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INDUCTION OF CYTOCHROME P450 ACTIVITIES BY POLYCHLORINATED BIPHENYLS IN ISOLATED MOUSE HEPATOCYTES

INFLUENCE OF *Ah*-PHENOTYPE AND IRON

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Abstract—Exposure of cultured primary hepatocytes from *Ah*-responsive male C57BL/10ScSn mice to a polychlorinated biphenyl (PCB) mixture (Aroclor 1254) at 0.1–20 μg/mL for up to 96 hr induced cytochrome P4501AI-mediated activity (ethoxyresorufin *O*-deethylase, EROD) up to 50-fold. In contrast, pentoxyresorufin *O*-dealkylase (PROD), which in some circumstances is a measure of phenobarbitone-induced cytochrome P450 isoenzymes, was induced only 5-fold. There were similar findings on EROD activities with the pure compounds 3,3′,4,4′,5,5′-hexachlorobiphenyl, 3,3′,4,4′,5,5′-hexabromobiphenyl and 3,3′,4,4′-tetrachlorobiphenyl(TCB) and also β-naphthoflavone but not with 2,2′,4,4′-TCB or phenobarbitone. The higher concentrations of Aroclor 1254 were also associated with cytotoxicity as estimated by release of alanine aminotransferase (ALT) into the medium. Unlike in C57BL/10ScSn hepatocytes induction of EROD and cytotoxicity was minimal in hepatocytes from the *Ah*-non-responsive strain DBA/2. Although *in vivo* the hepatic toxicity and carcinogenicity of polyhalogenated aromatics are markedly potentiated by iron, no enhancement of the cytotoxicity of Aroclor 1254 towards C57BL/10ScSn hepatocytes by iron was observed *in vitro*. However, iron caused decreased EROD activities and possibly cytochrome P4501AI (as judged by Western blotting) as *in vivo*. Even in the presence of iron and the haem precursor 5-aminolaevulinic acid (5-ALA) there was no development of uroporphyria in this system although this occurs with Aroclor *in vivo* and is enhanced by iron. Accumulation of uroporphyrin did occur after extended culture of C57BL/10ScSn hepatocytes on matrigel for 8 days in the presence of 5-ALA and Aroclor 1254 but again no potentiation by iron was observed. Thus, although culture of *Ah*-responsive and -non-responsive hepatocytes mimics some aspects of the mechanisms of *in vivo* toxicity of PCBs, there is some unknown associated influence of iron metabolism which cannot, as yet, be produced *in vitro* but which is of importance *in vivo*.

Polychlorinated biphenyl (PCB†) and polybrominated biphenyl (PBB) mixtures, originally produced commercially, are widespread environmental contaminants [1]. In the case of the PCBs, considerable deposits also remain to be disposed of as a result of their industrial use [2]. Some of the concern about these and related polyhalogenated chemicals centres on the fact that they cause hepatocellular carcinoma in rodents by a mechanism which is not yet understood [3]. Metabolism does not seem to be of primary importance and there is evidence for the promotion of background initiation events by inhibition of intracellular communication or down-regulation of growth action [3–5]. However, we have demonstrated recently that in C57BL/10ScSn mice, but not in DBA/2 mice, preloading the liver with iron massively potentiates the hepatocarcinogenicity of PCBs and hexachlorobenzene [6, 7]. This suggests that an additional mechanism is involved. One possibility is that the hepatocarcinogenicity is linked to the ability of these chemicals to cause a block in haem biosynthesis at uroporphyrinogen decarboxylase (hepatic uroporphyria) which is also potentiated by iron [8]. At the initial biochemical level the action of PCBs is thought to depend mainly on their chronic interaction with the *Ah* receptor leading to changes in the expression of many genes including *Cyp*1a-1 and *Cyp*1a-2 [9, 10]. In *Ah*-responsive mouse strains, such as C57BL/10ScSn, the receptor has a high affinity for polychlorinated aromatic hydrocarbons, whereas the receptor in the *Ah*-non-responsive DBA/2 strain appears to have a much lower affinity, although other regulatory influences may also be involved.

As one approach to investigate in detail the mechanism of the iron-enhanced toxicity of PCBs in mice, it seemed desirable to develop an *in vitro* model with isolated hepatocytes. A wealth of studies has demonstrated that primary hepatocytes can be utilized to investigate the cellular activities involved in the metabolism and cytotoxicity of xenobiotics [11]. Although rat hepatocytes maintained as primary monolayer cultures are frequently used, considerably more background genetic information is available

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† Abbreviations: PCB, polychlorinated biphenyl; PBB, polybrominated biphenyl; TCB, tetrachlorobiphenyl; HCB, hexachlorobiphenyl; HBB, hexabromobiphenyl; HBSS, Hanks' balanced salt solution; EGTA, ethylene glycol-bis(β-aminoethyl)*N,N′*-tetracetic acid; NTA, nitrilotriacetate; Fe-NTA, ferric NTA; EROD, ethoxyresorufin *O*-deethylase; PROD, pentoxyresorufin *O*-dealkylase; ALT, alanine aminotransferase; TTBS, Tween–Tris-buffered saline β-NF, β-napthoflavone; 5-ALA, 5-aminolaevulinic acid.

on mice [12]. Despite this there have been only a few studies investigating the *Ah* response *in vitro* with primary mouse hepatocytes [13–16]. As a first step towards the *in vitro* model the present study demonstrates that induction of CYP1A-mediated activity occurs after PCB treatment of hepatocytes from *Ah*-responsive C57BL/10ScSn mice but to a much less degree in hepatocytes from the *Ah*-non-responsive DBA/2 strain when maintained in monolayer culture. Subsequently, since liver toxicity and carcinogenicity of PCBs *in vivo* appear to correlate with the induction of CYP1A isoenzymes and yet are modulated by iron status [7], we have examined the interaction between PCBs and iron. In particular, we have investigated the influence of iron loading on CYP1A1 activity and cytotoxicity, and the influence of the iron chelator desferrioxamine. Finally, we have investigated whether uroporphyria can be caused in C57BL/10ScSn hepatocytes and if so whether it can be influenced by iron loading as it occurs *in vivo*.

MATERIALS AND METHODS

Animals. Male C57BL/10ScSn and DBA/2 mice (20–25 g) were bred on site and used at 8–10 weeks. Mice were fed breeder diet 3 expanded (Special Diet Services, Witham, U.K.) and maintained at 21° with a 12-hr light/dark cycle. Where indicated, iron was given by a single intraperitoneal injection as an iron–dextran complex (Imferon®, Fisons Ltd, Loughbrough, U.K.) at 600 mg iron/kg 1 week prior to perfusion. Control mice received an equivalent volume of dextran C solution in water (200 mg/mL).

Materials. Aroclor 1254 was originally a gift to Dr J. B. Greig from Monsanto (IL, U.S.A.). Firemaster BP-6, 2,2′,4,4′-tetrachlorobiphenyl (TCB), 3,3′,4,4′-TCB, 3,3′,4,4′,5,5′-hexachlorobiphenyl (HCB) and 3,3′,4,4′,5,5′-hexabromobiphenyl (HBB) were supplied by British Greyhound (Birkenhead, U.K.). Williams' E medium, foetal calf serum and glutamine were obtained from Flow Laboratories (Irving, U.K.). Gentamicin was from Gibco (Life technologies, Paisley, U.K.) and collagenase was purchased from Boehringer Mannheim (GmbH, Germany). Desferrioxamine was obtained from Ciba-Geigy (Summit, NJ, U.S.A.). All other chemicals were obtained from the Sigma Chemical Co. (Poole, U.K.) except 7-ethoxyresorufin and 7-pentoxyresorufin which were prepared as described previously [17]. Plasticware was obtained from Falcon (Becton Dickinson U.K., Oxford, U.K.).

Hepatocyte isolation and culture. Hepatocytes were isolated from control and iron-dosed mice using a modification of the method of Klaunig *et al.* [18]. Perfusion with calcium and magnesium-free Hanks' balanced salt solution (HBSS), containing 0.5 mM ethylene glycol-bis-(β-aminoethyl) *N,N′*-tetraacetic acid (EGTA) buffered with 0.2% sodium bicarbonate (pH 7.4) maintained at 37°, was carried out for 3 min. The solution was allowed to run to waste by an excision made through the sub-hepatic vena cava. After excision from the animal the liver was placed on a platform above a recirculating reservoir and the liver perfused for a further 3 min with EGTA-free HBSS. A collagenase–$CaCl_2$ solution consisting of 0.025% collagenase (w/v) plus 2.25 mM $CaCl_2$ dissolved in HBSS was perfused for 10 min. A perfusion rate of 10 mL/min and a perfusion temperature of 37° were maintained throughout the procedure. After perfusion, the liver was transferred to a sterile petri dish, the gall bladder removed and the hepatocytes released by gentle agitation with a glass rod into a small volume of Williams' medium E containing foetal calf serum (5% v/v), insulin (1 μM), hydrocortisone (0.1 mM), and gentamicin (50 mg/L). The resulting suspension was filtered through a nylon bolting cloth (pore size 125 μM) and centrifuged at 60 *g* for 3 min at room temperature. The cell pellet was resuspended in fresh medium and the procedure repeated twice. Approximately 70×10^6 cells were obtained from each liver with viabilities of approximately 80% (as assessed by Trypan blue exclusion). Cells from iron-treated mice had consistently lower viabilities. Cells were dispersed in Williams' medium E and allowed to seed at a density of 1.5×10^6 cells/60-mm dish. Where indicated, cells were plated in 60-mm culture dishes previously coated with matrigel [19], a tumour biomatrix which was prepared from the Engelbroth Holm-Swarm mouse tumour as described by Bissell *et al.* [20]. All cultures were maintained at 37° at 5% CO_2:95% O_2 in a humidified incubator. Non-attached cells were discarded after a 4-hr incubation period and the medium was renewed. Chemicals were added with the daily medium change as indicated in the figure legends. Water-insoluble chemicals were added in dimethyl sulphoxide, (maximum 2 μL/mL culture medium). Ferric nitrilotriacetate (Fe-NTA) solution was prepared according to the method of White and Jacobs [21]. Essentially, 500 mg of nitrilotriacetate disodium salt were dissolved in 100 mL ultra clean sterile water, 200 mg ferric nitrate added and the pH was adjusted to 7 with sodium bicarbonate. The final concentration of the ferric iron was 4.95 mM and the molar ratio of nitrilotriacetate to ferric iron was 4:1. Fe-NTA was added to the cells at a final concentration of 20 μM. Nitrilotriacetate (NTA) was used as a control treatment and added to the cells at 200 μM final concentration. These solutions were sterilized by filtration on 0.22-μm filter membranes. At the times indicated medium was collected and stored at −70°, cells were washed twice in phosphate-buffered saline and scraped into 0.2% (v/v) Emulgen 911, 20% (v/v) glycerol, 0.1 M phosphate buffer, pH 7.4 and also stored at −70°. When required, cell samples were thawed and sonicated for 12 sec using a Soniprobe (Dawe Instruments, U.K.).

Analyses. Fluorimetric determinations of ethoxyresorufin *O*-deethylase (EROD) and pentoxyresorufin *O*-depentylase (PROD) activities were performed using sonicated cells according to the method of Lubet *et al.* [22] with substrate concentrations of 2 μM ethoxyresorufin and 5 μM pentoxyresorufin, and cytochrome P450 was measured according to the method of Omura and Sato [23]. Protein was determined by the method of Lowry *et al.* [26] using bovine serum albumin as a standard. Alanine aminotransferase (ALT) activity in the medium was assayed using a kit from Sigma (Catalogue number 59–10).

Porphyrins were extracted from combined cells and medium by addition of 10% (v/v) 6 N HCl, the precipitated protein being removed by centrifugation. The porphyrin content of these samples was determined by fluorescence spectrometry on a Perkin–Elmer LS50 Luminescence Spectrometer and the results expressed in terms of total porphyrin content [25]. In the case of hepatocytes cultured on matrigel, porphyrins were extracted in the same manner. After centrifugation the supernatant fraction was injected on a reverse phase ODS 18 (Shandon, U.K.) HPLC column and the porphyrins separated as described [26]. Porphyrin data are presented as picomoles per dish since matrigel contributed considerable amounts of protein relative to cellular protein.

Iron content was quantified by an adaptation of the method of Carter [27]. Approximately 1–2 $\times 10^5$ cells (0.6 mL) were reduced with 0.6 mL ascorbic acid (20% in 0.2 N HCl) and allowed to stand at room temperature for 5 min. Protein was precipitated using 0.6 mL of trichloroacetic acid (11.3%), and the tubes were thoroughly mixed and capped. Samples were then heated to 90° for 10 min and centrifuged for 5 min to obtain a clear pellet. The supernatant fraction (1.5 mL) was removed to which was added 0.6 mL buffer solution (10% ammonium acetate) and 0.15 mL ferrozine reagent (75 mg ferrozine, 75 mg neocuproine/25 mL H_2O containing a drop of concentrated HCl). The samples were mixed well. After 5 min the absorbance of the magenta-coloured complex was measured at 562 nm against a water blank and a standard iron solution.

Gel electrophoresis and immunoblotting. Analysis of microsomal proteins was as described by Sinclair *et al.* [15] After centrifuging sonicated cells at 100,000 *g* for 45 min to obtain a total membrane fraction the pellet was homogenized in Emulgen buffer. Insoluble material was removed by centrifugation at 10,000 *g* for 8 min. Separation of microsomal proteins was carried out on a LKB 2050-001 Midget Electrophoresis Unit using the SDS–PAGE discontinuous system of Laemmli [28], with 4.8% stacking gel and 7.5% resolving gel, adding 10–15 μg microsomal protein/well. After separation, the resolved proteins were electrophoretically transferred to nitrocellulose according to the method of Towbin *et al.* [29]. A current setting of 100 mA was applied for 75 min at 4°. The western blots were washed three times for 5 min each in Tween–Tris-buffered saline (TTBS: 0.05% (v/v) Tween-20, 150 mM NaCl, 50 mM Tris–HCl, pH 8.5). To block non-specific binding, the blots were immersed overnight at room temperature in TTBS containing 3% (w/v) dried milk powder and 0.1% (w/v) NaN_3. After three 5-min washes in TTBS the blots were incubated for 2 hr at room temperature in TTBS containing 1% (w/v) gelatin and a 1:1000 dilution of the primary antibody directed to CYP1A1 (a gift from Dr C. R. Wolf which appears relatively specific for 1A1 [30, 31]). Blots were then washed three times in TTBS before incubating with a 1:3000 dilution of goat-anti-rabbit IgG-alkaline phosphate conjugate for 2 hr at room temperature. After washing in TTBS immunoreactive bands were revealed by incubating the blots for 5–10 min in a fresh solution of nitro blue tetrazolium (0.4 mM) and 5-bromo-4-chloro-3-indolyl phosphate (0.4 mM) in Tris-HCl (100 mM) pH 7.4 containing NaCl (100 mM) and $MgCl_2$ (5 mM). The reaction was stopped with PBS containing EDTA (20 mM).

RESULTS

Comparison of induction of cytochrome P450 activities in C57BL/10ScSn and DBA/2 hepatocytes by Aroclor 1254

The PCB mixture, Aroclor 1254, induced EROD activity, mediated mainly by CYP1A1 [30], in both C57BL/10ScSn and DBA/2 mouse hepatocytes in a concentration-dependent manner (Fig. 1a, b). However, inductions of EROD activity in DBA/2 cultures were markedly lower than those in C57BL/10ScSn hepatocytes. Maximal induction in C57BL/10ScSn hepatocytes was observed at 20 μg Aroclor/mL; at higher concentrations, Aroclor proved toxic to cells judged by their morphology, i.e. blebbing and detachment from the dish. Constitutive levels of EROD activity also appeared higher in C57BL/10ScSn cultures than in cells isolated from DBA/2 mice and remained so throughout the culture period, although for both strains activity declined with incubation time. In C57BL/10ScSn hepatocytes, EROD activity was induced 5-, 21-, 32-, 35- and 52-fold at 0.1, 1, 5, 10 and 20 μg Aroclor/mL, respectively, after 72 hr. In contrast, although PROD activity which is particularly mediated by CYP2B1 [30] was also increased in this strain, induction was only 1.2-, 1.3-, 1.7-, 3.3- and 5.5-fold at the same concentration levels and with the same incubation time (Fig. 1c). Interestingly, there was no significant induction of PROD activity after exposure of DBA/2 cells to Aroclor at any of the treatment levels (Fig. 1d). Total cytochrome P450 content decreased by 70% in untreated cultures from 0.75 to 0.17 nmol/mg protein during 24 hr of culture compared with that measured in freshly isolated C57BL/10ScSn hepatocytes. However, as the incubation period progressed the decrease in activity became very much slower, declining by approximately 30% to 0.12 nmol/mg protein in the last 72 hr of culture.

ALT leakage into the medium was measured to assess cytotoxic effects of Aroclor added to the hepatocytes. *In vivo* this has proved to be a serum marker of liver damage caused by polyhalogenated chemicals after iron overload [32]. Initial measurements were taken 24 hr after plating cells and indicated that ALT activity in the medium was high with all treatments and both mouse strains. This dropped universally to approximately 13 U/L at 48 hr and remained so at 72 hr, but then increased markedly in a dose-dependent manner at 96 hr with the C57BL/10ScSn hepatocytes (Fig. 2a). In contrast, in primary cultures of DBA/2 hepatocytes, which displayed weak induction of EROD activity at all concentrations, the cytotoxicity of Aroclor appeared to be minimal although displaying similar profiles between 24 and 48 hr (Fig. 2b). Hence, there appears to be a partial correlation between the induction of EROD activity by Aroclor and its cytotoxic effects, e.g. blebbing in the *Ah*-responsive strain especially at high concentrations.

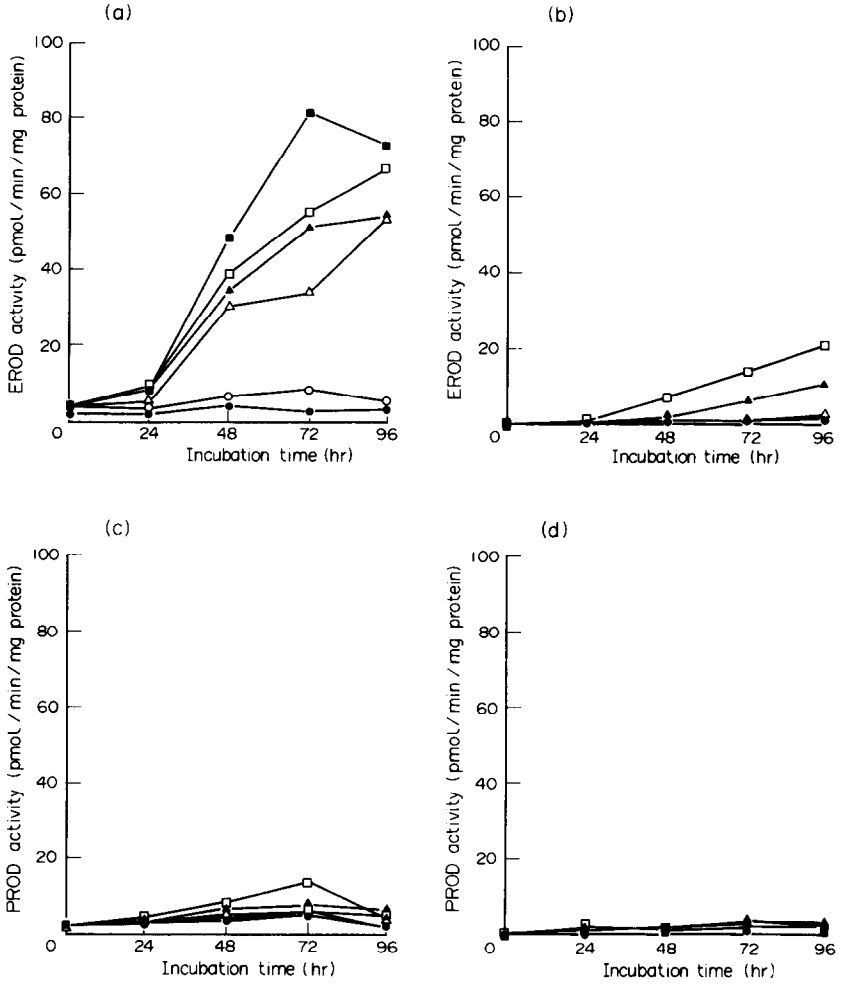

Fig. 1. Influence of Aroclor 1254 on EROD and PROD activities in isolated C57BL/10ScSn and DBA/2 hepatocytes. Cells were incubated for up to 96 hr with Aroclor 1254 at 0 (●), 0.1 (○), 1 (△), 5 (▲) 10 (□) and 20 (■) μg/mL of medium. EROD activity in (a) C57BL/10ScSn and (b) DBA/2 hepatocytes. PROD activity in (c) C57BL/10ScSn and (d) DBA/2 hepatocytes. Hepatocyte activities were estimated as described in Materials and Methods. Data points are the means from experiments with four different mice. SEMs are omitted for the sake of clarity but were usually less than ±20% of the mean.

For further studies it was decided to incubate all cultures for 72 hr with an Aroclor concentration of 10 μg/mL since at this time and concentration cells appeared healthy whilst still maintaining near peak levels of EROD activity without significantly high levels of ALT leakage.

Induction of P450 activities by individual halogenated biphenyls in C57BL/10ScSn hepatocytes

Table 1 summarizes the potency of individual halogenated biphenyls to induce EROD and PROD activities in hepatocytes isolated from C57BL/10ScSn mice 72 hr after incubation. Significant induction of EROD activity was observed by all except 2,4,2',4'-TCB, a known phenobarbitone-type inducer *in vivo* [33]. However, most induced activities were lower than that produced by the PCB and PBB mixtures Aroclor and Firemaster BP-6, respectively. The overall order of potency for the congeners studied was 3,3',4,4',5,5'-HBB > 3,3',4,4',5,5'-HCB > 3,3',4,4'-TCB > 2,2',4,4'-TCB. When cells were treated with β-naphthoflavone (β-NF), a non-halogenated inducer of CYP1A1 *in vivo* [34], EROD activity was markedly increased with no significant induction of PROD activity. PROD activity was not induced to a significant extent by any of the purified biphenyls examined or even phenobarbitone despite its being a good inducer of CYP2B1 activity *in vivo* [30]. It is thus possible that the induction of PROD observed with Aroclor was due to low activity with CYP1A1 or some other isoenzyme rather than CYP2B1. There was no change in cytotoxic damage at 72 hr as assessed by ALT leakage into the medium by any of the halogenated biphenyls examined.

Effect of iron on EROD induction by Aroclor 1254

The effect of iron was investigated by injecting

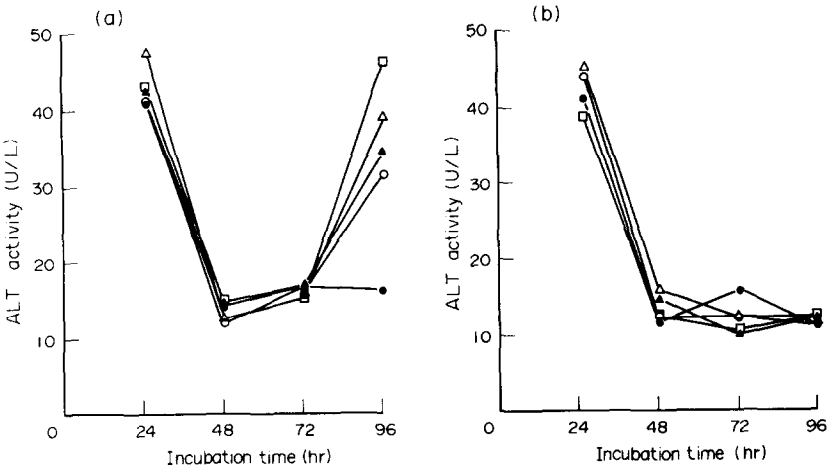

Fig. 2. Changes in ALT activity in the medium in which (a) C57BL/10ScSn and (b) DBA/2 hepatocytes were cultured in the presence of Aroclor 1254 as described in Fig. 1.

Table 1. Induction by halogenated biphenyls or EROD and PROD activities in hepatocytes isolated from C57BL/10ScSn mice

Indecer	EROD (pmol/min/mg protein)	PROD (pmol/min/mg protein)	ALT (U/L)
Control	1.62 ± 0.17	1.07 ± 0.12	20.0 ± 3.9
Aroclor 1254	29.89 ± 3.18*	2.51 ± 0.69	19.5 ± 1.4
Firemaster	27.60 ± 3.35*	2.22 ± 0.23*	17.7 ± 0.0
Phenobarbitone	1.06 ± 0.52	1.54 ± 0.58	20.6 ± 2.9
β-NF	6.44 ± 0.44*	1.30 ± 0.35	21.2 ± 2.0
2,2',4,4'-TCB	0.76 ± 0.23	0.76 ± 0.23	20.0 ± 0.2
3,3',4,4'-TCB	9.74 ± 1.73*	1.29 ± 0.17	36.7 ± 8.1
3,3',4,4',5,5'-HCB	19.44 ± 1.10*	2.26 ± 0.58	36.7 ± 8.1
3,3',4,4',5,5'-HBB	29.84 ± 3.46*	1.96 ± 0.40	18.9 ± 4.0

Hepatocytes were cultured for 72 hr as described in Materials and Methods. Chemicals were added at the following concentrations: Aroclor 1254, 10 μg/mL; Firemaster BP-6, 10 μg/mL; phenobarbitone, 2 mM; β-naphthoflavone, 2 μM; 2,2',4,4'-TCB, 3,3',4,4'-TCB, 3,3',4,4',5,5'-HCB and 3,3',4,4',5,5'-HBB, 10 μg/mL.

Values are means ± SEM for hepatocytes from three mice.

* Significantly different from control, $P < 0.05$ (Student's paired t-test).

mice with an iron–dextran complex 1 week prior to hepatocyte isolation. In freshly isolated iron-loaded hepatocytes (35.3 ± 3.5 μg iron/10^6 cells) there was significantly lower cytochrome P450 and EROD activity ($P < 0.05$) compared with their control counterparts (1.4 ± 0.3 μg iron/10^6 cells) although there was no apparent difference in PROD activity (Table 2). Similarly, 72 hr after isolation there was a significant reduction in EROD activity but no reduction in PROD activity compared with controls. However, in cultures treated with Aroclor there was essentially no difference in EROD between iron-loaded and control hepatocytes (Table 2).

A comparison was made between the hepatocytes loaded with iron *in vivo* and those in which iron was added to cells *in vitro* as Fe-NTA (20 μM). This method resulted in a gross iron content (29.6 ± 1.5 μg iron/10^6 cells) similar to that observed with hepatocytes from mice given iron *in vivo*. Again iron caused a significant depression of EROD activity relative to dimethyl sulphoxide-treated controls (data not shown). In the presence of Aroclor both EROD and PROD were again reduced but this also occurred with NTA. Because of the difficulty in distinguishing Fe-NTA from NTA effects due to the acquisition of endogenous iron, studies along this line were not pursued.

Effect of desferrioxamine on EROD and PROD induction

The iron chelator desferrioxamine was added to primary cultures of C57BL/10ScSn hepatocytes after isolation and added with the daily medium change thereafter. Its presence caused total P450 levels (not shown) and EROD and PROD activities to fall to undetectable levels (Table 3). However, in iron-

Table 2. Effects of *in vivo* iron overload on EROD and PROD activities in C57BL/10ScSn hepatocytes treated with Aroclor 1254

Treatment		Culture time	EROD	PROD
In vivo	*In vitro*	(hr)	(pmol/min/mg protein)	(pmol/min/mg protein)
—	—	0	4.54 ± 0.17	1.68 ± 0.85
Iron	—	0	1.67 ± 0.20*	1.22 ± 0.12
—	—	72	1.62 ± 0.17	1.07 ± 0.12
Iron	—	72	0.60 ± 0.34*	1.80 ± 0.26
—	Aroclor	72	29.72 ± 2.71	6.03 ± 0.96
Iron	Aroclor	72	25.35 ± 4.70	4.73 ± 0.62*

Mice received iron–dextran by i.p. injection (600 mg Fe/kg) or an equivalent volume of dextran solution (200 mg/mL) 1 week prior to perfusion. Isolated hepatocytes were cultured for 72 hr as described in Materials and Methods. Aroclor 1254 was added to the cells at a final concentration of 10 μg/mL.

Values are means ± SEM for hepatocytes from 3 to 4 mice.

* Significantly different from corresponding hepatocytes not loaded with iron, $P < 0.05$ (Student's paired *t*-test).

Table 3. Effects of desferrioxamine on EROD and PROD activities in iron-loaded C57BL/10ScSn hepatocytes treated with Aroclor 1254

Treatment		EROD	PROD
In vivo	*In vitro*	(pmol/min/mg protein)	(pmol/min/mg protein)
—	—	1.48 ± 0.09	2.47 ± 0.87
—	Desferrioxamine	ND	ND
Iron	—	1.16 ± 0.31	1.42 ± 0.44
Iron	Desferrioxamine	1.42 ± 0.95	2.12 ± 0.16
—	Aroclor	29.72 ± 2.71	5.90 ± 0.61
—	Aroclor Desferrioxamine	ND	ND
Iron	Aroclor	25.35 ± 4.70	4.73 ± 0.62
Iron	Aroclor Desferrioxamine	2.67 ± 1.34	1.03 ± 0.62

Mice received dextran solution or iron–dextran (600 mg Fe/kg) 1 week prior to isolation of hepatocytes. Isolated hepatocytes were cultured for 72 hr as described in Materials and Methods. Cells were treated with 10 μg/mL Aroclor 1254 and 10 mM desferrioxamine.

Values are means ± SEM for hepatocytes from 3 to 4 mice.

ND, not detectable.

loaded hepatocytes, there proved to be no significant reduction in these activities after treatment with desferrioxamine. From these results and those shown in Table 2 it appears that iron overload causes a loss of EROD activity over 72 hr but this depletion does not occur in the presence of desferrioxamine. When Aroclor and desferrioxamine were simultaneously added to control cultures, EROD and PROD activities again fell to undetectable levels. However, these activities were detectable in iron-loaded hepatocytes treated with Aroclor and desferrioxamine although the magnitudes of induction were only marginally higher than those observed in iron-loaded cultures treated with desferrioxamine only.

Immunoblotting for CYP1A1

To confirm the induction of CYP1A1, control and iron-loaded hepatocytes were incubated with Aroclor 1254, β-naphthoflavone or phenobarbitone and the microsomes examined by immunoblotting with a polyclonal antibody to CYP1A1. This antibody revealed a dominant CYP1A1 band in those hepatocytes treated with Aroclor or β-NF, its expression being greater in the former (Fig. 3). A similar pattern was observed in hepatocytes pretreated with iron except that levels of this isoenzyme were noticeably diminished. This band was not detected in control hepatocytes or those treated with phenobarbitone. Hence, the overall order of CYP1A1 content as detected by immunoblotting was Aroclor > Aroclor/Fe; β-NF > β-NF/Fe. Induction of CYP1A2 was very low; this isoenzyme was weakly detected only in those hepatocytes, with or without iron, treated with Aroclor (results not shown). Although a CYP1A1

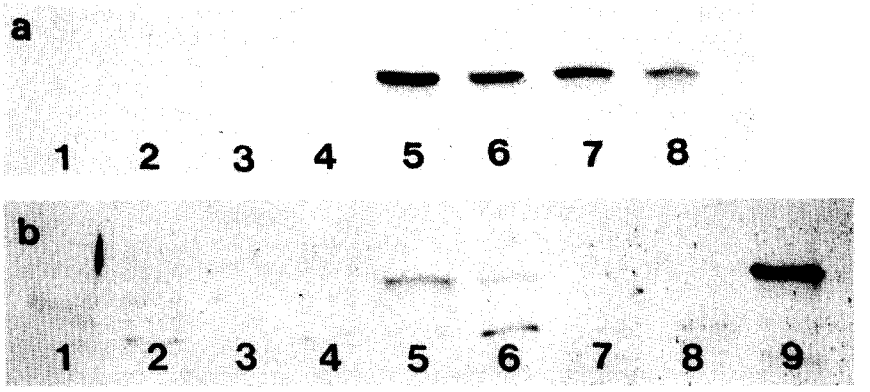

Fig. 3. Immunoblot of microsomal proteins from hepatocytes isolated from (a) C57BL/10ScSn and (b) DBA/2 mice. Hepatocytes were isolated from control mice or mice preloaded with iron. Microsomes were probed with an antibody against CYP1A1. Lane 1, control/phenobarbitone; 2, iron/phenobarbitone; 3, control/control; 4, iron/control; 5 control/Aroclor; 6, iron/Aroclor; 7, control/β-NF; 8, iron/β-NF; 9, 2 pmol rat CYP1A1 standard. Blots from DBA/2 microsomes were incubated for alkaline phosphatase colour development for longer than those from C57BL/10ScSn microsomes to maximize sensitivity.

Table 4. Porphyrin accumulation after 72 hr in C57BL/10ScSn hepatocytes treated with Aroclor 1254 and 5-ALA

Treatment		Porphyrin levels (nmol/dish)
In vivo	*In vitro*	
—	—	0.7 ± 0.1
Iron	—	0.2 ± 0.0*
—	Aroclor	1.8 ± 0.1
Iron	Aroclor	0.6 ± 0.1*
—	5-ALA	1.8 ± 0.2
Iron	5-ALA	1.6 ± 0.2
—	Aroclor/5-ALA	2.0 ± 0.1
Iron	Aroclor/5-ALA	0.9 ± 0.1*

Mice received dextran solution or iron–dextran (600 mg Fe/kg) 1 week prior to isolation of hepatocytes. Aroclor 1254 added to cultures at a final concentration of 10 μg/mL and 5-ALA at 25 μg/mL. Cells and medium were harvested at 72 hr, the samples sonicated and porphyrins extracted and their accumulation determined by fluorimetry as described in Materials and Methods.

Values are means ± SEM for hepatocytes from three mice.

* Significantly different from corresponding hepatocytes not treated with iron *in vivo*.

induction was observed in Aroclor 1254-treated hepatocytes from DBA/2 mice it was to a much lower degree than seen in C57BL/10ScSn hepatocytes (Fig. 3).

Accumulation of porphyrins in cultures treated with Aroclor 1254 and 5-aminolaevulinic acid (5-ALA)

The accumulation of porphyrins at 72 hr in this culture system was investigated by the addition of the haem precursor 5-ALA, a known enhancer of the porphyric process in mice *in vivo* [35], to hepatocytes during culture. Porphyrin accumulation, as detected by fluorimetry, occurred only to a small extent in both control and iron-loaded hepatocytes but was consistently lower in the latter (Table 4). A more detailed examination by HPLC (results not shown) revealed that this difference was due to variation in coproporphyrin not uroporphyrin accumulation; the iron-loaded hepatocytes accumulating consistently less coproporphyrin than their control counterparts. Neither Aroclor nor 5-ALA, either separately or in combination, had any marked enhancing influence on porphyrin accumulation. Hence, the *in vivo* response [7, 8] could not be reproduced in this *in vitro* system.

In a recent study, Sinclair *et al.* [15] have reported that uroporphyria can be produced by 3,3′,4,4′,5,5′-HCB and 5-ALA *in vivo* with mouse hepatocytes by the use of matrigel, an extracellular matrix isolated from a mouse tumour, which enables a prolonged culture period to be attained and other cytochrome P450 isoenzymes to be expressed. This system was utilized here to investigate uroporphyrin accumulation in the presence of Aroclor and 5-ALA with control and iron-loaded C57BL/10ScSn hepatocytes. Table 5 shows their effect on the accumulation of uroporphyrin and heptacarboxyporphyrin after 8 days culture on matrigel. Uroporphyrin and heptacarboxyporphyrin levels were significantly higher than those detected at 72 hr in previous experiments; thus, the use of matrigel did indeed allow the accumulation of porphyrins. This increase in porphyrin content could be detected to a lesser extent at 5 days (data not shown). Treatment with Aroclor or 5-ALA alone yielded small and again equivalent increases in uroporphyrin. However, in none of the studies was there any potentiation by previous iron loading. Coproporphyrin was detected in all treatments but levels were not significantly higher than those detected at 72 hr.

DISCUSSION

The synergism between iron and PCBs to produce hepatic toxicity and also liver cancer in *Ah*-responsive

Table 5. Porphyrin accumulation by hepatocytes obtained from control or iron-treated mice and cultured on matrigel for 8 days in the presence of 5-ALA and Aroclor 1254

In vitro treatment	Iron treatment	Uroporphyrin accumulation* (nmol/dish)	Coproporphyrin accumulation (nmol/dish)
Control	−	ND	ND
	+	ND	ND
5-ALA	−	ND	0.41 ± 0.05
	+	0.72 ± 0.23	0.81 ± 0.31
Aroclor	−	0.40 ± 0.22	0.14 ± 0.03
	+	0.23 ± 0.23	0.53 ± 0.09
5-ALA + Aroclor	−	6.46 ± 2.7	0.69 ± 0.49
	+	8.02 ± 2.4†‡	0.64 ± 0.05

C57BL/10ScSn mice received dextran or iron–dextran (600 mg Fe/kg) as described in Materials and Methods 1 week prior to isolation of hepatocytes following perfusion of the liver.

* Includes heptacarboxylic porphyrin.

† Significantly different from iron/Aroclor group.

‡ Not significantly different from 5-ALA/Aroclor group.

Porphyrins were estimated by HPLC.

ND, Not detected.

C57BL/10ScSn mice but not in *Ah*-non-responsive DBA/2 mice is a novel system in chemically induced carcinogenesis [6, 7]. The mechanism of this synergistic interaction is not clear but one possibility is that it is related to the development of uroporphyria [36]. This may involve an iron-catalysed oxidative pathway and there is much circumstantial evidence suggesting a role in this process for CYP1A1 isoenzymes [8].

Although there are many investigations which could be carried out into the mechanism and progression of this carcinogenesis system *in vivo*, particular types of mechanistic studies require the development of appropriate *in vitro* systems. As a first step to this goal, cultured isolated primary hepatocytes from C57BL/10ScSn mice but not those from DBA/2 mice have been shown here to be inducible for CYP1A1-mediated activity by both pure and mixtures of PCBs. At the higher concentrations of PCBs, and apparently subsequent to induction of CYP1A1, there was marked cytotoxicity which again did not occur with DBA/2 hepatocytes. Thus, the results demonstrate that as far as the *in vitro* cytotoxicity of PCBs is concerned there is a correlation with the *Ah*-phenotype and associated induction of CYP1A1. Whether changes in gene expression commonly associated with liver carcinogenesis can also be found remains to be determined. However, despite causing cytotoxicity the PCB mixture Aroclor 1254, even at the highest concentration, did not cause the accumulation of uroporphyrin that is observed *in vivo* after chronic treatment. This may suggest that prolonged exposure is required which is not possible using the culture system for 3–4 days employed in the majority of this work. Recently, porphyrin accumulation by mammalian hepatocytes (from *Ah*-responsive C57BL/6J mice) has been carried out by culturing them on matrigel, a membrane preparation isolated from a mouse tumour, for up to 11 days [15]. In the present work, we were able to confirm these findings after a culture period of 8 days. Clearly, the use of matrigel has potential for future investigations into this problem if the *in vivo* influence of iron can be reproduced *in vitro* (see below).

In vivo, iron overload potentiates markedly the hepatotoxic and carcinogenic properties of Aroclor 1254 in mice [6, 7]. With isolated hepatocytes no such enhancement of either cytotoxicity or the accumulation of porphyrins, even on matrigel, occurred with cells from iron-loaded liver. Therefore, with respect to iron, cultured hepatocyte systems did not reproduce *in vivo* findings.

Although the mechanism of uroporphyria caused by polyhalogenated aromatic chemicals such as PCBs is thought to depend on the activity of CYP1A isoenzymes [8, 15, 36] in conjunction with iron, it seems a conundrum that iron overload depresses the levels of cytochrome P450 and some associated monooxygenase levels induced by PCBs in these mice *in vivo* (A. G. Smith, unpublished data). Similar findings occurred *in vitro* in the studies reported here. Depression of cytochrome P450 by iron overload is well known and usually assumed to be non-selective due to a number of events including lipid peroxidation of the microsomal system [37, 38]. However, the details of how this occurs or whether it is the only explanation have not been fully investigated. Indeed, it might be argued that the loss of cytochrome P450 in the presence of iron overload does not appear compatible with the proposed role of *Cyp*1a-1 and its protein product in the toxicity and carcinogenicity of PCBs and the potentiation by iron. One explanation might be that it is the inactivation of cytochrome P450 in the presence of iron which produces toxic and porphyrinogenic species *in vivo* but not as yet *in vitro*.

It is also evident from the results that although iron overload caused a decline in cytochrome P450-catalysed activities in primary culture this loss was

not seen in the presence of the iron chelator desferrioxamine. This observation may indicate a dual role for desferrioxamine. Firstly, it may act by chelating excess free iron that may otherwise have become involved in the autoxidation of cell membranes and associated enzymes. Secondly, since desferrioxamine binds only free iron and perhaps some loosely bound by proteins (not that which is in haem), its depletion of constitutive levels of EROD and PROD can only be due to chelation of iron in the medium or cells which would otherwise have been channelled into P450 synthesis and other cellular processes [39, 40]. This implies that there is a constant (if declining) *de novo* synthesis of cytochrome P450 occurring in isolated hepatocytes up to 72 hr after isolation. Presumably chelation is incomplete under conditions of excess iron and it could therefore be postulated that free unbound iron is rapidly channelled into P450 synthetic pathways; hence, one does not observe the loss in P450-catalysed activities.

In summary, *in vitro* systems with cultured mouse hepatocytes have been developed which are applicable to the investigation into the mechanisms of the toxicity and carcinogenicity of polyhalogenated aromatic chemicals *in vivo*. However, in one important aspect the cultured hepatocytes did not reflect *in vivo* findings since they showed only a partial response to iron overload. Further investigations are in progress to determine why iron did not enhance cytotoxicity and the induction of uroporphyria *in vitro* yet caused depletion of cytochrome P450 as is observed *in vivo*. These studies should aid our understanding of the *in vivo* processes.

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