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Uroporphyria and hepatic carcinogenesis induced by polychlorinated biphenyls–iron interaction: Absence in the *Cyp1a2(−/−)* knockout mouse

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Abstract

Aryl hydrocarbon receptor ligands, such as polychlorinated biphenyls (PCBs), cause inhibition of the heme biosynthesis enzyme, uroporphyrinogen decarboxylase; this leads to uroporphyria and hepatic tumors, which are markedly enhanced by iron overload in C57BL/10 and C57BL/6 strains of mice. *Cyp1a2(−/−)* knockout mice were used to compare the effects of CYP1A2 expression on uroporphyria and liver carcinogenesis. PCBs in the diet (100 ppm) of *Cyp1a2(+/+)* wild-type mice caused hepatic uroporphyria, which was strongly increased by iron–dextran (800 mg Fe/kg). In contrast, uroporphyria was not detected in *Cyp1a2(−/−)* knockout mice, although expression of CYP1A1 and CYP2B10 was greatly induced. After 57 weeks on this diet, hepatic preneoplastic foci and tumors were seen in the *Cyp1a2(+/+)* mice; numbers and severity were enhanced by iron. No foci or tumors were detected in *Cyp1a2(−/−)* mice, although evidence for other forms of liver injury was observed. Our findings suggest a link not only between CYP1A2, iron metabolism, and the induction of uroporphyria by PCBs, but also with subsequent hepatocarcinogenesis.

Keywords: *Cyp1a2(−/−)* mice; PCBs; Iron; Porphyria; Carcinogenesis

The risk of cancer is known to be increased by environmental chlorinated ligands of the aromatic hydrocarbon receptor (AHR), such as polychlorinated biphenyls (PCBs) and dioxin. This knowledge is based on limited human occupational evidence of these chemicals acting as nonspecific multi-site carcinogens, tumors induced experimentally in rodents, and extrapolations based on mechanistic knowledge of AHR as a transcription factor [1]. The rodent tumors, frequently of the liver, are types that have not normally been associated with human exposure to PCBs and dioxins [1,2]. In general, chemicals like PCBs are not strongly mutagenic, nor do they react with DNA at significant levels [3–5], and studies have been devoted to understanding the properties of PCBs in promoting spontaneously initiated cells in the liver [6]. The promoting potential of PCBs falls short, however, of explaining their ability to be complete hepatocarcinogens.

Another common feature of chlorinated AHR ligands is their ability to cause dysfunction of hepatic heme synthesis at the uroporphyrinogen decarboxylase step, with massive accumulation of oxidised heme precursors (uroporphyrins) leading to a porphyria (in this case uroporphyria). The mechanism is unknown, but there is strong evidence that implicates aspects of oxidative stress and synergistic involvement of iron metabolism [7]. In mice, prior administration of iron enhances both uroporphyria and liver carcinogenesis induced by

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PCBs and by another uroporphyria-inducing agent, hexachlorobenzene [7–9]. It is plausible that there is a specific link between hepatic porphyria and the process of carcinogenesis. In support of this proposition, *Fech(m1/Pas)* mice—that develop hepatic protoporphyria as a consequence of a homozygous mutation in the gene for ferrochelatase, which inserts iron into protoporphyrin IX at the last step in heme synthesis—develop advanced hepatic tumors [10].

Considerable evidence demonstrates that, in the C57BL/6J mouse, CYP1A2 can play a role in the process leading to uroporphyria—but a role that does not involve simple oxidation of exogenous chemicals [11]. A line of C57BL/6J mice having a disruption in the *Cyp1a2* gene that has been used to explore toxic and carcinogenic processes [12–15] showed no induction of porphyria following administration of the potent and weak AHR ligands, dioxin and hexachlorobenzene, respectively, even with the additional stimulation of iron overload [16–18]. We hypothesised that, if the mechanisms of porphyria and hepatic tumor development were linked, PCB-treated C57BL/6J *Cyp1a2(−/−)* mice would be resistant not only to the development of porphyria but also to liver neoplasia; furthermore, we postulated that these mice would remain resistant—even when given an iron overload.

Materials and methods

Mice and treatment. Generation of the *Cyp1a2(−/−)* knockout mouse line, originally from a mixture of the C57BL/6J and 129/SvJ inbred strains, has been described [12]. The *Cyp1a2(−/−)* genotype was subsequently backcrossed into the C57BL/6J strain to a theoretical level of >99.8% and would be expected to have fewer than 90 genes of 129/SvJ origin; thus, C57BL/6JOla mice (Harlan, Bicester, UK) were used as the *Cyp1a2(+/+)* controls. Male *Cyp1a2(+/+)* and *Cyp1a2(−/−)* mice (6–8 weeks old) were administered the PCB mixture Aroclor 1254 (Monsanto, St. Louis, USA) in RM1 diet (0.01%, SDS, Witham, UK), with or without a prior dose of iron–dextran (800 mg Fe/kg, Sigma, Poole, UK) for 7 weeks, or up to 57 weeks, under Home Office licence 80/1329. The latter period was chosen in comparison with a study on the effects in C57BL/6 heterozygous null p53 [*Trp53(+/−)*] mice in which there was no influence of the loss of a *Trp53* allele on hepatic tumor incidence (unpublished data). Mice were housed in negative pressure isolators at 21 ± 1 °C with a 12 h–12 h light–dark cycle. Mice were culled by cervical dislocation. Livers from mice after 7 weeks were frozen in liquid nitrogen to await analyses. Total liver from the 57-week study was fixed in neutral buffered formalin and embedded in paraffin wax. Standard histologic sections 5 μm thick were prepared and stained with hematoxylin and eosin. Iron content was determined by Perl's stain.

Western immunoblot analysis. SDS electrophoresis and Western blotting of microsomal proteins were performed as reported previously [19] using chemiluminescence detection (Amersham Biosciences, Amersham, UK) and primary antibodies from the following sources: CYP1A1/1A2 detection with a goat antibody against the rat enzymes (Gentest, Woburn, MA); CYP2B10 detection with rabbit anti-human CYP2B6 (Chemicon, Temecula, CA). Rat microsomes expressing human CYP2B6 (74.4% amino-acid similarity with mouse CYP2B10) gave a single band, but with a slightly lower molecular weight. Quantitation was achieved by densitometry and based on the density of blot bands, relative to corresponding regions in control samples.

Porphyrin assay. Uroporphyrin levels in tissues were estimated using spectrofluorometry and expressed as nmol of uroporphyrin/g of liver [20]. Significance was assessed by one-way analysis of variance.

Results and discussion

Importance of CYP1A2 expression in uroporphyria

We confirmed by Western immunoblotting that the CYP1A2 protein was detectable in *Cyp1a2(+/+)* wild-type mice (C57BL/6) at constitutive levels, but could not be detected in *Cyp1a2(−/−)* mice—even after prolonged exposure of Western blots to the detection system (Fig. 1A). Mice of both genotypes received a subcutaneous dose of iron and were administered the PCB mixture in the diet. After 7 weeks, all PCB-treated *Cyp1a2(+/+)* mice showed marked induction of hepatic CYP1A2 protein levels, as well as the CYP1A1 and CYP2B10 proteins (Fig. 1B). In *Cyp1a2(−/−)*, mice CYP1A1 and CYP2B10 proteins were induced by the PCB mixture to the same, or even greater levels, compared with that in the *Cyp1a2(+/+)* (Figs. 1C and E); in contrast, even under these highly inducing conditions, CYP1A2 protein was not detected in any of the *Cyp1a2(−/−)* mice (Figs. 1B and D).

Livers were analysed for uroporphyrin content after 7 weeks, and *Cyp1a2(+/+)* mice were found to exhibit marked hepatic porphyria—especially if pre-dosed with iron. No elevation of porphyrin levels whatsoever was detected in the *Cyp1a2(−/−)* mice, even with the additional stimulus of iron overload (Table 1). These data indicate that the block in heme biosynthesis, at uroporphyrinogen decarboxylase caused by PCBs acting through the AHR in wild-type mice, did not occur in the knockout mice.

Influence of CYP1A2 absence on carcinogenesis

When exposure of mice to the PCBs was continued for 57 weeks, livers from *Cyp1a2(+/+)* mice showed the clear presence of large preneoplastic foci. The carcinogenesis response, like the porphyria, was particularly striking in those mice that had received a pre-dose of iron, with half the animals showing adenomas (Table 1). In contrast to these findings with the *Cyp1a2(+/+)* mice, no foci or adenomas were observed in the livers of *Cyp1a2(−/−)* mice—even in those that had also received iron (Table 1, Fig. 2). Tissue was not fluorescent due to porphyrins and there was no evidence of pigment in the sections under polarized light. Intriguingly, however, other changes such as cellular hypertrophy, moderate focal inflammation, and bile duct proliferation were similar, or even greater, in the knockout mice compared with that in the wild-type, and enhanced by iron in both

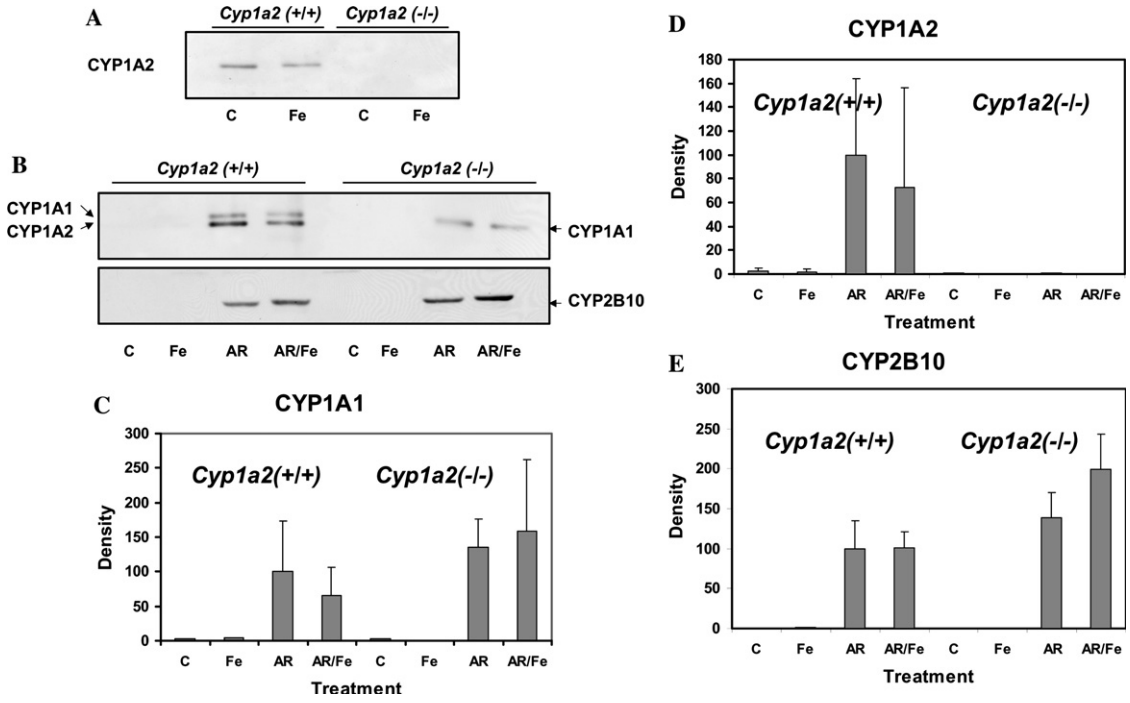

Fig. 1. Expression of hepatic cytochrome P450 proteins as detected by Western immunoblotting in C57BL/6J *Cyp1a2(+/+)* and *Cyp1a2(−/−)* mice. (A) No CYP1A2 protein was detected in null mouse liver, even after prolonged exposure to the chemiluminescence detection system, whereas constitutive levels were found in untreated and iron-treated wild-type mice. (B) Induction of CYP1A1, 1A2, and 2B10 proteins, following 7 weeks of daily exposure to Aroclor 1254 in the diet (0.01%). Time of exposure of the blot to the chemiluminescence detection system was considerably less than in (A), so that constitutive levels of CYP1A2 in wild-type were barely observed. (C,E) In mean analyses from four mice per group, the CYP1A1 and CYP2B10 protein levels were markedly induced in *Cyp1a2(+/+)* and *Cyp1a2(−/−)*, mice as shown by relative densities following Western blotting. (D) CYP1A2 levels were 37-fold higher than constitutive levels with the PCBs, and 27-fold with PCBs + Fe, in the *Cyp1a2(+/+)* mice when compared with respective controls. No CYP1A2 was detected in any of the *Cyp1a2(−/−)* mice.

Table 1
Incidence and severity of porphyria, hepatic foci, and tumors in *Cyp1a2(+/+)* and *Cyp1a2(−/−)* mice after chronic exposure to PCBs

Genotype	Treatment		Porphyria at 7 weeks	Findings up to 57 weeks			
	Iron	Aroclor	Uroporphyrin (nmol/g liver)	Average survival (weeks)	Liver % body wt	Mice with foci	Mice with adenomas
Cyp1a2(+/+)	−	−	0.4 ± 0.1	56	6.7 ± 1.0	0/5	0/5
Cyp1a2(+/+)	+	−	0.3 ± 0.1	57	5.8 ± 0.5	0/5	0/5
Cyp1a2(+/+)	−	+	11.9 ± 18.9	56	12.2 ± 2.3	1/10	1/10
Cyp1a2(+/+)	+	+	219 ± 173[a]	52	20.9 ± 4.4	10/10[b]	5/10
Cyp1a2(−/−)	−	−	0.3 ± 0.1	57	5.0 ± 0.3	0/5	0/5
Cyp1a2(−/−)	+	−	0.4 ± 0.2	57	5.9 ± 0.3	0/5	0/5
Cyp1a2(−/−)	−	+	0.4 ± 0.2	57	10.1 ± 2.0	0/10	0/10
Cyp1a2(−/−)	+	+	0.4 ± 0.2	56	15.2 ± 2.2	0/10	0/10

Findings in control and iron-dosed mice were comparable with those for other studies [8,9]. No hepatocellular tumors in iron-dosed groups have been detected.

[a] Significantly different from controls based on five mice per group ± SD ($p < 0.05$).

[b] Total number of foci was greater in the PCB plus iron group than in the PCB treated group, 38:21, respectively.

strains (Fig. 2). This unique observation differentiates the present model from those in which adaptive proliferation associated with chronic general toxicity is thought to play a major role [21].

Comparison with rats

The potential role of porphyria in the carcinogenic process in rats is often not discussed, but, as with the mouse models, there seems to be evidence for a link between these processes. Female rats are predominantly more sensitive than males to the development of liver porphyria caused by PCBs, dioxins, hexachlorobenzene, and related chemicals [7,22–24]. Again, in many studies, females have a greater propensity than males to develop liver tumors with these agents, which is not seen with other hepatocarcinogens (for a review, see [7]). In detailed carcinogenesis studies in rats with PCBs—in which tumors occur earlier

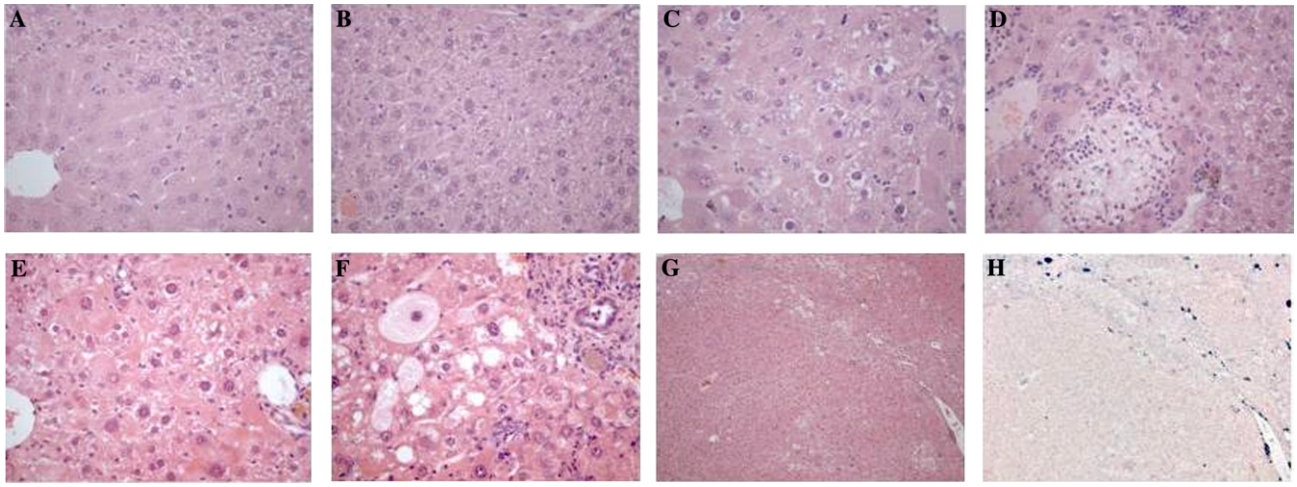

Fig. 2. Histopathology of hepatic effects of Aroclor 1254 in C57BL/6J *Cyp1a2(+/+)* and *Cyp1a2(−/−)* mice and the additional influence of initial iron-loading. (A) In *Cyp1a2(+/+)* mice administered Aroclor 1254 alone, at 7 weeks there was marked centrilobular hepatocellular hypertrophy. Affected hepatocytes were characterised by variably expanded granular eosinophilic cytoplasm and enlarged vesicular nuclei; this was associated with increased mitotic activity, cellular vacuolation, individual cell necrosis, and slight inflammation. (Inflammation and degeneration were more marked at 57 weeks.) (B) In *Cyp1a2(−/−)* mice at 7 weeks, there was marginal hepatocellular hypertrophy without significant degeneration or inflammation. (By 57 weeks, this was more marked, and some small foci of chronic inflammation and lipofuscin were present.) (C) Non-neoplastic changes were accentuated in all mice given iron prior to Aroclor 1254. In wild-type mice the enlarged hepatocytes were more variable in size and showed more degenerative changes than those without iron. There was more widespread inflammation and mitoses. (D) In *Cyp1a2(−/−)* mice at 7 weeks of PCBs + iron, a more severe pattern of pathology than Aroclor 1254 alone was similarly observed. Although cellular hypertrophy was less marked than wild-type, the inflammation was severe and associated with extensive zonal hepatocellular necrosis, possibly more than in the wild-type. (E,F) After 57 weeks in both *Cyp1a2(+/+)* and *Cyp1a2(−/−)* mice, respectively, the pathology was more pronounced than at 7 weeks—although the inflammatory process remained more marked in *Cyp1a2(−/−)* mice and was associated with inflammation, swelling of hepatocytes, and bile duct proliferation in portal tracts. (G) Hepatocellular foci of eosinophilic or clear-cell type and hepatocellular tumors were observed only in Aroclor or Aroclor + iron groups; they were more numerous in mice treated with both Aroclor + iron than with Aroclor alone. (H) Perl's stain demonstrated that the larger foci and adenomas excluded iron. None was observed in *Cyp1a2(−/−)* mice.

in females—porphyrin levels also were correlated with the differential tumorigenic response [23]. Iron accumulation in hepatocytes was observed in females, but not males, prior to tumor appearance [25], suggesting a role for iron in the carcinogenesis response. Hepatic carcinogenesis in rats caused by hexachlorobenzene is also enhanced by iron loading [26].

Relevance to humans

What is the relevance of these rodent findings for humans? Chlorinated aromatic chemicals related to PCBs cause porphyria in humans as well as rodents [7]. Perhaps more importantly, these chemicals are used in animal model systems to mimic a similar human hepatic disorder, porphyria cutanea tarda (PCT), which occurs sporadically following mild or moderate liver injury as a consequence of prolonged alcohol or estrogenic drug use. There is strong evidence for a role for aspects of iron metabolism in this disorder [27,28]. One of the features of PCT now established is an association with an elevated incidence of hepatocellular carcinoma that cannot be ascribed solely to concomitant cirrhosis [29–31]. Interestingly, there are more than 60-fold differences in hepatic CYP1A2 activity between individuals in human populations [32,33]. Many DNA variant sites, in and around the *CYP1A2* gene, have been described in detail [34,35], but—at the present time—none of these polymorphic sites has explained unequivocally the large interindividual differences in human constitutive CYP1A2 expression.

As noted above, CYP1A2 has been shown to be essential for the development of uroporphyria in several rodent models of the disease. Such experiments performed in the genetic absence of CYP1A2, however, do not allow predictions about small differences in the CYP1A2 levels that might be sufficient to support the development of uroporphyria. To this end, a recent study [36] demonstrated that striking differences in the severity of porphyria can be achieved by altering the levels of hepatic CYP1A2 within 3- to 4-fold of normal. Therefore, we would predict that those humans having the highest levels of hepatic CYP1A2, especially under the appropriate adverse circumstances, would be most susceptible to developing PCT. The present study would suggest that these same humans might also be the most susceptible to the development of primary liver cancer.

Mechanism of possible link between porphyria and hepatic carcinogenesis

The present mouse experiments suggest that there is either an oxidative mechanism, implicating iron

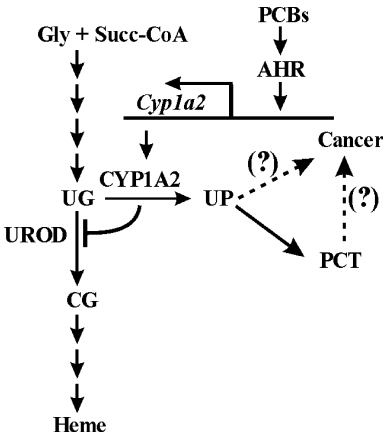

Fig. 3. Simplified scheme showing the possible interrelationships between PCBs, the AHR, CYP1A2, porphyria, and liver neoplasia. Glycine (Gly) and succinate acetyl-CoA (Succ-CoA) are combined to enter the heme biosynthetic pathway, which includes uroporphyrinogen (UG) and coproporphyrinogen (CG); it is not known how CYP1A2 is involved in the inhibition of uroporphyrinogen decarboxylase or the role of iron. Increased CYP1A2 levels, induced by PCBs by way of the AHR up-regulating the mouse *Cyp1a2* gene, are associated with uroporphyrin (UP) formation, which leads to uroporphyria (the similar disorder, porphyria cutanea tarda, PCT, in humans). Whether UP or PCT is directly in the pathway to liver cancer [denoted by **(?)**] remains to be established.

metabolism, which is common between this type of porphyria and hepatic carcinogenesis involving CYP1A2, or that porphyria itself contributes to neoplasia (Fig. 3). Porphyrins occurring in these livers may be the cause of tumor development. Alternatively, carcinogenesis may be the consequence of some other aspect of heme synthesis dysfunction. Many endogenous products produced abnormally are potentially carcinogenic [37]. Some drugs that induce protoporphyria in mice also cause subsequent liver cancer [38]. Both in drug-induced porphyria and the protoporphyric *Fech(m1/Pas)* mutant mouse, severe disturbances of gene expression in liver metabolism are observed—including those associated with toxic bile acids [39,40].

Conclusions

In summary, neither uroporphyria nor carcinogenesis was detected in the *Cyp1a2(−/−)* knockout mice, but both diseases were induced by PCBs in wild-type mice, especially in combination with iron overload. We would suggest that—although PCBs and related chemicals are undoubtedly promoters in carcinogenesis assays—development of porphyria may be a factor in their complete tumorigenic action in rodents. Exposure to PCBs at levels that do not cause marked porphyria in rodents or humans might be considered unlikely to cause hepatic cancer.

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