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# POLYCYCLIC AROMATIC HYDROCARBONS CAUSE HEPATIC PORPHYRIA IN IRON-LOADED C57BL/10 MICE: COMPARISON OF UROPORPHYRINOGEN DECARBOXYLASE INHIBITION WITH INDUCTION OF ALKOXYPHENOXAZONE DEALKYLATIONS

Jean E. Francis and Andrew G. Smith*

MRC Toxicology Unit, Medical Research Council Laboratories,
Woodmansterne Road, Carshalton, Surrey SM5 4EF, UK

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**SUMMARY:** Multiple doses of β-naphthoflavone to iron-loaded C57BL/10ScSn mice for 6 weeks caused inhibition of hepatic uroporphyrinogen decarboxylase and a porphyria indistinguishable from that previously only reported for polyhalogenated aromatic chemicals. β-Naphthoflavone and other polycyclic aromatic hydrocarbon inducers of cytochrome $P_1$-450-mediated ethoxyphenoxazone deethylation (ethoxyresorufin deethylase), benzo[a]pyrene, benz[a]anthracene, dibenz[ah]anthracene, 3-methylcholanthrene and α-naphthoflavone, also gave porphyria when fed. Isosafrole was inactive but by both methods phenobarbital produced a small but significant inhibition of the decarboxylase. The results demonstrate a toxic action of polycyclic aromatic hydrocarbons which probably does not involve reactive metabolites. 

Certain polyhalogenated chemicals such as hexachlorobenzene (HCB) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) are well known to cause in man and animals an hepatic porphyria similar to the human disease sporadic porphyria cutanea tarda believed to be initiated by consumption of alcohol or other drugs (1). There is a depression of uroporphyrinogen decarboxylase activity in both syndromes and apparently roles for iron and cytochrome $P_1$-450 (1-3). In some mouse strains, the porphyrogenic effects of the potent cytochrome $P_1$-450 inducing agent, TCDD, can be enhanced by preloading with iron (4). Iron will also sensitise C57BL/10 mice to the porphyrogenic action of the much weaker inducer HCB (5,6). There appears to be no direct link between metabolism of HCB and the development of porphyria (6-8). A much discussed mechanism is that oxygen becomes activated by cytochrome $P_1$-450, rather than by related isozymes, and the species produced (perhaps superoxide anion radical) reacts with iron leading to a chronic oxidative, toxic, process (3,9). It is envisaged that this process would be particularly pronounced

* To whom correspondence should be addressed.

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with inducers of cytochrome $P_1$-450 that are themselves poor substrates. If this theory is correct there seems no reason why any nonhalogenated inducer of this cytochrome should not have the potential to cause porphyria in animals. However, so far, the classical inducers of this type, the polycyclic aromatic hydrocarbons such as 3-methylcholanthrene and β-naphthoflavone, have not been shown to have this effect (3).

Here we demonstrate for the first time that chronic exposure of iron-loaded mice to polycyclic aromatic hydrocarbon inducers of cytochrome $P_1$-450 will indeed cause inhibition of uroporphyrinogen decarboxylase and an hepatic porphyria which is identical to that previously considered unique to the actions of HCB, TCDD and related environmental chemicals.

## MATERIALS AND METHODS

**Chemicals** Phenobarbital was from BDH, isosafrole (cis+trans) from Fluorochem and Imferon (50 mg iron/ml) from Fisons. Pentacarboxyporphyrin I was from Porphyrin Products. All other chemicals were purchased from Aldrich or Sigma.

**Mice and treatments** Male C57BL/10ScSn mice were bred in these laboratories and used at 7-10 weeks old. Animals were given iron (600 mg/kg) as Imferon (12 ml/kg) 3 days before further treatment. Mice not given Imferon received the dextran base (200 mg/ml). Chemicals were given in separate doses by oral intubation or i.p. injection as indicated in Tables 1-3. For these experiments mice were kept on bedding. In feeding experiments, after an initial oral dose, chemicals were fed at a level of 500 ppm in the diet which also contained 2% arachis oil. These mice were kept on grids (to avoid any polychlorinated chemicals in bedding material) in a negative pressure isolator. All treatments consisted of 4 mice per group and results are ± S.E.M. Urine samples were obtained from each mouse and pooled.

**Analyses** Livers were homogenised in 0.25M sucrose (1:4 v/v) except where indicated below. Porphyrin contents of livers were determined by fluorescence spectroscopy and are expressed in terms of uroporphyrin (10). Uroporphyrinogen decarboxylase activity was estimated from the conversion of pentacarboxyporphyrinogen I to coproporphyrinogen I (6). Assay of the inhibitor of the decarboxylase was based on preincubating a heat-treated extract of liver prepared in water with control liver supernatant before assaying residual decarboxylase activity (11). Uroporphyrins in liver and urine were analysed by h.p.l.c. using a 5μm ODS-1 column (25cm x 4.6mm i.d.) and a gradient of 0.05M lithium citrate (pH 3.0) and methanol (12). Creatinine was estimated with Sigma Diagnostic kit 555A. Protein was determined as in Lowry et al. (13). Ethoxy-, pentoxy- and benzyloxy-phenoxazone O-dealkylations were measured on 9,000 g liver supernatants prepared in 0.25M sucrose, in the presence of dicumarol (11,14,15). Cytochrome P-450 was measured as in Omura and Sato (16).

## RESULTS

Iron-loaded mice were given β-naphthoflavone 5 days a week. The level of uroporphyrins in the urine began to rise after 3-4 weeks and by six weeks was 100-fold higher than control (Fig. 1). Analyses of liver showed a considerable depression of uroporphyrinogen decarboxylase activity and a rise in porphyrin concentrations (Table 1). Heat-treated extracts of the livers

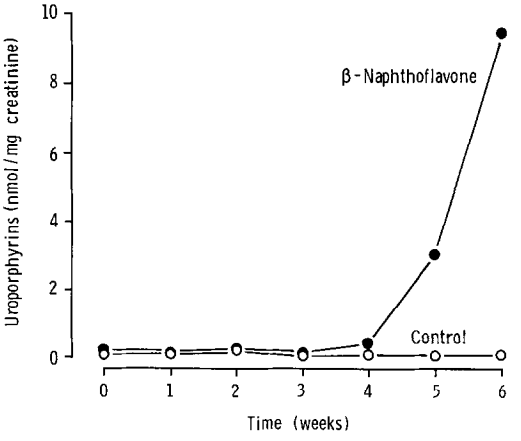


Fig. 1. Uroporphyrins in the pooled urines from iron-loaded mice given repeated oral doses of β-naphthoflavone as described in Table 1.

inhibited decarboxylase activity of liver supernatant from untreated mice as reported for TCDD, HCB, etc (11,17,18). Interestingly, in similar experiments phenobarbital also caused a significant, albeit small, decrease in the activity of decarboxylase whereas isosafrole was inactive (Table 1).

To determine whether other polycyclic aromatic hydrocarbons besides β-naphthoflavone could cause porphyria we first tested their ability to induce ethoxyphenoxazone O-deethylation (ethoxyresorufin deethylase) an indicator of cytochrome $P_1$-450 or P-450c-mediated activity (14,19-21) (Table 2). After a single dose, benzo[a]pyrene, benz[a]anthracene,

**Table 1**

Influence of multiple doses of phenobarbital, isosafrole, and β-naphthoflavone on hepatic uroporphyrinogen decarboxylase activity of C57BL/10 mice with iron-overload

| Treatment[a] | Decarboxylase (pmol/min per mg protein) | Inhibitor Assay (pmol/min per mg protein) | Porphyrin levels (nmol/g tissue) |
|---|---|---|---|
| Control saline | 37.6 ± 0.3 | 32.0 ± 0.9 | 0.34 ± 0.01 |
| Control oil | 38.3 ± 0.8 | 32.7 ± 0.5 | 0.58 ± 0.03 |
| Phenobarbital | 34.4 ± 0.2* | 28.5 ± 0.4** | 0.35 ± 0.01 |
| Isosafrole | 39.3 ± 0.4 | 30.5 ± 0.4** | 0.43 ± 0.05 |
| β-Naphthoflavone | 3.2 ± 0.9* | 2.8 ± 0.6* | 297 ± 48** |

[a] Mice which had received iron (600mg/kg) were given by gavage saline (10ml/kg), oil (10ml/kg), phenobarbital (80mg/kg) in saline, isosafrole (150mg/kg) or β-naphthoflavone (150mg/kg) in oil 5 days a week for 6 weeks. Total approximate doses were phenobarbital 2.5g/kg, isosafrole 4.5g/kg and β-naphthoflavone 5g/kg.

*Significantly different from the control group $P<0.001$ or ** $P<0.05$.

Table 2

Induction of hepatic alkoxyphenoxazone dealkylations

| Inducer[a] | Alkoxyphenoxazone O-dealkylations (pmol/min per mg protein) | | | Total cytochrome P-450 (pmol/mg protein) |
|---|---|---|---|---|
| | Ethoxy- | Pentoxy- | Benzyloxy- | |
| Saline | 5 ± 1 | 4 ± 1 | 14 ± 3 | 458 ± 25 |
| Oil | 5 ± 1 | 4 ± 1 | 14 ± 1 | 379 ± 21 |
| Phenobarbital | 11 ± 2 | 92 ± 11 | 494 ± 65 | 773 ± 18 |
| Isosafrole | 13 ± 2 | 5 ± 1 | 78 ± 22 | 572 ± 41 |
| Pyrene | 7 ± 1 | 4 ± 1 | 12 ± 3 | 469 ± 19 |
| Benzo[a]pyrene | 814 ± 94 | 3 ± 1 | 45 ± 4 | 709 ± 65 |
| Benz[a]anthracene | 404 ± 19 | 3 ± 1 | 58 ± 6 | 695 ± 65 |
| Dibenz[ah]anthracene | 821 ± 40 | 3 ± 1 | 84 ± 4 | 542 ± 80 |
| 3-Methylcholanthrene | 615 ± 55 | 3 ± 1 | 50 ± 8 | 782 ± 68 |
| α-Naphthoflavone | 29 ± 4 | 3 ± 1 | 35 ± 6 | 585 ± 47 |
| β-Naphthoflavone | 331 ± 24 | 4 ± 1 | 65 ± 6 | 724 ± 31 |

[a] Mice were given a 100mg/kg dose of inducer in oil (10ml/kg) by i.p. injection and killed after 2 days except for phenobarbital which was given in two doses of 50mg/kg in saline on successive days.

dibenz[ah]anthracene, 3-methylcholanthrene and β-naphthoflavone induced this activity by 66 to 160-fold: α-naphthoflavone was apparently considerably less active than β-naphthoflavone. Pyrene was without effect but the monoaromatics isosafrole and phenobarbital showed a 2 to 3-fold induction. The induction of pentoxy- and benzyloxyphenoxazone O-dealkylations (indicators of other induced cytochrome P-450 isozymes) were consistent with results in rats (21). Of all the inducers only phenobarbital significantly induced depentylation activity. O-Debenzylation was selectively induced by isosafrole although phenobarbital induced the greatest absolute level and polycyclic aromatic hydrocarbons caused moderate increases.

All of these chemicals were given to mice in food or drinking water (phenobarbital). At 6 weeks mice fed β-naphthoflavone showed a 24% inhibition of uroporphyrinogen decarboxylase (controls 39.1±0.1, β-naphthoflavone 29.7±0.2 pmol/min per mg protein) but no significant rises in porphyrin concentrations. After 10 weeks most of those polycyclic aromatics that markedly induced ethoxyphenoxazone dealkylation caused a pronounced inhibition of the decarboxylase, high accumulation of porphyrins in the liver and the

Table 3

Hepatic porphyria induced in iron-loaded mice after consumption of polycyclic aromatic hydrocarbons for 10 weeks

| Treatment | Liver | | Urine |
|---|---|---|---|
| | Decarboxylase (pmol/min per mg protein) | Porphyrins (nmol/g tissue) | Uroporphyrins (nmol/mg creatinine) |
| Control[a,b] | 38.6 ± 0.6 | 0.56 ± 0.02 | 0.02 |
| Control[a] | 38.6 ± 0.1 | 0.50 ± 0.06 | 0.04 |
| Control[b] | 39.1 ± 0.3 | 1.22 ± 0.08 | 0.03 |
| Control | 39.1 ± 0.7 | 0.99 ± 0.10 | 0.03 |
| Phenobarbital[b] | 21.1 ± 7.2** | 8.42 ± 3.98 | 4.13 |
| Isosafrole | 37.9 ± 0.3 | 0.43 ± 0.05 | 1.10 |
| Pyrene | 37.5 ± 0.5 | 1.15 ± 0.10 | 1.69 |
| Benzo[a]pyrene | 10.8 ± 2.6* | 9.96 ± 2.93** | 5.87 |
| Benz[a]anthracene | 2.4 ± 0.9* | 158 ± 28** | 5.27 |
| Dibenz[ah]anthracene | 0.9 ± 0.1* | 257 ± 23* | 19.01 |
| 3-Methylcholanthrene | 0.7 ± 0.2* | 565 ± 58* | 41.53 |
| α-Naphthoflavone | 5.5 ± 0.4* | 71.6 ± 17.3** | 3.00 |
| β-Naphthoflavone | 1.6 ± 0.4* | 383 ± 71** | 9.60 |
| β-Naphthoflavone[a] | 36.9 ± 0.7 | 0.49 ± 0.0** | 1.37 |

All groups of mice received a subcutaneous injection of iron (600mg/kg) and 2% oil in their diet except where indicated; [a] no iron, [b] no oil. Chemicals were given in the diet (500 ppm) or in drinking water (phenobarbital, 500 ppm) after an initial oral dose (100-150mg/kg). Urine samples from each mouse were pooled.

* Significantly different from appropriate control $P<0.001$; ** $P<0.05$.

excretion of uroporphyrins in the urine (Table 3). β-Naphthoflavone, benz[a]anthracene, dibenz[ah]anthracene and 3-methylcholanthrene were particularly active. Without iron, β-naphthoflavone had little porphyrogenic effect. Benzo[a]pyrene was less effective than might have been expected from Table 2. Pyrene and isosafrole did not cause significant inhibition of the decarboxylase. As observed in the first experiment phenobarbital caused some inhibition of uroporphyrinogen decarboxylase and the beginnings of porphyria despite a low induction of deethylation (Table 2). Fig. 2 illustrates representative h.p.l.c. chromatograms of porphyrins in the liver and urine of mice after treatment with phenobarbital or dibenz[ah]anthracene. The proportion of uroporphyrin I to uroporphyrin III increased with the severity of the porphyria (6).

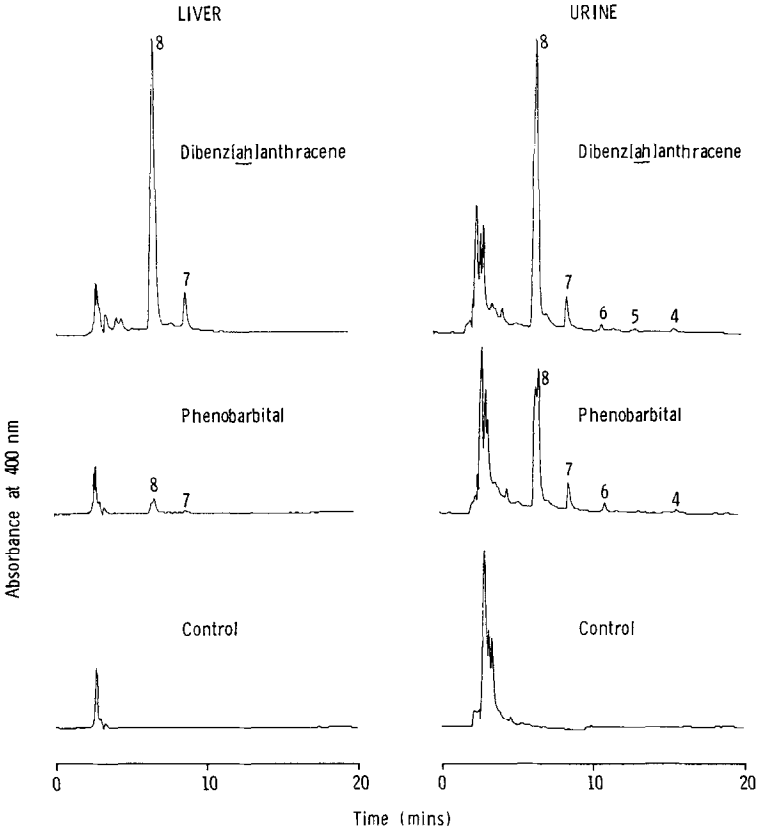


Fig. 2. H.p.l.c. of porphyrins in the liver and urine from iron-loaded mice fed dibenz[ah]anthracene (500ppm) or given phenobarbital in the water (500ppm) for 10 weeks as described in Table 3. 8 uroporphyrins I and III, 7 heptacarboxyporphyrins, 6 hexacarboxyporphyrins, 5 pentacarboxyporphyrins, 4 coproporphyrins. Chromatograms represent injections equivalent to 2mg of liver or 50µg of urinary creatinine.

## DISCUSSION

The demonstration that polycyclic aromatic hydrocarbons cause an hepatic porphyria in iron-loaded mice further strengthens the case for a mechanism of uroporphyrinogen decarboxylase inhibition which involves uncoupling of the cytochrome $P_1$-450 system and not a metabolite of the chemical. Phenobarbital may exert porphyrogenic effects through uncoupling of other induced cytochrome P-450 isozymes or by greater induction of cytochrome $P_1$-450 in a chronic period than seen in Table 2. This potential of phenobarbital, a nonhalogenated and nonpolyaromatic chemical, to cause porphyria is of considerable interest in the context of human porphyria cutanea tarda. The effect of benzo[a]pyrene was poorer than expected from Table 2 perhaps due to very rapid metabolism. Polycyclic aromatic hydrocarbons such as 3-methylcholanthrene and β-naphthoflavone are good inducers of cytochrome $P_1$-450 but they are also good substrates and high doses for prolonged periods

are required to see any porphyrogenic effects. In contrast, although the halogenated chemical HCB is not a particularly good inducer (6) its metabolism is extremely slow (6,8) and induction can be maintained for a long time after a single dose (6). TCDD combines both potent, persistent induction of cytochrome $P_1$-450 and slow metabolism (4). The hypothesis that uncoupling of cytochrome $P_1$-450, particularly in the absence of substrate or presence of a poor substrate, causes porphyria may imply that this isozyme differs from other cytochrome P-450 isozymes in this respect. For the equivalent isozyme in rats (cytochrome P-450c) there is evidence which might support this theory (21).

How would release of active oxygen cause porphyria? Liver microsomes from chick embryos pre-treated with 3-methylcholanthrene in the presence of NADPH and 3,4,3',4'-tetrachlorobiphenyl oxidise uroporphyrinogen I to the porphyrin (23). In hepatocyte cultures this could lead to the accumulation of uroporphyrins (6,23). However, in experimental animals porphyrogenic chemicals seem to cause the accumulation of uroporphyrins by inhibition of uroporphyrinogen decarboxylase (1,2,6). In rats and mice there is evidence for the formation of an inhibitor (11,17,18). Our studies suggest that if there is an inhibitor which is a porphyrin it is more hydrophilic than uroporphyrin (11). Possibly a chronic oxidative process arises, enhanced by iron (perhaps by the conversion of superoxide anion radical to hydroxyl radical), which causes the formation of an hydroxylated or N-oxidised (24) porphyrin that is a potent inhibitor of the decarboxylase. Studies are in progress to examine these and other possibilities. If a chronic oxidative process can occur by the combination of an uncoupled cytochrome $P_1$-450 system and iron it is tempting to speculate whether it also plays a part in the hepatocarcinogenesis of polyhalogenated aromatic chemicals (25).

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