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Protection against iron-induced uroporphyria in C57BL/10ScSn mice by the peroxisome proliferator nafenopin

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Administration of some polyhalogenated aromatic chemicals to C57BL/6J and C57BL/10ScSn mice causes a depression of uroporphyrinogen decarboxylase activity in the liver resulting in uroporphyria perhaps by an oxidative process [1]. Depletion of hepatic iron stores or iron overload significantly modulate the response of mice to chemicals of this type such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [2–4], polychlorinated and polybrominated biphenyls [5] and hexachlorobenzene [6, 7]. This experimental uroporphyria closely resembles the iron-linked human disorder porphyria cutanea tarda, which may occur in some patients with moderate liver damage prompted by excessive intake of alcoholic beverages or other drugs [8]. The connection between the experimental and human uroporphyrias has been strengthened by the demonstration that some non-halogenated chemicals and drugs cause hepatic uroporphyria in iron-loaded C57BL/6J or C57BL/10ScSn mice [9–11]. In fact, the most recent experiments demonstrate that in these strains of mice, inhibition of hepatic uroporphyrinogen decarboxylase and moderate uroporphyria can eventually occur under the influence of iron overload alone or in conjunction with 5-aminolaevulinic acid and be maintained for many months [1, 5, 11–13]. Thus in mice, the influence of the drugs and chemicals could be viewed as potentiation of a malfunction of iron metabolism. All of these compounds are inducers of the microsomal cytochrome P450 system and most of them of the P450IA isoenzymes [1, 9, 10]. In the present work, we have investigated the effects of nafenopin which like other peroxisome proliferators [14–16] induces a novel form of cytochrome P450 termed P450IVA1. This isoenzyme is very active in the metabolism of endogenous substrates of cytochrome P450 including fatty acids and steroids [14–16]. In contrast to the other chemicals so far studied, nafenopin protected mice against developing uroporphyria during iron overload.

Materials and Methods

Chemicals. Iron–dextran solution (Imferon; 50 mg/mL Fe, 200 mg/mL dextran) was purchased from Fisons plc (Loughborough, U.K.). Dextran C (the 5000 mol. wt dextran used in the manufacture of Imferon) was a gift from Fisons. Nafenopin (2-methyl-2-[*p*-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]propionic acid) was a gift from Ciba Geigy Ltd (Basel, Switzerland). Ethoxy-, pentoxy- and benzyloxyresorufins (more correctly the alkoxyphenoxazones) were prepared as described previously [17]. Pentacarboxyporphyrin I, uroporphyrins and other porphyrin standards were from Porphyrin Products (Logan, U.S.A.). [1-^{14}C]Lauric acid (26 mCi/mmol 98% pure) was from Amersham International plc (Amersham, U.K.).

Animals and treatments. Male C57BL/10ScSn mice (7–10 weeks old) were bred on site. Mice received Imferon (12 mL/kg 600 mg Fe/kg) or the equivalent volume of dextran solution by s.c. injection and then were fed powdered Breeder diet No. 3 (Special Diet Services, Witham, U.K.) with or without 0.025% nafenopin as indicated. Experiment A: one week after receiving iron–dextran or dextran mice were fed nafenopin for 15 weeks. Experiment B: mice were fed nafenopin-containing diet from week 16 to week 32 after receiving iron–dextran or dextran. Animals were killed by cervical dislocation. Samples of urine were obtained from individual mice onto plastic Petri dishes and pooled for analysis.

Biochemical assays. Porphyrins in urine samples were analysed by reverse phase HPLC as described previously [9] and are expressed relative to creatinine content which was estimated using Sigma Diagnostic kit 555A. Livers were homogenized in 0.25 M sucrose (1:5 w/v) and total liver porphyrins were determined by spectrofluorimetry and expressed in terms of uroporphyrin [18]. Non-haem

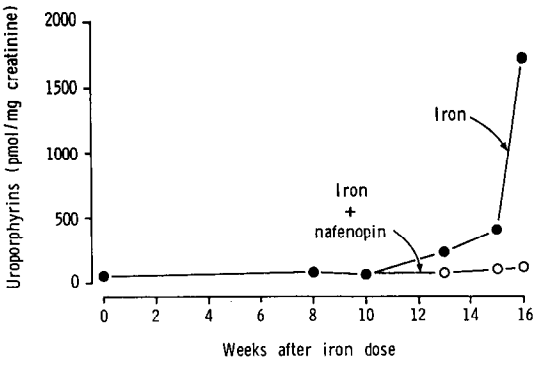

Fig. 1. Uroporphyrin levels in the urine from C57BL/10ScSn mice with iron overload and fed control or a nafenopin diet. Points represent analyses of pooled urine samples from five or six mice and are expressed per milligram of creatinine. The mice are those reported as Experiment A in Table 1. Nafenopin was fed one week after iron injection. Mice which did not receive iron and were fed either a control or nafenopin diet had uroporphyrin levels which were not significantly different from the iron/nafenopin group shown above.

iron concentrations were estimated as in Smith *et al.* [5]. Microsomes were obtained from 10,000 *g* × 15 min fractions by centrifugation at 100,000 *g* for 60 min. Uroporphyrinogen decarboxylase activity was determined on the resulting cytosol with pentacarboxyporphyrinogen I as substrate and activities are expressed as picomoles of coproporphyrinogen I formed per minute per milligram of protein [19]. Microsomes were washed with 0.15 M KCl before estimations of ethoxy-, pentoxy- and benzyloxyresorufin dealkylase activities [20], lauric acid 11- and 12-hydroxylations [21] and cytochrome P450 [21].

Results and Discussion

Administration of a single dose of iron–dextran to C57BL/10ScSn mice caused uroporphyria to develop after a delay of several months (Experiment A). This is illustrated in Fig. 1 by the elevation of urinary uroporphyrin levels from 13 weeks onwards so that by 16 weeks levels were 45-fold greater than control mice given the dextran carrier (Fig. 2). In contrast, mice with iron overload did not develop uroporphyria if they subsequently received a diet containing nafenopin. Sixteen weeks following administration of iron uroporphyrin levels in urine from mice also given nafenopin were still low and little different from mice fed nafenopin without prior iron treatment (Figs 1 and 2). Analysis of livers from Experiment A confirmed the marked elevation in porphyrin levels in mice administered iron and fed a control diet for 16 weeks (130-fold) whereas porphyrin concentrations were unaltered in those fed nafenopin (Table 1). Other experiments have shown that if feeding of nafenopin-containing diet was maintained, the mice were protected against the onset of uroporphyria for at least 80 weeks (Smith and Lake, unpublished data). As expected from the hepatic porphyrin data, at 16 weeks uroporphyrinogen decarboxylase activity was significantly depressed (>75%) in the iron group fed only control diet whereas there was no difference between iron–dextran and dextran treated mice fed nafenopin (Table 1). However, nafenopin treatment by itself caused a depression in measurable uroporphyrinogen decarboxylase activity (about 30%). Whether this depression of decarboxylase activity was due to inhibition of the enzyme, as occurs in hexachlorobenzene-induced uroporphyria [22], or the result of a change in gene expression remains to be determined. Despite the modest decrease in uroporphyrinogen decarboxylase activity with nafenopin this did not result in an elevation of liver porphyrin levels.

In a separate study (Experiment B), mice were left on control diet for 16 weeks after iron dosing and then fed a control or nafenopin diet for a further 16 weeks (Table 1). Those mice which received a control diet throughout the 32-week experiment developed a marked depression of uroporphyrinogen decarboxylase activity (3% of control) and elevated porphyrin levels (233-fold). In this experiment administration of nafenopin from 16 weeks onwards was only partially successful in reversing the porphyric process. Decarboxylase activity was increased to a small extent but there was a more significant decrease in the accumulation of porphyrins (Table 1). These results are compatible with previous findings that once hepatic uroporphyria is established, its reversal is a difficult and slow process [8, 23, 24].

The development of uroporphyria in mice is usually presumed to be associated with induction of the microsomal mixed function monooxygenase system, particularly with the IA forms of cytochrome P450 [1, 9–11, 25, 26], and leading to an inhibitor of the decarboxylase [1]. However,

Table 1. Effects of nafenopin on the development of iron-induced uroporphyria in C57BL/10ScSn mice

Dietary regime	Iron treatment	Uroporphyrinogen decarboxylase (pmol/min/mg protein)	Hepatic porphyrins (nmol/g liver)	Non-haem iron (μg/g liver)
Experiment A				
Control (5)	−	38.7 ± 1.0	0.6 ± 0.1	77 ± 8
Control (6)	+	9.0 ± 1.7*	83.5 ± 30.4*	2087 ± 72*
Nafenopin (6)	−	22.0 ± 1.3	0.7 ± 0.1	48 ± 8
Nafenopin (6)	+	23.1 ± 0.3†	0.6 ± 0.1†	1712 ± 169*
Experiment B				
Control (3)	−	33.4 ± 1.3	0.7 ± 0.2	24 ± 6
Control (4)	+	1.0 ± 0.3*	161.2 ± 19.0*	1599 ± 165*
Nafenopin (4)	−	25.0 ± 1.2	0.5 ± 0.1	20 ± 3
Nafenopin (4)	+	4.7 ± 2.3*	38.7 ± 22.1†	1229 ± 89*

Mice received dextran solution (2.4 g/kg) or iron–dextran (600 mg Fe/kg) as a single i.p. injection. Experiment A: nafenopin (0.025% diet) was administered after one week for 15 weeks. Experiment B: nafenopin was administered after 16 weeks for a further 16 weeks. Values in parentheses are the number of mice per group. Results are means ± SE.

* Significantly different from group not given iron $P < 0.05$.

† Significantly different from iron group fed control diet $P < 0.05$.

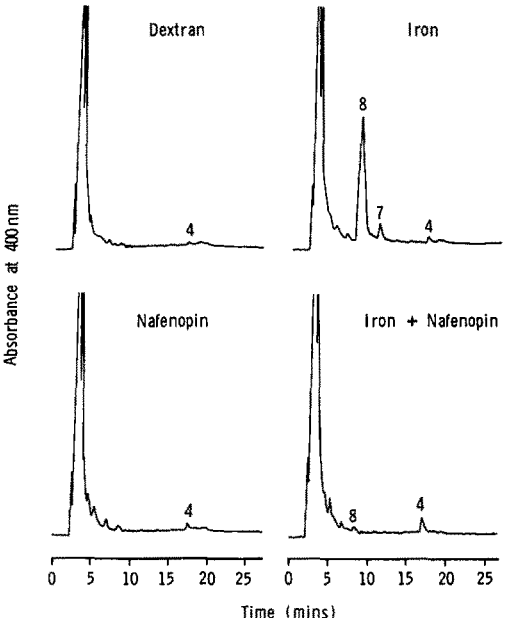

Fig. 2. HPLC traces of urine samples from mice given dextran or iron–dextran and fed a control or 0.025% nafenopin diet for 15 weeks as shown in Table 1 and Fig. 1. 4, coproporphyrin; 7, heptacarboxylic porphyrin; 8, uroporphyrin I and III isomers. Porphyrins were analysed by reverse phase (5 μm ODS—1 column) with a 0.05 M lithium citrate (pH 3.0)–methanol gradient. Detection was by absorbance at 400 nm and full scale deflection was 0.05 A.

the finding that iron overload can cause porphyria [12] either alone or in combination with aminolaevulinate in the drinking water [13], indicates that induction of cytochrome P450IA isoenzymes (as opposed to constitutive levels) is not an absolute requirement. Consequently, the influences of iron and nafenopin treatments on hepatic microsomal monooxygenase activities were determined in Experiments A and B (Table 2). Iron caused a depletion of total cytochrome P450 as reported previously [7, 27] which was still evident after 32 weeks. This was reflected by a 50% depression of ethoxyresorufin deethylase activity (associated with cytochrome P450IA) but there was much less effect on pentoxy- and benzyloxyresorufin dealkylation (indicative of IIB cytochrome P450 species [17, 28]) and lauric acid 11-hydroxylation and 12-hydroxylation (catalysed by cytochrome P450IVA1 [14–16]). In keeping with previous observations in the rat, nafenopin markedly increased the 11- and particularly the 12-hydroxylation of lauric acid [14–16], but either slightly depressed or did not affect alkoxyresorufin dealkylations. Combination treatments of iron and nafenopin led to no significant changes in 12-hydroxylase activity from nafenopin treated mice but a tendency for increases in benzyloxyresorufin dealkylation. In Experiment B, ethoxyresorufin dealkylation in the iron/nafenopin group was as severely depressed as in the microsomes from mice fed a control diet after iron whereas in Experiment A nafenopin appeared to afford some degree of protection.

Clearly from these studies, continuous exposure of iron loaded C57BL/10ScSn mice to nafenopin prevents both the marked inhibition of uroporphyrinogen decarboxylase and the hepatic accumulation and urinary excretion of uroporphyrins. In contrast, other chemicals that have been studied appear to enhance the influence of iron [6, 9–11].

Table 2. Hepatic microsomal monooxygenase activities in iron-induced uroporphyric mice

Dietary regime	Iron treatment	Cytochrome P450 (pmol/mg protein)	Ethoxy-	Pentoxy-	Benzyloxy-	Lauric acid hydroxylations	
			resorufin dealkylations (pmol/min/mg protein)			11-hydroxylase (nmol/min/mg protein)	12-hydroxylase
Experiment A							
Control	–	803 ± 20	51 ± 7n	20 ± 5	85 ± 13	0.48 ± 0.11	0.65 ± 0.13
Control	+	493 ± 20*	25 ± 3*	16 ± 2†	123 ± 19†	0.33 ± 0.06†	0.57 ± 0.13†
Nafenopin	–	665 ± 21	30 ± 2*	14 ± 1*	51 ± 1*	1.35 ± 0.32*	7.46 ± 1.94*
Nafenopin	+	579 ± 54*	31 ± 1*	31 ± 2	171 ± 12*	1.63 ± 0.13*	7.26 ± 0.94*
Experiment B							
Control	–	946 ± 62	40 ± 6	7 ± 3	42 ± 13	0.52 ± 0.05	0.61 ± 0.17
Control	+	412 ± 10*	13 ± 3*	5 ± 2	43 ± 14†	0.35 ± 0.05†	0.57 ± 0.05†
Nafenopin	–	922 ± 74	37 ± 4	8 ± 2	36 ± 5	2.81 ± 0.12*	13.50 ± 0.92*
Nafenopin	+	487 ± 178*	14 ± 4*	9 ± 3	96 ± 22*	2.19 ± 0.52*	11.43 ± 0.26*

Mice were treated as in Materials and Methods and were those in Table 1. Results are means ± SE.
* Significantly different from the group fed control diet and not given iron $P < 0.05$.
† Iron group significantly less than the iron/nafenopin group $P < 0.05$.

Unfortunately for therapeutic possibilities the uroporphyria, once established, is obviously difficult to reverse by administration of nafenopin. Although both cytochrome P450IA isoenzymes and iron metabolism are implicated in the development of uroporphyria [1, 8], it is a conundrum that iron overload depletes hepatic cytochrome P450 [27]. Curiously, the loss of total cytochrome P450 with iron treatment in the present work was not expressed as a uniform loss of activities. Ethoxyresorufin deethylase activity which is mainly indicative of the presence of cytochrome P450IA, was preferentially decreased. This was especially evident in Experiment B in which uroporphyria was more severe and subsequent administration of nafenopin was only partially successful in affording protection. Perhaps during hepatic iron overload resulting in loss of cytochrome P450 [27] and inhibition of the decarboxylase, P450IA is particularly vulnerable whereas P450IIB1 and P450IVA1 are resistant. Nafenopin could protect from uroporphyria by inducing cytochrome P450IVA1. The presence of this isoenzyme might somehow prevent an oxidative process occurring which can inactivate other isoenzymes and also causes uroporphyrinogen decarboxylase inhibition. Alternatively, nafenopin may prevent uroporphyria by inducing protective systems elsewhere in the cell perhaps associated with peroxisome proliferation [29]. Whether other proliferators of peroxisomes have the same protective effect as nafenopin remains to be determined.

In summary, we have studied the influence of the cytochrome P450IVA1 inducer nafenopin, on the development of uroporphyria in C57BL/10ScSn mice caused by iron–dextran. In contrast to other inducers of cytochrome P450 studied, e.g. halogenated aromatics, polycyclic hydrocarbons and phenobarbital, nafenopin protected mice from developing uroporphyria. However, once the porphyria was established the influence of nafenopin was greatly reduced.

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