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CYP3A-Inducing Agents and the Attenuation of Uroporphyrin Accumulation and Excretion in a Rat Model of Porphyria Cutanea Tarda

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ABSTRACT. An experimental model of porphyria cutanea tarda (PCT) can be achieved in 3 weeks by a single injection of a mixture of polychlorinated biphenyls (Aroclor 1254) into iron-loaded female Fischer 344 rats maintained continuously on δ-aminolevulinic acid-supplemented drinking water. In this model, daily treatment with 5-pregnen-3β-ol-20-one-16α-carbonitrile (pregnenolone 16α-carbonitrile) attenuated uroporphyrin and heptacarboxylporphyrin accumulation and excretion by 75%. Pregnenolone 16α-carbonitrile treatment had only a minor effect on hepatic iron stores, and it had no effect on the induction of CYP1A activities by Aroclor 1254. In the absence of Aroclor 1254, pregnenolone 16α-carbonitrile had no effect on the accumulation and excretion of highly carboxylated porphyrins. Attenuation of porphyrin accumulation could also be demonstrated with daily troleandomycin treatment. Troleandomycin increased CYP3A-dependent erythromycin demethylase activity, but to a lesser extent than pregnenolone 16α-carbonitrile. Much of the CYP3A induced by troleandomycin was sequestered as a catalytically inactive metabolic-intermediate complex. In the absence of Aroclor 1254, troleandomycin had no effect on the accumulation and excretion of highly carboxylated porphyrins, nor did troleandomycin alter the induction of CYP1A by Aroclor 1254. The results suggest that the major attenuation of hepatic accumulation and urinary excretion of uro- and heptacarboxylporphyrins in the rat PCT model by pregnenolone 16α-carbonitrile and troleandomycin is due to an enhancement of CYP3A catalytic activity. BIOCHEM PHARMACOL **60**;9:1325–1331, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. porphyria cutanea tarda; uroporphyria; CYP3A induction; troleandomycin; pregnenolone-16α-carbonitrile

PCT§ is the most common form of porphyria in humans. The disorder is characterized clinically by hepatic siderosis and a photosensitive dermatosis. Biochemically, PCT is characterized by the accumulation of uroporphyrin and heptacarboxylporphyrin in the liver and the excretion of these compounds in the urine. PCT has been associated with mutations of the uroporphyrinogen decarboxylase gene, which result in decreased enzymatic activity [1]. However, not everyone carrying a mutation develops PCT, nor do all PCT patients carry a uroporphyrinogen decarboxylase gene mutation, suggesting other, possibly environmental, influences. PCT has been associated with accidental chemical exposures and chronic drug exposures, but here again the association is incomplete. Not all persons exposed to porphyrinogenic chemicals develop PCT, nor do all PCT patients have documented exposure to such chemicals. Rodent models of PCT that have been developed generally rely on treatment with chlorinated aromatic compounds. Iron-dextran-treated female Fischer 344 rats given a single exposure to a mixture of polychlorinated biphenyls (Aroclor 1254) developed the biochemical features of PCT in 3 weeks if maintained continuously on δALA-supplemented drinking water [2]. The presence of CYP1A2 clearly is required for the development of experimental PCT in mice [3], but additional factors appear to contribute to the expression of PCT [4–6]. Alcohol abuse, hepatitis C, estrogen use, and hemochromatosis all have been associated with PCT in humans [7, 8], yet a change in only a single implicated factor cannot adequately account for the sporadic appearance of PCT. A common theme of the multiple factors implicated is their ability to disturb the normal biochemistry and function of the liver.

Many drugs and other chemicals disturb cytochrome P450 concentration and activity in the liver. Foremost among the cytochrome P450 isozymes affected are those in the CYP3A family, a family that contributes, at least in part, to the metabolism of over 60% of the drugs that undergo cytochrome P450-dependent oxidative metabolism [9]. Agents from a wide range of pharmacological classes induce CYP3A, including macrolide antibiotics such as troleandomycin, anticonvul-

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§ *Abbreviations*: PCT, porphyria cutanea tarda; PCB, polychlorinated biphenyls (Aroclor 1254); P450-CO, carbon monoxide-detectable cytochrome P450; i.g., intragastric(ally); and δALA, δ-aminolevulinic acid.

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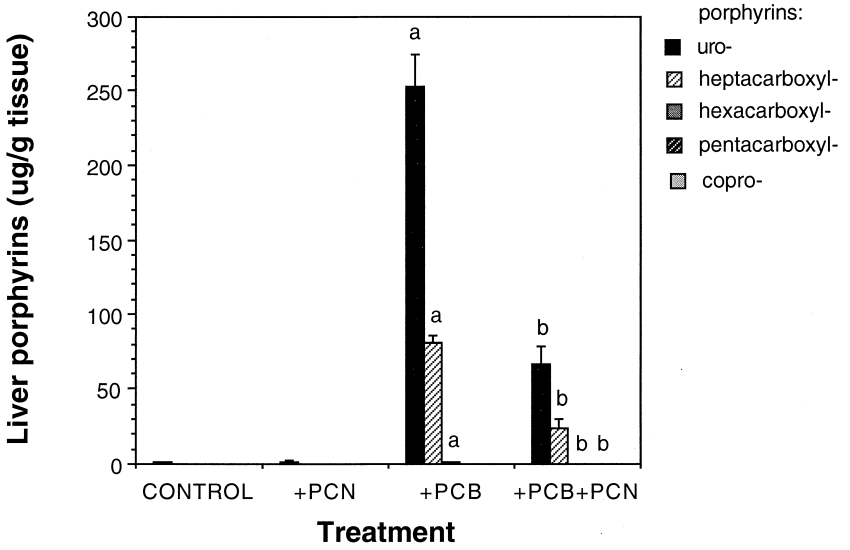

FIG. 1. Effect of treatment with 5-pregnen-3β-ol-20-one-16α-carbonitrile (PCN) and Aroclor 1254 (PCB) on hepatic porphyrin content in iron-dextran-treated, δ-aminolevulinic acid-supplemented (Fe/δALA) rats. All values are means, with error bars indicating the SEM. N = 7 for all groups except PCB+PCN, where N = 6. The uro- and heptacarboxylporphyrin concentrations for the Control and +PCN groups were 0.72 ± 0.06, 0.10 ± 0.05 and 1.41 ± 0.64, 0.21 ± 0.08 μg/g tissue, respectively. Hexacarboxyl-, pentacarboxyl-, and coproporphyrin concentrations were 0 ± 0 for all treatment groups except hexacarboxylporphyrins of 1.06 ± 0.12 μg/g tissue (+PCB) and 0.48 ± 0.48 μg/g tissue (+PCB+PCN) and pentacarboxylporphyrin of 0.05 ± 0.03 μg/g tissue (+PCB+PCN). Key: (a) significantly different from the Control (Fe/δALA) group, $P < 0.05$; and (b) significantly different from the Control, PCB (Aroclor 1254, Fe/δALA), and PCN (PCN, Fe/δALA) groups, $P < 0.05$.

sants, glucocorticoids such as dexamethasone, and azole antifungal agents. With some drugs, induction of CYP3A is also accompanied by induction of several other drug-metabolizing enzymes, as well as a multidrug resistance transporter, MDR1. We report here on the effects of two CYP3A-inducing agents, which do not induce MDR1 as a complicating factor, in the rat model of PCT. The two agents, troleandomycin and 5-pregnen-3β-ol-20-one-16α-carbonitrile (pregnenolone-16α-carbonitrile), differ with respect to the extent to which the induction of CYP3A is accompanied by increased enzyme activity, since the major fraction of the CYP3A induced by troleandomycin is catalytically inactive due to its sequestration as a stable metabolic-intermediate complex. Inactivation of CYP3A by troleandomycin cotreatment has been held responsible for the decreased porphyrinogenic effect of hexachlorobenzene in rats, an effect originally attributed to the inhibited formation of the tetrachloro-1,4-benzoquinone metabolite [10], but later to a metabolite or reactive intermediate earlier in the biotransformation pathway [11]. If reactive intermediates generated from a mixture of chlorinated biphenyls (Aroclor 1254) are similarly responsible for its porphyrinogenic effect, an exacerbation of the porphyrinogenic effect would be anticipated from a pure CYP3A inducer (pregnenolone-16α-carbonitrile) as compared with an attenuating effect by an inhibitory inducer (troleandomycin).

MATERIALS AND METHODS

Mature female Fischer 344 rats from Simonson Laboratories Inc. were maintained on water containing δALA (2 g/L) neutralized to pH ~7, and with free access to food (Prolab RMH 3000). All animals received iron-dextran (400 mg iron/kg, i.p.) 22 days prior to being killed. Some animals received Aroclor 1254 (63 mg/kg, i.p., in 0.6 mL of corn oil vehicle) 21 days prior to being killed; others received only corn oil vehicle. Additional groups of animals were treated daily with pregnenolone 16α-carbonitrile (25 mg/kg, i.g.) or troleandomycin (500 mg/kg, i.g.). All chemicals used for the treatment of animals were obtained from the Sigma Chemical Co. except for Aroclor 1254, which was obtained from the Monsanto Chemical Co. Urine was collected from individual animals 1 day before they were killed and was analyzed for porphyrins. At killing, livers were removed, and small pieces were analyzed for total porphyrin and iron content. The remaining liver was homogenized in 0.25 M sucrose, and microsomal fractions were prepared by differential centrifugation for the determination of cytochrome P450 concentration and CYP1A (ethoxyresorufin and methoxyresorufin dealkylations) and CYP3A (erythromycin *N*-demethylation) isozyme activities. Cytochrome P-450 determination and ethoxyresorufin and methoxyresorufin dealkylation assays were performed as described previously [2]. CYP3A-dependent erythromycin demethylase activity was determined from the formaldehyde produced [12] in 3-, 6-, and 10-min incubations containing 1 mM erythromycin, 2 mg/mL of microsomal protein, and 1.5 mM NADPH in 50 mM Tris–chloride buffer, pH 7.4, containing 150 mM KCl and 10 mM $MgCl_2$. Cytochrome P450 metabolic-intermediate complex content was determined directly and indirectly following treatment of microsomes with 50 μM potassium ferricyanide [13]. Hepatic iron content was determined by atomic absorption spectrometry following digestion of 100 μg of liver tissue in nitric/perchloric acid (5:2) at 100° for 9 hr.

Statistical analyses were performed using ANOVA, and differences between the groups were assessed by Fisher's Protected Least Significant Difference multiple range test. Differences were considered significant at P values of less than 0.05.

RESULTS

The female rat PCT model [2] is characterized by excessive accumulation of uro- and heptacarboxylporphyrins in the liver and their excretion in the urine. The accumulated liver porphyrins present on day 21 for animals treated with

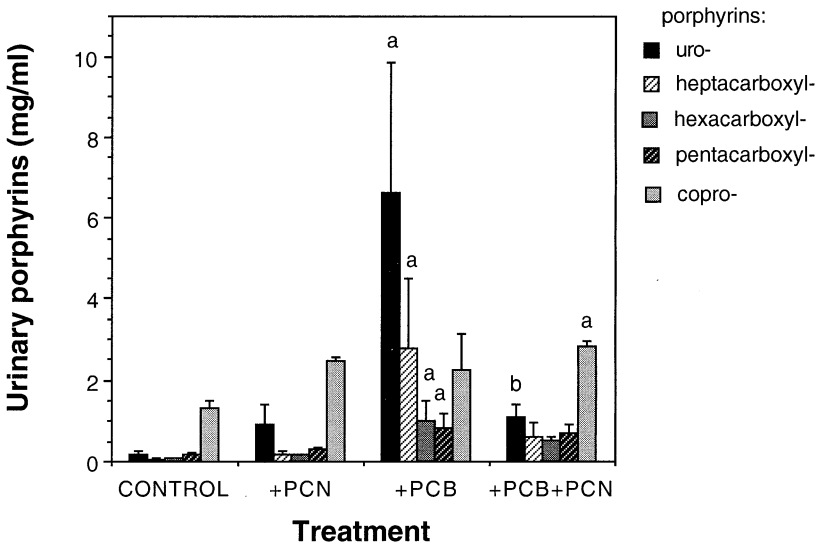

FIG. 2. Urinary porphyrin profile after treatment with 5-pregnen-3β-ol-20-one-16α-carbonitrile (PCN) and Aroclor 1254 (PCB) in iron-dextran-treated, δ-aminolevulinic acid-supplemented (Fe/δALA) rats. All values are means, with error bars indicating the SEM. N = 5 for all groups except PCB+PCN, where N = 4. Key: (a) significantly different from the Control (Fe/δALA) group, $P < 0.05$; and (b) significantly different from the PCB group (Aroclor 1254, Fe/δALA), $P < 0.05$.

pregnenolone 16α-carbonitrile, Aroclor 1254, and controls are shown in Fig. 1. In the absence of Aroclor 1254, pregnenolone 16α-carbonitrile treatment did not affect hepatic porphyrin content significantly. Pregnenolone 16α-carbonitrile treatment reduced the accumulated hepatic porphyrins by 73% in the animals treated with Aroclor 1254, although the 27% remaining still consisted predominantly of uro- and heptacarboxylporphyrins. The changes in the renal excretion of highly carboxylated porphyrins with pregnenolone 16α-carbonitrile treatment paralleled the effect on hepatic concentrations, a drastic reduction in uroporphyrin and heptacarboxylporphyrin (Fig. 2).

All experimental groups received the same dose of iron-dextran, but the hepatic iron content in animals treated with Aroclor 1254 was 26% less than in animals not receiving Aroclor 1254 (Fig. 3). Treatment with pregnenolone 16α-carbonitrile also reduced the hepatic iron content (18% less than control), and there was an almost additive effect (39% reduction) when both pregnenolone 16α-carbonitrile and Aroclor 1254 were part of the treatment regimen. The reductions notwithstanding, the iron content of all treatment groups receiving iron-dextran was an order of magnitude higher than in animals receiving their iron from their normal diet, where a hepatic iron concentration of 0.14 ± 0.02 μg/mg liver was found.

The effect of pregnenolone 16α-carbonitrile alone, and together with Aroclor 1254, on cytochrome P450 concentration and isozyme activities is shown in Fig. 4. Pregnenolone 16α-carbonitrile alone significantly induced cytochrome P450 concentration (56%) due to its ability to induce CYP3A, as demonstrated by an elevation of erythromycin *N*-demethylase activity from barely detectable (0.08 nmol/mg/min) to 1.59 nmol/mg/min. In the PCT model where Aroclor 1254 had induced CYP1A activities (ethoxyresorufin and methoxyresorufin *O*-dealkylations), the pregnenolone 16α-carbonitrile induction of CYP3A-dependent erythromycin *N*-demethylase activity was slightly (13%) attenuated. The CYP1A induction by Aroclor 1254 was unaffected by pregnenolone 16α-carbonitrile treatment.

The addition of troleandomycin treatment to the regimen required to generate experimental PCT also produced a major attenuation (78% reduction) of hepatic porphyrin accumulation (Fig. 5) and a 75% decrease in the urinary excretion of uroporphyrin and heptacarboxylporphyrin (Table 1). With troleandomycin treatment, CYP3A-dependent erythromycin *N*-demethylase activity was increased from barely detectable (0.06 ± 0.01 nmol/mg/min) to 0.46 ± 0.07 nmol/mg/min (Fig. 6). This activity was only 29% of that seen with pregnenolone 16α-carbonitrile

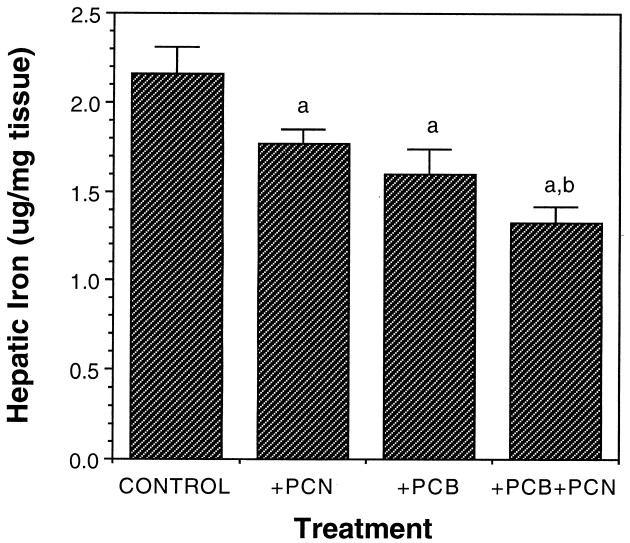

FIG. 3. Hepatic iron concentration following treatment with 5-pregnen-3β-ol-20-one-16α-carbonitrile (PCN) and Aroclor 1254 (PCB) in iron-dextran-treated, δ-aminolevulinic acid-supplemented (Fe/δALA) rats. All values are means, with error bars indicating the SEM. N = 7 for all groups except PCB+PCN, where N = 6. Key: (a) significantly different from the Control (Fe/δALA) group, $P < 0.05$; and (b) significantly different from the PCN group (PCN, Fe/δALA), $P < 0.05$.

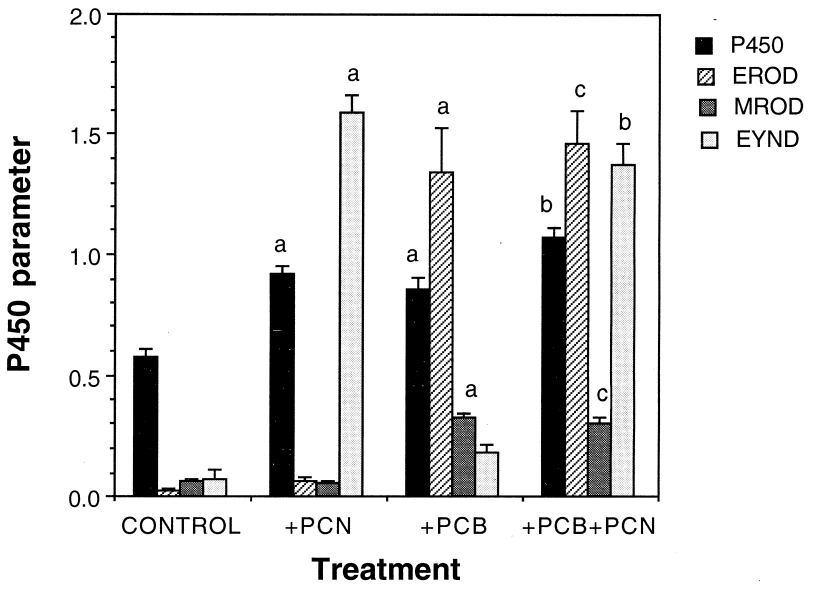

FIG. 4. Hepatic cytochrome P450 profile after treatment with 5-pregnen-3β-ol-20-one-16α-carbonitrile (PCN) and Aroclor 1254 (PCB) in iron-dextran-treated, δ-aminolevulinic acid-supplemented (Fe/δALA) rats. All values are means, with error bars indicating the SEM. N = 7 for all groups except PCB+PCN, where N = 6. Key: (a) significantly different from the Control (Fe/δALA) group, $P < 0.05$; (b) significantly different from the Control, PCB (Aroclor 1254, Fe/δALA), and PCN (PCN, Fe/δALA) groups, $P < 0.05$; and (c) significantly different from the Control and PCN groups, $P < 0.05$. Key: P450, cytochrome P450; EROD, ethoxyresorufin O-deethylase; MROD, methoxyresorufin O-demethylase; and EYND, erythromycin N-demethylase.

treatment (1.59 ± 0.07 nmol/mg/min). With Aroclor 1254 co-treatment, the troleandomycin induction of CYP3A activity appeared slightly attenuated (0.39 ± 0.08 nmol/mg/min), but this was not statistically significant. Similarly, CYP1A induction by Aroclor 1254 appeared to be slightly attenuated by troleandomycin treatment, but this did not achieve statistical significance. Thus, neither troleandomycin nor pregnenolone 16α-carbonitrile treatment affected CYP1A induction by Aroclor 1254, and Aroclor 1254 did not affect CYP3A induction by either agent. The smaller elevation in CYP3A activity with troleandomycin as compared with pregnenolone 16α-carbonitrile results from much of the induced cytochrome P450 being present as a catalytically inactive metabolic-intermediate complex, a complex that can be dissociated by mild ferricyanide treatment of the microsomes. The complex exhibits a distinctive 455 nm absorbance maximum (with a known extinction coefficient), and loss of this absorbance following ferricyanide treatment allows its direct quantitation. By this method 2.86 and 1.75 nmol/mg microsomal protein of troleandomycin-derived metabolic-intermediate complex were present in the absence and presence of concurrent Aroclor 1254 treatment, respectively (Table 2). Ferricyanide treatment of microsomes from troleandomycin-treated rats also restored the ability of the complexed cytochrome P450 to catalyze erythromycin *N*-demethylation. Gains of 2.13 and 1.68 nmol/mg/min of this activity for the two sets of microsomes, respectively, were realized. A third method for gauging the amount of complex present is to quantitate the gain in cytochrome P450 capable of binding carbon monoxide once the complex is destroyed by mild ferricyanide treatment. By this method 2.20 and 2.19 nmol

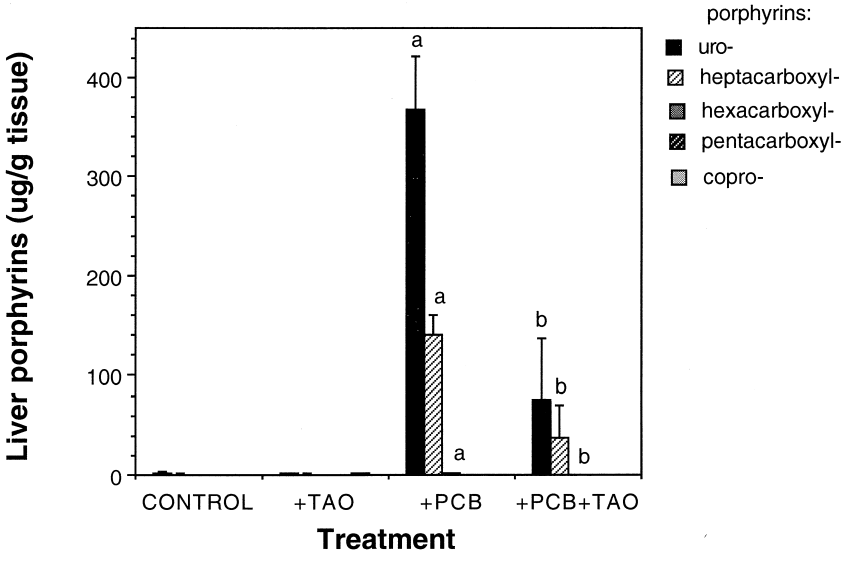

FIG. 5. Hepatic porphyrin content after treatment with troleandomycin (TAO) and Aroclor 1254 (PCB) in iron-dextran-treated, δ-aminolevulinic acid-supplemented (Fe/δALA) rats. All values are means, with error bars indicating the SEM, N = 6. The uro- and heptacarboxylporphyrin concentrations for the Control and +TAO groups were 2.59 ± 0.94, 0.80 ± 0.37 and 1.65 ± 0.62, 0.75 ± 0.50 μg/g tissue, respectively. Hexacarboxyl-, pentacarboxyl-, and coproporphyrin concentrations were 0 ± 0 μg/g tissue for all treatment groups except hexacarboxylporphyrins of 1.73 ± 0.32 μg/g tissue (+PCB) and 0.44 ± 0.44 μg/g tissue (+PCB+TAO), pentacarboxylporphyrin of 0.22 ± 0.22 μg/g tissue (+PCB), and coproporphyrins of 0.42 ± 0.42 μg/g tissue (Control), 0.92 ± 0.44 μg/g tissue (+TAO), and 0.18 ± 0.18 μg/g tissue (+PCB). Key: (a) significantly different from the Control (Fe/δALA) group, $P < 0.05$; and (b) significantly different from the PCB group (Aroclor 1254, Fe/δALA), $P < 0.05$.

TABLE 1. Highly carboxylated porphyrins present in the urine after 20 days of treatment in the rat PCT model

Treatment*	Uroporphyrin (mg/mL urine)	Heptacarboxylporphyrin (mg/mL urine)
Control (2)	1.46, 2.04	0.28, 0.00
Troleandomycin (3)	0.70 ± 0.18	0.14 ± 0.02
PCB (3)	7.96 ± 1.56†	3.34 ± 1.18†
PCB + troleandomycin (3)	2.20 ± 0.97‡	0.47 ± 0.19‡

Values are means ± SEM when N = 3, and actual values when N = 2.

*Numbers in parentheses indicate the number of animals examined per group.

†Significantly different from the Control group (iron-dextran and δALA), $P < 0.05$.

‡Significantly different from the PCB group (Aroclor 1254, iron-dextran, and δALA), $P < 0.05$.

cytochrome P450-CO/mg were present in the two sets of microsomes (i.e. without and with concurrent Aroclor 1254 treatment) from troleandomycin-treated animals. Trace amounts of complex found in microsomes of animals not receiving troleandomycin (Control and Aroclor 1254 groups) probably arose from small errors inherent to the manipulations and methods. Although there was less than complete agreement between metabolic-intermediate complex loss, erythromycin *N*-demethylase activity gain, and cytochrome P450-CO binding gain following ferricyanide treatment of microsomes, the observations combined suggest that slightly greater amounts of CYP3A are present as a complex when troleandomycin is given alone than when it is given to Aroclor 1254-treated animals.

DISCUSSION

Two agents that have in common the ability to induce CYP3A significantly attenuated the accumulation of highly carboxylated porphyrins in the liver and their excretion in the urine in a rat model of PCT. This attenuation appeared to be independent of differences in the induction properties of the two agents, occurring whether the majority of the induced CYP3A was in a catalytically active state (with pregnenolone 16α-carbonitrile induction), or was present as a catalytically inactive metabolic-intermediate complex (with troleandomycin induction). Studies have shown the necessity of CYP1A2 for the development of uroporphyria in the mouse [3], and it might be anticipated that the additional burden of heme production when inducing multiple P450 isozymes in the rat model could reduce the amount of CYP1A present. However, CYP3A induction by either pregnenolone 16α-carbonitrile or troleandomycin did not alter CYP1A catalytic activities. Hepatic iron status is a known positive effector for the development of porphyria. It is unlikely that the small (13%) lowering of iron concentration by pregnenolone 16α-carbonitrile in this iron-overloaded rat model of PCT would account for such a large attenuation of porphyrin accumulation and excretion. The hepatic iron concentration is still an order of magnitude higher than in animals obtaining their iron from normal dietary sources. Why pregnenolone 16α-carbonitrile treatment results in a lowering of hepatic iron is not known. Liver iron is determined as total iron, and any use of storage iron for additional cytochrome P450 formation would result in no net change.

Induction of proteins other than cytochrome P450s, particularly the multidrug transporter P-glycoprotein [14, 15], by CYP3A-inducing agents is well documented, but this has no known function in regulating intracellular concentrations of porphyrins. Regardless, neither troleandomycin nor pregnenolone 16α-carbonitrile induces this protein in rats [16]. Other liver enzymes induced by pregnenolone 16α-carbonitrile and troleandomycin include UDP-glucuronosyltransferases [17] and, for pregnenolone 16α-carbonitrile, sulfotransferases [18], but the only known role for these enzymes in tetrapyrrole biosynthesis and metabolism is in the glucuronidation of bilirubin, the final heme degradation product. One remaining explanation for

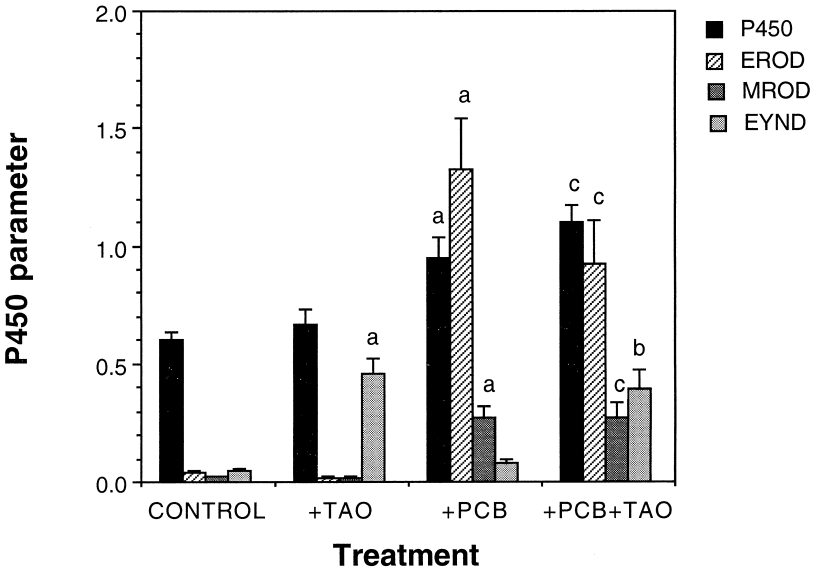

FIG. 6. Hepatic cytochrome P450 profile after treatment with troleandomycin (TAO) and Aroclor 1254 (PCB) in iron-dextran-treated, δ-aminolevulinic acid-supplemented (Fe/δALA) rats. All values are per milligram of microsomal protein, nanomoles per milligram for cytochrome P450, and nanomoles per milligram per minute for enzyme activities. Values are means, with error bars indicating the SEM, N = 6. Key: (a) significantly different from the Control (Fe/δALA) group, $P < 0.05$; (b) significantly different from the Control and PCB (Aroclor 1254, Fe/δALA) groups, $P < 0.05$; and (c) significantly different from the Control and TAO (TAO, Fe/δALA) groups, $P < 0.05$. Key: P450, cytochrome P450; EROD, ethoxyresorufin O-deethylase; MROD, methoxyresorufin O-demethylase; and EYND, erythromycin N-demethylase.

TABLE 2. Quantification of cytochrome P450 present as a metabolic-intermediate (MI) complex as revealed by *in vitro* ferricyanide treatment

Animal treatment	MI complex loss* (nmol/mg)	EYND gain* (nmol/mg/min)	P450-CO gain* (nmol/mg)
Control	0.00 ± 0.01 (3)	0.09 ± 0.05 (3)	0.03 ± 0.04 (6)
Troleandomycin	2.86 ± 0.06† (3)	2.13 ± 0.29† (6)	2.20 ± 0.16† (6)
PCB	0.01 ± 0.02 (3)	0.08 ± 0.04 (4)	0.08 ± 0.06 (6)
PCB + troleandomycin	1.75 ± 0.10‡ (3)	1.68 ± 0.35§ (6)	2.19 ± 0.33§ (6)

Values are per mg microsomal protein and are means ± SEM. The numbers in parentheses indicate the number of animals per group.

*Losses and gains following treatment of microsomes with 50 μM ferricyanide, as described in Materials and Methods.

†Significantly different from the Control group (iron-dextran and δALA), $P < 0.05$.

§Significantly different from both Control and PCB (Aroclor 1254, iron-dextran, and δALA) groups, $P < 0.05$.

‡Significantly different from Control and PCB and troleandomycin (troleandomycin, iron-dextran, and δALA) groups, $P < 0.05$.

the common effects of troleandomycin and pregnenolone 16α-carbonitrile may relate to the induction of CYP3A activity. Even the small increase in CYP3A activity seen with troleandomycin induction, where the majority of the induced enzyme is catalytically inactive, is sufficient to attenuate the porphyria. Such an explanation has additional appeal because it is more difficult to develop porphyria in male rats [2, 19, 20], the sex that expresses higher basal levels of CYP3A [21]. Troleandomycin treatment inhibited the development of uroporphyria in two studies where a hexachlorobenzene-treated rat model was utilized [10, 11], and the effect was attributed to an inhibition of hexachlorobenzene metabolism. We obtained similar results of uroporphyria attenuation by troleandomycin in our PCB-treated rat model. However, we found a similar attenuation of the porphyric response by pregnenolone 16α-carbonitrile, a compound not possessing any of the cytochrome P450 inhibitory characteristics of troleandomycin. Inhibition of any CYP3A-dependent metabolism of chlorinated aromatic compounds, which has been suggested elsewhere, is therefore an unlikely explanation for the attenuation effect. CYP3A induction, not inhibition, is the effect that both agents have in common, but how the induction response results in attenuation is unclear. There appears to be no interplay of CYP3A induction with CYP1A2 induction, the presence of which is essential for the development of uroporphyria in mice [3]. Possible interplay with the expression or function of rat gender-specific cytochrome P450 isozymes remains an uninvestigated possibility, since it is more difficult to induce uroporphyria in male rats in the Aroclor 1254 model. Effects of troleandomycin and pregnenolone 16α-carbonitrile not related to cytochrome P450s remain as possible explanations for the uroporphyria-attenuating effect.

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