate dehydrogenase). Uroporphyrinogen I was prepared from uroporphyrin I (0.75 nmol/μL in 60 mM dithiothreitol) by reduction with 5% sodium amalgam and diluted 1:1 (v/v) with 10 mM dithiothreitol/0.05 M HEPES buffer (pH 7.5). Incubations were initiated by the addition of 20 μL of uroporphyrinogen solution (final concentration 3 μM) and the formation of uroporphyrin monitored by fluorescence spectroscopy (excitation 397 nm, emission 617 nm) for up to 15 min.

Ethoxy-, pentoxy- and benzyloxyresorufin dealkylase activities of microsomes were estimated with 3, 10 and 5 μM concentrations of substrate, respectively, at 25°C by fluorescence spectroscopy [25]. NADPH-stimulated chemiluminescence of microsomes in the presence of lucigenin was performed by a method modified from that described by Urquhart and Elder [26] using an LKB 1250 luminometer with integrator and recorder. Incubations consisted of 0.46 mL 50 mM Tris–HCl buffer (pH 7.5), 10 μL of lucigenin solution (0.4 mg/mL) and 20 μL of microsomal suspension, which were incubated together for 1 min before the addition of 10 μL of NADPH (0.2 mg). Maximum response (mV) occurred within 2 min and this value was used to give a representative indication of total chemiluminescence produced by different microsomal preparations over longer time periods. This method is thought to detect preferentially superoxide anion radical but there is no doubt that lucigenin oxidation occurs to some extent with other oxidizing species such as H_2O_2 [27].

Immunoblotting. SDS/polyacrylamide-gel electrophoresis of microsomes was performed as described by Laemmli [28] using 7.5% gels. After separation, proteins were transferred to nitrocellulose sheets using the semi-dry method of Peferoen [29]. After blocking non-specific binding with 2.5% horse serum/0.5% gelatin solution in PBS (w/v) overnight, blots were incubated successively for 3 hr with rabbit polyclonal antibodies to specific forms of cytochrome P450 and with alkaline phosphatase conjugated goat anti-rabbit IgG. After washes with PBS, immunoreactive bands were revealed by incubating blots with naphthol AS-Mx (0.2 mg/mL) and Fast Blue BB (1 mg/mL) in 0.2 M Tris–HCl (pH 8.2). Some cross reactivity was observed between antibodies against cytochromes P450IA1 and P450IA2 but this did not interfere in analyses due to the resolution of the isoenzymes during electrophoresis.

Immunocytochemistry. Acetone-fixed paraffin-embedded sections of liver were incubated with polyclonal antibodies to rat cytochrome P450 isoenzymes and specific binding revealed as in Foster *et al.* [30]. Sections were counterstained with haematoxylin.

RESULTS

Sex difference in uroporphyria development

Exposure of male and female rats to HCB (0.02% of the diet) for 15 weeks illustrated the marked sex difference in the development of uroporphyria (Table 1). The sex difference in uroporphyrinogen decarboxylase inhibition and porphyria development was still very evident after 6 months [10], although males do eventually develop uroporphyria [9]. A distinction between the sexes was also apparent after administration (0.005%) of PCBs (Aroclor 1254) and PBBs (Firemaster BP-6). However, these halogenated biphenyl mixtures are more potent than

Table 1. Comparison of the development of uroporphyria in male and female F344 rats after exposure to hexachlorobenzene, polychlorinated biphenyls and polybrominated biphenyls

Diet	Sex	Liver (% body wt)	Plasma ALT (I.U./L)	Liver porphyrins (nmol/g tissue)	Uroporphyrinogen decarboxylase inhibitor activity (pmol/min/mg protein)	
Control	Male	3.7 ± 0.1	68 ± 3	0.82 ± 0.09	34.9 ± 0.2	(0)
Control	Female	3.5 ± 0.1	53 ± 3	0.76 ± 0.11	35.0 ± 0.3	(0)
Hexachlorobenzene	Male	4.9 ± 0.1†	57 ± 2	1.24 ± 0.12†	29.8 ± 0.7†	(15)
Hexachlorobenzene	Female	4.5 ± 0.1*†	52 ± 2	1268 ± 269*†	2.4 ± 0.7*†	(93)
Aroclor 1254 (0.005%)	Male	5.9 ± 0.1†	49 ± 4	21.0 ± 7.4†	26.5 ± 1.1†	(24)
Aroclor 1254 (0.005%)	Female	4.5 ± 0.2*†	53 ± 5	692 ± 140*†	4.2 ± 0.8*†	(88)
Aroclor 1254 (0.02%)	Male	7.1 ± 0.2†	230 ± 60†	1.88 ± 0.28†	31.2 ± 0.3†	(11)
Aroclor 1254 (0.02%)	Female	6.7 ± 0.3†	223 ± 46†	1.86 ± 0.27†	32.1 ± 0.5†	(8)
Firemaster BP-6	Male	7.6 ± 0.1†	49 ± 5	8.47 ± 2.67†	19.3 ± 3.5†	(45)
Firemaster BP-6	Female	6.3 ± 0.2*†	54 ± 3	335 ± 104*†	2.5 ± 0.5*†	(93)

Male and female F344 rats (10 weeks old) were fed diets containing HCB (0.02%), Aroclor 1254 (0.005 and 0.02%) or Firemaster BP-6 (0.005%) for 15 weeks. Plasma ALT, hepatic porphyrin levels (expressed in terms of uroporphyrin) and the ability of liver extracts to inhibit mouse uroporphyrinogen decarboxylase were estimated as described in Materials and Methods. Values in parentheses are % inhibition compared with control extracts. Results are means ± SE of 4 rats per group.

* Significantly different from males $P < 0.05$ as assessed by Student's *t*-test.

† Significantly greater than appropriate control groups $P < 0.05$.

HCB and some degree of porphyria was observed in males. The development of porphyria was not associated with an elevation of plasma ALT as occurs in mice [19, 31]. A greater exposure (0.02%) to Aroclor 1254 for 15 weeks resulted in severe hepatic toxicity, some females becoming highly jaundiced, but there was no accumulation of uroporphyrin in the livers of either sex. In this case, plasma ALT levels were significantly raised in both males and females reflecting the observed severe liver damage (Table 1). The time courses of porphyria and liver damage were not studied in more detail, but it appears that porphyria is only sustained under moderate toxic conditions. One explanation might be that the haem biosynthetic pathway becomes depressed with marked toxicity leading to a lack of precursors required for porphyrin accumulation.

TCDD is the most potent ligand known for the *Ah*-receptor, involved in the induction of cytochrome P450IA isoenzymes, and also the most effective at causing uroporphyria in female rats [2, 32, 33]. In the present study, administration of TCDD (25 μg/kg body weight) at the start of the experiment and subsequently after a further 4 and 8 weeks, did not result in porphyria at 15 weeks in either sex, but produced considerable depression of body weight gain (results not shown). Similar problems with single large doses of TCDD have been reported [33]. After 30 weeks of 1 μg TCDD/kg per week, mild uroporphyria developed in the livers of female rats (12.3 ± 10.6 nmol porphyrin/g liver), but not males (0.82 ± 0.15 nmol/g). No changes in porphyrin levels were observed after 15 weeks. This time scale was more in agreement with the findings of Cantoni *et al.* [32] than those of Goldstein *et al.* [33]. However, the three studies involved different strains of rats. Because of these difficulties in producing uroporphyria with TCDD, further studies were not conducted.

Uroporphyrinogen decarboxylase inhibitor

Some investigations have indicated that the depression of uroporphyrinogen decarboxylase activity in rodents may be due to the generation of an inhibitor [22, 34, 35]. Heat-treated porphyric livers from female rats exposed to HCB or the polyhalogenated biphenyls were purified by passage through SEP-PAK C_{18} cartridges. All extracts markedly inhibited uroporphyrinogen decarboxylase activity of mouse liver cytosol (Table 1). Inhibition of the murine enzyme was used for direct comparisons with previous work [22], but extracts from porphyric animals (not controls) also inhibited the rat enzyme by >90% (results not shown). Analysis of inhibitory extracts by HPLC confirmed that most of the uroporphyrins and heptacarboxylic porphyrins present in original liver homogenates had been removed (>95%) by adherence to precipitated protein and by passage through SEP-PAK C_{18} cartridges. Either no inhibitory activity or significantly less, was exhibited by extracts from livers of treated males or from those female rats administered a high dose of Aroclor 1254 and in which porphyria was not evident (Table 1). Significant inhibition of mouse hepatic uroporphyrinogen decarboxylase was also observed with extracts from livers of TCDD-treated female rats (34%), whereas <10% occurred with male extracts.

When uroporphyrins I and III were added to control female liver homogenates to a level equivalent to 10 nmol/g of liver, followed by heat treatment and passage through SEP-PAK C_{18} cartridge, the resulting extracts did not inhibit mouse hepatic uroporphyrinogen decarboxylase. Thus, as observed previously with mice [22], the inhibitory activity of weakly porphyric liver extracts was not the result of products formed from uroporphyrins during sample preparation. When much higher concentrations of

uroporphyrin I or III (equivalent to 500 nmol/g of liver) were added, extracts inhibited uroporphyrinogen decarboxylase to a small but variable extent (0–20%). Thus the main proportion of the inhibitory factor from highly porphyric livers appeared to be formed *in vivo*, but a minor part could be ascribed to the generation of inhibitor(s) *in vitro*. Further studies on the inhibitory potential of extracts from porphyric livers are in progress.

To explore further the nature of *in vitro* generated inhibitors, heat-treated extracts of porphyric livers were examined by HPLC using a more polar mobile phase than was routinely employed for porphyrin analysis. A peak was observed which was more polar than uroporphyrins and was not formed by heating uroporphyrin I in water, the method used for preparation of inhibitor fractions. However, a similar product not retained by a SEP-PAK C_{18} cartridge was observed if uroporphyrin I (100 μM) was heated at 90°C for 5 min with control liver supernatant (40,000 *g* for 30 min). Under our conditions of HPLC [22] this product had a retention time similar to that of iron-uroporphyrin I, but could well be an oxidized uroporphyrin [18]. When tested against mouse liver cytosol, iron-uroporphyrin I was found to be a relatively poor inhibitor of the decarboxylase (100 μM caused a 25% inhibition) and similar in potency to the parent porphyrin.

Microsome-catalysed oxidation of uroporphyrinogen

Male rat liver microsomes in the presence of NADPH oxidise uroporphyrinogen to uroporphyrin, especially after preinduction of cytochromes P450IA1 and P450IA2 by single large doses of 3-methylcholanthrene or HCB [17]. Oxidation of uroporphyrinogen has been reported to be catalysed particularly by P450IA2 isoenzyme [17] and by-products may play a role in uroporphyrinogen decarboxylase inhibition [18].

In the present work, microsomes were prepared from rats administered HCB at a porphyrogenic dose (for females) for 5 or 15 weeks. Microsomal oxidation of uroporphyrinogen I was induced 1.8- to 2.5-fold. Induction was not as great as observed by others after a single dose of HCB, although in the present work control values were much higher than in previous studies [17]. Greater induction (4-fold) was observed after 5 weeks of treatment with the chlorinated biphenyls (Table 2). Under these particular conditions there was no greater propensity of induced microsomes from females than males to oxidize uroporphyrinogen. In fact, at 15 weeks oxidation by both control and HCB-induced microsomes was significantly less with females (Table 2). With chick embryo liver microsomes the presence of 3,3′,4,4′-tetrachlorobiphenyl (6.8 μM) is required for oxidation [17, 36, 37]. With Aroclor-induced microsomes from male rats 3,3′,4,4′-tetrachlorobiphenyl (6.8 μM; 2 μg/mL) had no effect on the oxidation rate as observed previously with microsomes from 3-methylcholanthrene or β-naphthoflavone treated rats [17, 36]. On the other hand 3,3′,4,4′,5,5′-hexachlorobiphenyl (3.3 μM) caused a 65% inhibition. The general cytochrome P450 inhibitor metyrapone (1 mM) was relatively inefficient (48% inhibition) whereas 9-hydroxyellipticine (5 μM), a potent inhibitor of cytochrome P450IA activities [23], was also highly effective in preventing uroporphyrinogen oxidation (100% inhibition).

Induction of cytochrome P450

Cytochrome P450 activities and isoenzymes were compared in male and female rats after exposure to HCB and the polyhalogenated biphenyls. Microsome-catalysed dealkylations of pentoxyresorufin and ethoxyresorufin are useful indicators of the mono-oxygenase activities of cytochromes P450IIB1 and P450IA1, respectively, although not completely specific [20, 38]. Dealkylation of benzyloxyresorufin is carried out by a number of isoenzymes, but is mainly indicative of phenobarbital-inducible cytochrome P450 forms [20]. After 5 or 15 weeks of HCB diet total cytochrome P450 and pentoxy- and benzyloxyresorufin dealkylase activities were markedly induced in both sexes but were either greater in males than females or there were no significant differences (Table 3). Treatment of rats for 5 weeks with PCBs and PBBs gave similar results. In contrast, under all regimes, ethoxyresorufin deethylation was significantly greater in females than in males. The halogenated biphenyls were much more potent inducers of ethoxyresorufin dealkylation than HCB, whereas other dealkylase activities were induced to a similar or lower level (Table 3). Control values of ethoxyresorufin deethylase were also significantly greater in females at 5 weeks, as first reported by Burke *et al.* [39]. In summary, NADPH-dependent mono-oxygenase activities of microsomal preparations demonstrated that the polyhalogenated chemicals may induce greater levels of hepatic cytochrome P450IA1 in females than in male rats, whereas the reverse is true for P450IIB1.

Microsomal proteins from HCB-induced rats were also examined by immunoblotting with a panel of polyclonal antibodies to cytochrome P450 isoenzymes and NADPH–cytochrome P450 reductase [20]. Forms P450IIC6 (marginally inducible by phenobarbital) and the male specific isoenzyme P450IIC11 were not induced by HCB under the porphyrogenic regime employed (results not shown). In contrast, the major phenobarbital inducible form P450IIB1 was markedly induced (Fig. 1a), but to a greater degree in males, in agreement with pentoxy- and benzyloxyresorufin dealkylase activities (Table 3), although it should be noted that the antibody to P450IIB1 shows some cross reactivity with other P450IIB isoenzymes [20]. HCB also induced the 3-methylcholanthrene-inducible forms, namely P450IA1 and P450IA2 [15, 40], but to a significantly greater degree in females than males after both 5 and 15 weeks (Fig. 1b). The halogenated biphenyls were not such good inducers of P450IIB1 as HCB (0.02% of the diet), but even at a lower dose (0.005%), both Aroclor 1254 and Firemaster BP-6 caused a much larger induction of cytochromes P450IA1 and P450IA2. With Aroclor 1254, induction again appeared to be greater in females, but with the PBBs such a distinction was not apparent at this particular time. Samples of microsomes shown in Fig. 1 were compared on the basis of equivalent amounts of original liver rather than equal amounts

Table 2. Oxidation of uroporphyrinogen I by hepatic microsomes

Treatment	Sex	5 weeks		15 weeks	
		Cytochrome P450 (nmol/mg protein)	Uroporphyrinogen oxidation (pmol/min/mg protein)	Cytochrome P450 (nmol/mg protein)	Uroporphyrinogen oxidation (pmol/min/mg protein)
Control	Male	0.81 ± 0.06	19.8 ± 0.6	0.78 ± 0.02	22.7 ± 1.2
Control	Female	0.75 ± 0.05	18.4 ± 1.1	0.74 ± 0.06	15.3 ± 0.8*
Hexachlorobenzene	Male	1.43 ± 0.06†	41.4 ± 2.6†	1.93 ± 0.23†	45.5 ± 3.5†
Hexachlorobenzene	Female	1.22 ± 0.09†	46.5 ± 4.7†	0.95 ± 0.07‡	27.3 ± 1.9*†
Aroclor 1254	Male	1.69 ± 0.04†	79.1 ± 1.9†	ND	ND
Aroclor 1254	Female	1.72 ± 0.06†	85.2 ± 3.4†	ND	ND

Male and female rats were given food containing HCB (0.02%) or Aroclor 1254 (0.005%) for 5 or 15 weeks as indicated. The preparation of microsomes and their oxidation of uroporphyrinogen I after the addition of NADPH are described in Materials and Methods. Results are means ± SE (N = 4).

* Significantly less than males ($P < 0.01$).

† Significantly greater than appropriate control group ($P < 0.01$).

ND, not determined.

of microsomal protein. This allowed a direct comparison between treatments without the complications of differences due to increases in protein content observed with potent inducers of the microsomal system. HCB and the polyhalogenated biphenyls did not induce NADPH–cytochrome P450 reductase which was present at higher levels in males than females (results not shown).

Some hypotheses of the mechanism of uroporphyria invoke cytochrome P450-mediated generation of reactive oxygen species as a consequence of uncoupling P450IA2 [5, 17, 36, 37, 41]. NADPH-stimulated production of reactive oxygen species by HCB-induced mouse liver microsomes using lucigenin-enhanced chemiluminescence has been reported [26]. This method was employed in the present work to determine whether the sex difference in uroporphyrin development in rats could be partly explained by a difference in the production of reactive oxygen species due to uncoupling components of the microsomal system. Formation of oxidizing species was induced by 5 and 15 weeks of HCB treatment and 5 weeks of the polyhalogenated biphenyls (Table 3). These increases in apparent production of oxidizing species were not directly proportional to total cytochrome P450 content, illustrating that particular forms of cytochrome P450 may have had a greater propensity to become uncoupled than others. However, there was no evidence that such a process was more likely to occur with female microsomes than with those from males; in fact, chemiluminescence was usually greater with the latter (Table 3).

Immunocytochemistry

Measurement of microsomal mono-oxygenase activities or the corresponding cytochrome P450 isozymes by immunoblotting indicates average distributions across liver lobules. However, uroporphyria does not develop uniformly amongst liver cells. To compare the distribution of cytochrome P450 isoenzymes in liver sections from males and females after porphyrogenic diets, tissue sections were examined with polyclonal antibodies. P450IIB1 was found to be only weakly staining in control male liver, whereas it was strongly induced in centrolobular areas of male rats administered HCB for 30 weeks (Fig. 2a–c) after which time there was still a marked sex difference in uroporphyria [10]. This isoenzyme was induced to a lesser extent in females. With antibody to P450IA1 no isoenzyme was detectable in control male liver, but small amounts were observed in the centrilobular areas of female control tissue (Fig. 2d). Treatment with HCB caused intense and widespread induction in female liver, although it was most pronounced in the centrilobular regions (Fig. 2f). In contrast, induction was considerably less and was more diffuse in male rats despite the prolonged HCB diet (Fig. 2e). Similar results were observed with antibody to P450IA2. Shorter exposures to HCB (5 and 15 weeks) and polyhalogenated biphenyls (5 weeks) gave similar results.

DISCUSSION

Possible mechanisms which produce uroporphyria in humans, avian systems and rodents and the involvement of cytochrome P450 have been recently discussed in depth by many authors [2, 5, 17, 22, 31, 36, 41, 42]. Here discussion is restricted mainly to aspects pertinent to uroporphyria development in rats. Previous studies showed that female rats develop uroporphyria much more readily than males after exposure to HCB [6–9], with associated selective depression of hepatic uroporphyrinogen decarboxylase [10]. This was confirmed in the present work, which also demonstrated that the marked sexual dimorphism also occurs with PCBs, PBBs, and probably TCDD. Although some evidence is consistent with active metabolites of HCB causing porphyria in rats [43], *in vivo* and *in vitro* comparisons between the sexes have not shown a marked potential of females to metabolize HCB [21, 23, 44]. An active metabolite hypothesis is also more difficult as an explanation for the sex difference in the response of rats to the more potent agents, polyhalogenated

Table 3. Induction of hepatic microsomal alkoxyresorufin dealkylations and lucigenin-enhanced chemiluminescence

Diet	Time (weeks)	Sex	Cytochrome P450 (nmol/mg protein)	EROD (pmol/min/mg)	PROD (pmol/min/mg)	BROD (pmol/min/mg)	Chemiluminescence (max mV/mg)
Control	5	Male	0.95 ± 0.02	20 ± 1	3 ± 1	15 ± 2	444 ± 67
Control	5	Female	0.81 ± 0.03†	42 ± 3*	2 ± 1	10 ± 1†	304 ± 19
Hexachlorobenzene	5	Male	1.79 ± 0.09	390 ± 6	533 ± 59	1992 ± 225	1985 ± 152
Hexachlorobenzene	5	Female	1.51 ± 0.05†	703 ± 47*	464 ± 68	1209 ± 214†	687 ± 65†
Aroclor 1254	5	Male	1.76 ± 0.07	1800 ± 281	140 ± 36	729 ± 116	1821 ± 127
Aroclor 1254	5	Female	1.49 ± 0.05	3092 ± 239*	84 ± 5†	529 ± 10	823 ± 44†
Firemaster BP-6	5	Male	1.82 ± 0.06	2386 ± 81	728 ± 67	2667 ± 165	2656 ± 89
Firemaster BP-6	5	Female	1.69 ± 0.07	3433 ± 85*	378 ± 34†	1453 ± 172†	1134 ± 89†
Control	15	Male	0.93 ± 0.03	54 ± 3	6 ± 5	19 ± 2	1159 ± 77
Control	15	Female	0.77 ± 0.02†	41 ± 9	5 ± 1	9 ± 1†	530 ± 32†
Hexachlorobenzene	15	Male	1.98 ± 0.12	363 ± 42	334 ± 12	1059 ± 148	2858 ± 137
Hexachlorobenzene	15	Female	1.39 ± 0.01†	633 ± 22*	170 ± 20†	747 ± 179	1724 ± 243†

Male and female F344 rats were fed diets of HCB (0.02%), Aroclor 1254 (0.005%) or Firemaster BP-6 (0.005%) for 5 or 15 weeks as indicated. Dealkylations and chemiluminescence (NADPH stimulated oxidation of lucigenin) were performed as described in Materials and Methods. Results are means ± SE of 4 rats per group.

* Significantly greater than males ($P < 0.005$).

† Significantly less than males ($P < 0.05$).

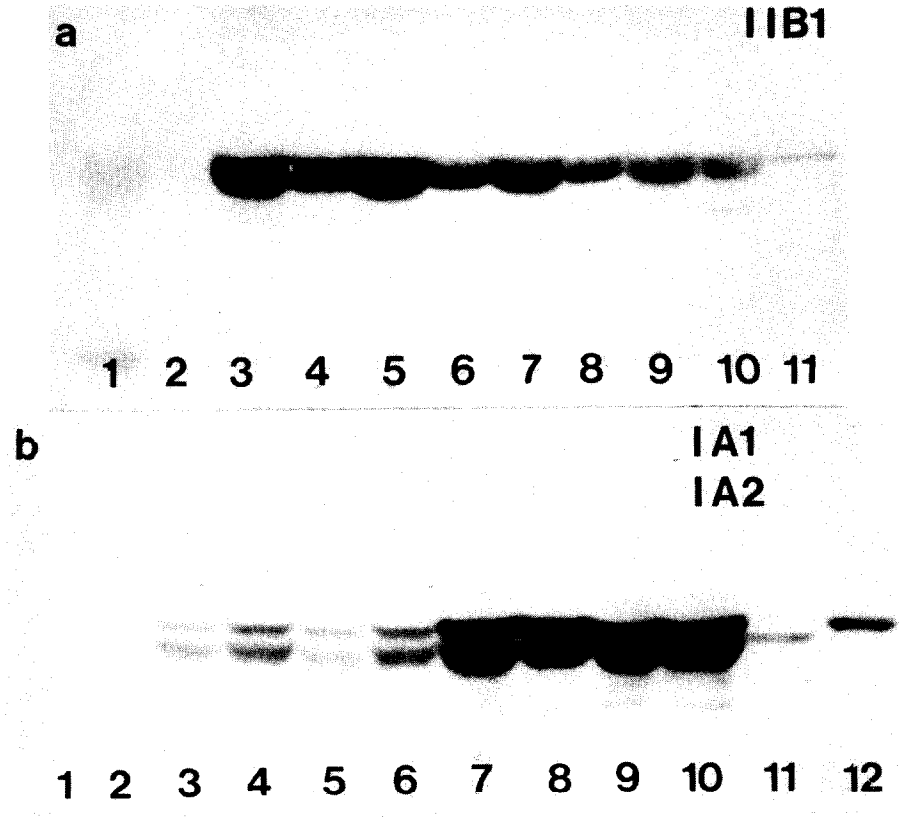

Fig. 1. Immunoblots of microsomal proteins from rats fed hexachlorobenzene or polyhalogenated biphenyls. Microsomal proteins (10 μL suspension = 2.5 mg of wet liver), from rats treated with HCB (0.02%) for 5 or 15 weeks, Firemaster BP-6 (0.005%) or Aroclor 1254 (0.005%) for 5 weeks, were probed with (a) a polyclonal antisera against cytochrome P450IIB1 or (b) a mixture of antisera against cytochromes P450IA1 and P450IA2. Lane 1, control male; 2, control female; 3, HCB male 5 weeks; 4, HCB female 5 weeks; 5, HCB male 15 weeks; 6, HCB female 15 weeks; 7, Firemaster male; 8, Firemaster female; 9, Aroclor male; 10, Aroclor female. Lane 11 in (a) 40 ng purified P450IIB1; lane 11 in (b) 40 ng P450IA2; lane 12 40 ng P450IA1.

biphenyls and TCDD. For instance, to develop porphyria female rats require only a total of 16–45 μg/kg of TCDD (which is poorly metabolized) administered over many weeks [32, 33].

A number of laboratories have proposed microsome-mediated oxidative hypotheses unifying the inhibition of uroporphyrinogen decarboxylase and uroporphyria in humans and experimental systems. In one proposal cytochrome P450 (particularly P450IA2) would oxidize uroporphyrinogen to uroporphyrin with the possible formation of other products that might inhibit the decarboxylase [5, 17, 18, 36, 37]. However, simple oxidation of uroporphyrinogen to uroporphyrin cannot be the whole story, since the data in Table 2 illustrate that microsomes from female rats administered HCB or Aroclor at porphyrogenic levels did not oxidize uroporphyrinogen to a greater extent than males. In fact at 15 weeks when porphyria had developed, the oxidation of uroporphyrinogen by female microsomes was significantly less than by males. In addition, the production of oxidizing species by NADPH-stimulated female microsomes was not greater than that by male microsomes. Thus it seems unlikely that this is the reason for the pronounced sex-linked development of uroporphyria. Even so, the inhibitor of uroporphyrinogen decarboxylase detected in the livers of porphyric rats might be an oxidized uroporphyrinogen related to those porphyrins recently described [45, 46] which are formed in the presence of polyhalogenated aromatics under conditions not yet understood.

Despite the apparent lack of a propensity for induced female microsomes to oxidize uroporphyrinogen, ethoxyresorufin deethylase activity (the mono-oxygenase activity associated with cytochrome P450IA1) was always significantly greater in females after HCB or halogenated biphenyl treatment. Similar results were obtained for P450IA1 and P450IA2 by immunoblotting studies, and for HCB especially showed a more marked sex difference. In contrast, both mono-oxygenase activities and immunological studies indicated that induction of cytochrome P450IIB1 was greater in the male. Although this is consistent with the hypothesis that initiation of uroporphyria is somehow linked to the chronic induction of cytochromes P450IA1 and P450IA2, the sex difference in induction alone may not be sufficient

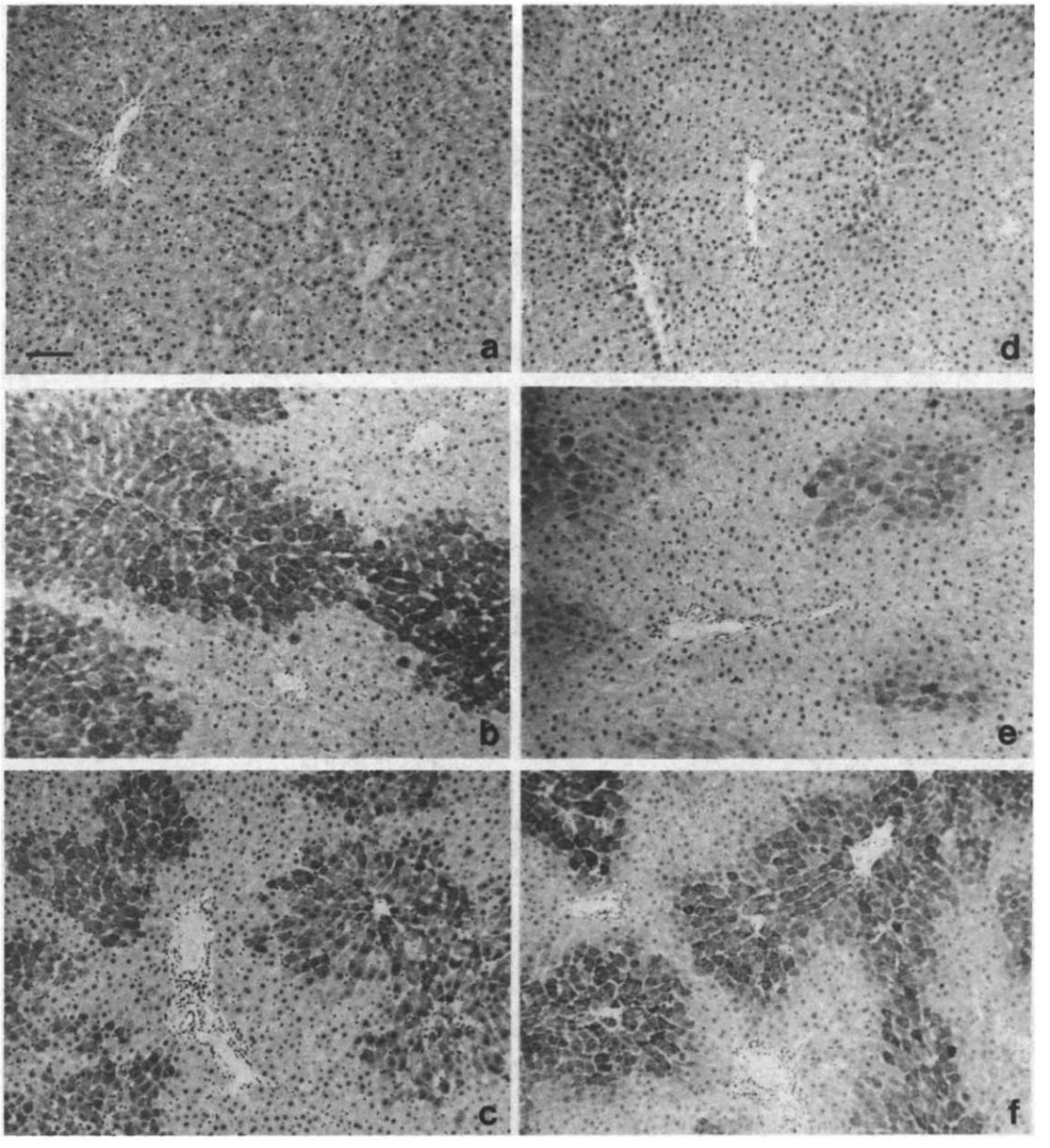

Fig. 2. Immunocytochemical localization of cytochrome P450 isoenzymes. Male and female rats were administered hexachlorobenzene for 30 weeks and then acetone-fixed liver sections were stained with polyclonal antibodies against P450IIB1 or P450IA1 (see Materials and Methods). (a) Control male; (b) HCB male; (c) HCB female—all stained with antibody to P450IIB1. (d) Control female; (e) HCB male; (f) HCB female—all stained with antibody to P450IA1. Scale bar = 100 μm.

to explain the marked susceptibility of females to HCB. However, it should be noted that the difference between centrilobular hepatocytes may be more marked than is apparent from estimates of whole liver. Whether there is a similar sex difference in cytochrome P450IA1 and P450IA2 induction with chronic administration of nonhalogenated aromatic chemicals which do not give porphyria in rats requires investigation. In addition, cytochrome P450IA2 is known to bind tightly polyhalogenated aromatics, such as TCDD and 3,3′,4,4′,5,5′-hexachlorobiphenyl, inhibiting its catalytic activity [47]. What influence this has, if any, on the porphyric process *in vivo* in female rats also requires investigation.

What other factor could contribute to the sex differences in porphyria development? There is considerable evidence implicating iron metabolism in the pathogenesis of both human and experimental uroporphyrias [2, 5, 19, 48–50]. The mechanism of iron action may involve catalysis of hydroxyl radical formation or perhaps the production of reactive iron-oxygen species [51] which react with uroporphyrinogen or some other susceptible target to form

an inhibitor of the decarboxylase [5]. Iron potentiates some forms of oxidative hepatic damage due to HCB in rodents [31, 52]. Fe-EDTA greatly enhanced the rate of oxidation of uroporphyrinogen by control and HCB-induced microsomes, although there was still no difference between the sexes (J. E. Francis and A. G. Smith, unpublished data). However, hepatic iron storage and turnover are significantly greater in the female rat than in the male [53], as are the levels of vitamin E and glutathione peroxidase [54]. HCB causes an accumulation of iron in the lysosomes of female rats [55]. In the present work induction of cytochromes P450IA1 and P450IA2 by HCB was mainly centrilobular (where the porphyria commences [56]) and was persistently greater in females. Perhaps these cytochrome P450 isoenzymes can be associated with a malfunction of iron metabolism which is enhanced by polyhalogenated aromatic chemicals [2, 5, 57]. Studies further to those so far conducted [57] are required to explore this possibility.

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