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IDENTIFICATION OF THE HEPATIC CYTOCHROME P-450 ISOZYMES INDUCED AND DECREASED BY PICLORAM

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Abstract—Microsomes from male rats treated with picloram (100 mg/kg/day) for 7 days showed a 48% decrease in 16α-hydroxylase activity when incubated with (4-^{14}C) androstenedione. These data are consistent with the assertion that picloram decreases the titer of hepatic male specific cytochrome P-450h. Several lines of evidence suggested that picloram is an inducer of hepatic cytochrome P-450d in male rats. First, SDS polyacrylamide gel electrophoresis revealed an intensified hepatic microsomal polypeptide (MW 54,000) following picloram pretreatment. This polypeptide co-migrated with protein bands which were correspondingly intensified after pretreatment with known inducers of cytochrome P-450d (3-methylcholanthrene and isosafrole). Second, no increase in the binding of metyrapone to picloram treated microsomes was noted compared with controls, suggesting no increase in phenobarbital-inducible forms of cytochrome P-450. Third, hepatic microsomes from picloram treated rats activated 2-amino-3-methylimidazo [4, 5-f] quinoline (a cytochrome P-450d mediated catalysis) causing a 5-fold increase in the number of induced *Salmonella typhimurium* TA98 revertant colonies formed compared with control microsomes. Fourth, the binding of *n*-octylamine to hepatic microsomes from picloram-treated rats showed, like microsomes from 3-methylcholanthrene-treated rats, an increase in the proportion of high-spin cytochrome P-450 present. Cytochrome P-450d is known to be a high spin haemoprotein.

Picloram (4-amino-3,5,6-trichloropicolinic acid) is a widely used systemic herbicide, effective in the control of most broad-leaved plants. In a previous study [1] we showed that administration of picloram to male and female rats altered hepatic cytochrome P-450 mediated monooxygenase activity. Specifically, picloram induced ethoxyresorufin and ethoxycoumarin-*O*-deethylation in male and female rats and decreased aldrin epoxidation in male rats only. To determine which hepatic cytochrome P-450 isozymes were involved in these changes, microsomes from picloram-treated male rats were (a) assayed for steroid hydroxylase activity using (4-^{14}C) androst-4-ene-3,17-dione (androstenedione), (b) subjected to sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE), (c) assayed for metyrapone and *n*-octylamine binding, and (d) incubated with 2-amino-3-methylimidazo[4,5-f] quinoline and benzo[a]pyrene to examine metabolic activation to mutagenic intermediates as measured by *Salmonella typhimurium* TA 98 reversion.

MATERIALS AND METHODS

Chemicals. Technical picloram (92–96% purity) lot no. TA 840406-A136 was a generous gift from Dow Chemicals Australia Limited. (4-^{14}C) Androst-4-ene-3,17 dione was obtained from Amersham Australia, Sydney, NSW; 3-methylcholanthrene (3-MC), *n*-octylamine, benzo[*a*]pyrene (B[*a*]P), unlabelled androst-4-ene-3,17-dione, 6β-hydroxy- and 16α-hydroxyandrost-4-ene-3,17-dione were obtained from Sigma Chemical Company (St Louis, MO); 7α-hydroxyandrost-4-ene-3,17-dione was obtained from Professor D. N. Kirk and the MRC Steroid Reference Collection, Queen Mary's College, London, U.K. 16β-Hydroxyandrost-4-ene-3,17-dione was prepared as described [2]. Isosafrole was obtained from Eastman Kodak Company (Rochester, NY) and redistilled prior to use. Metyrapone was purchased from Aldrich Chemical Company (Milwaukee, WI). Chemicals for electrophoresis were obtained from Bio Rad (Sydney, NSW). Enzymes and cofactors were purchased from Sigma Chemical Company and Boehringer Mannheim (Sydney, NSW). 2-Amino-3-methylimidazo[4,5-f] quinoline (IQ) was kindly donated by Dr T. Sugimara, National Cancer Centre Research Institute, Tokyo, Japan.

Animals. Male Sprague–Dawley rats (body wt 220–400 g) from the University of Sydney Animal House were allowed free access to food and water during these studies. Picloram (100 mg/kg) dissolved in dimethylsulfoxide (DMSO: 1 ml/kg) was administered i.p. daily to rats for 7 days. 3-MC (40 mg/kg) and isosafrole (150 mg/kg) were dissolved in corn oil and administered i.p. daily for 3 days. Controls received the same volume of vehicle. Rats were

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Table 1. Effects of picloram administration on androstenedione hydroxylation in male rat liver microsomes

	Hydroxylase activity			
	nmol product/min/mg protein			
Microsomes	16α-OH.A	6β-OH.A	7α-OH.A	16β-OH.A
Control	1.28 ± 0.18*	0.46 ± 0.02	0.18 ± 0.02	0.11 ± 0.01
Picloram†	0.62 ± 0.15‡	0.44 ± 0.09	0.20 ± 0.01	0.10 ± 0.02
% of control	48	96	116	91

* Values represent means ± SE; N = 6.
† Picloram administered i.p. daily (100 mg/kg) for 7 days; controls received DMSO (vehicle) 1 ml/kg.
‡ Significantly different from control $P < 0.05$.

sacrificed 24 hr after final dosing. The number of animals used in each experiment (N) ranged from 3 to 6. Microsomes were prepared as described [1].

Protein and enzyme assays. Microsomal protein was estimated by the modified Lowry method of Chaykin [3]. Androstenedione hydroxylase activity was determined as described [2] following the method of Gustafsson and Ingelman-Sundberg [4].

Optical difference spectroscopy. Cytochrome P-450 content was determined according to the method of Omura and Sato [5] with modifications [1]. Metyrapone complexation with dithionite reduced hepatic microsomal cytochrome P-450 was determined following Liu and Franklin [6] using an extinction coefficient of 68.5 mM^{-1} cm^{-1} at 446 nm. The binding of *n*-octylamine with hepatic microsomal cytochrome P-450 was determined essentially as described by Jefcoate *et al.* [7]. The final concentration of *n*-octylamine was 2 mM in the sample cuvette, as 1 mM did not appear to be completely saturating.

Mutagenicity assays. IQ (final concentration 0.2 mM) was incubated with 50 mg of microsomal protein as described [8] and then assayed for mutagenicity towards *Salmonella typhimurium* TA98. Activation of B[*a*]P by microsomal fractions was measured by direct plate incorporation [9]. Briefly, 5 μg B[*a*]P was incubated with control or picloram-treated microsomes (25 μg–2 mg/plate), an NADPH generating system and *Salmonella typhimurium* TA98. The number of revertant colonies was counted after 3 days incubation at 37°.

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE). Microsomal fractions were prepared for electrophoresis by incubation with 4% sodium dodecylsulphate and 5% 2-mercaptoethanol as described by Toftgaard *et al.* [10]. Molecular weight markers were phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), and carbonic anhydrase (31,000). Stained gels were scanned using a Hoefer GS-3000 scanning densitometer interfaced with an Apple IIe.

Statistics. All results were evaluated by an analysis of variance and compared using the least significant difference. Statistical significance was set at $P < 0.05$. When sample variances were non-homogeneous the Behrens–Fisher test was employed to approximate t'.

RESULTS AND DISCUSSION

Effect of picloram on microsomal androstenedione hydroxylase activities

The hydroxylation of the C-19 steroid androstenedione has been shown to be stereo- and regioselectively catalysed by different cytochrome P-450 isozymes [11]. In control (untreated) male rats, 16α-hydroxylation is associated with cytochrome P-450h and 16β-hydroxylase activity with the phenobarbital inducible forms cytochrome P-450 b/e. As can be seen in Table 1 administration of picloram to male rats resulted in a significant decrease in the 16α hydroxylation pathway to 48% of control. This suggests that a decrease of the male specific isozyme cytochrome P-450h is associated with picloram pretreatment. In a previous study [1] we showed that a sex difference existed in the response of aldrin epoxidation to picloram administration. In contrast to males, female rats showed no decrease in microsomal aldrin epoxidase activity when administered picloram. Further, in agreement with other studies [12], a 10-fold difference in aldrin epoxidation was noted between male and female control rats. A possible explanation for these observations is provided by the androstenedione 16α-hydroxylase data. A similar decrease in aldrin epoxidase acivity (46%) [1] and androstenedione 16α-hydroxylase activity (48%) was observed following the picloram pretreatment regimen. Thus, it is feasible that the male-specific cytochrome P-450h is active in both of these monooxygenase pathways. Accordingly, control male rats with a 10-fold greater titer of cytochrome P-450h [11] than female control rats exhibit much higher basal aldrin epoxidase activity [1]. Decreases in cytochrome P-450h content in males result in significant decreases in aldrin epoxidation while in females little or no change is noted.

Sodium dodecylsulphate polyacrylamide gel electrophoresis of microsomal proteins

In an attempt to identify the cytochrome P-450 isozyme(s) involved in the induction of ethoxyresorufin-*O*-deethylase by picloram noted in our previous study [1] hepatic microsomes from picloram-treated rats were subjected to SDS–PAGE (Fig. 1). Gels of picloram-treated microsomes were scanned

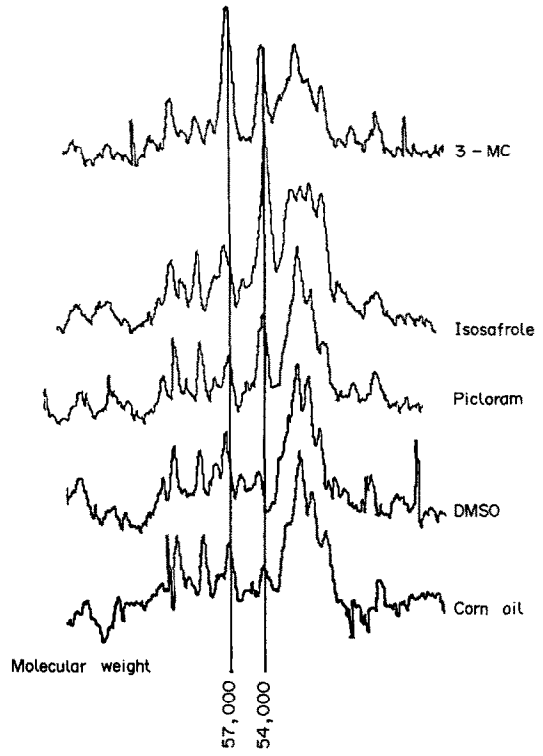

Fig. 1. Densitometric profile of stained proteins on SDS–polyacrylamide gels in the cytochrome P-450 molecular weight range. 60 μl of treated microsomes, containing 15 μg of protein were placed in each well. Treatments, 3-MC = 3-methylcholanthrene, DMSO = dimethylsulphoxide.

in the cytochrome P-450 molecular weight range 47,000–58,000 [13] and compared with 3-MC and isosafrole induced microsomes. Densitometric analysis (Fig. 1) showed that both 3-MC and isosafrole treatments resulted in the increased protein staining of two bands of molecular weights 57,000 and 54,000 compared with controls. 3-MC has been shown to induce two cytochrome P-450 isozymes namely cytochromes P-450c and P-450d. Cytochrome P-450c has a molecular weight range of 56–58,000 while cytochrome P-450d has a range of 52–54,000 [14, 15]. Isosafrole has been shown to induce four cytochrome P-450 isozymes: principally cytochrome P-450d and to a smaller extent, cytochromes P-450c, P-450b/e and P-450 PCN-E, in that order [2]. Figure 1 shows a large increase in peak height and area at molecular weight 54,000 present in microsomal fractions from isosafrole-treated rats, consistent with induction of cytochrome P-450d. Figure 1 also reveals that picloram-induced microsomes show a substantial increase in peak height and area at 54,000 daltons which is suggestive of cytochrome P-450d induction. Cytochrome P-450d induction in picloram treated microsomes is consistent with increases in ethoxyresorufin-*O*-deethylase activity noted in our previous study [1] as cytochrome P-450d has high ethoxydeethylase activity in reconstituted systems [16].

Metyrapone binding

Cytochrome P-450 forms P-450b, P-450 PCN-E and P-450d have similar molecular weights [14] and thus are not well resolved by SDS–PAGE. It may be possible therefore that the increased protein staining band of MW 54,000 noted in microsomes from picloram-treated rats may be due to increases in titers of not only cytochrome P-450d but also P-450b/e and P450 PCN-E. The androstenedione hydroxylase data (Table 1) suggest otherwise. No increases in cytochrome P-450b/e mediated 16β-hydroxylase activity or the P-450 PCN-E catalysed 6β-hydroxylase pathway were observed arguing against increased levels of these isozymes. To confirm that cytochrome P-450b/e and P-450 PCN-E were not elevated after picloram administration, we compared the binding of metyrapone to control and picloram-induced microsomes. Metyrapone binds with dithionite-reduced cytochrome P-450 to produce a stable ferrocytochrome–metyrapone complex which absorbs maximally at 446 nm [6]. Lui and Franklin [6] have shown that this complex is formed between two sub-populations of cytochrome P-450; the first being a constitutive form(s) and the second, a form(s) inducible by phenobarbital. It is likely that the second population comprises both cytochrome P-450b/e and P-450 PCN-E, as administration of cytochrome P-450 PCN-E inducers substantially increases metyrapone binding [17]. As can be seen in Table 2, no difference in the percentage of cytochrome P-450 bound to metyrapone was observed between control and picloram-treated microsomes. This suggests that no phenobarbital/pregnenolone 16α-carbonitrile inducible forms are increased after administration of picloram to male rats and confirms the findings of the androstenedione hydroxylase data.

Mutagenesis

The results of the electrophoresis, androstenedione metabolism and metyrapone binding experiments suggest induction of cytochrome P-450d by picloram. However, the possibility remains that

Table 2. Effects of picloram administration on the binding of metyrapone to dithionite-reduced male hepatic microsomes

Microsomes	% Cytochrome P-450 bound to metyrapone
Control	26.0% ± 1.0*
Picloram†	24.1% ± 1.2

* Values represent means ± SE; N = 4.

† Picloram administered i.p. daily (100 mg/kg) for 7 days; controls received DMSO (vehicle) 1 ml/kg.

Table 3. Activation of IQ by hepatic microsomes from control or picloram treated male rats

Microsomes	N	No. of TA 98 revertants/μg protein
Control (DMSO)	3	85.84 ± 5.94*
Picloram†	3	436.96 ± 112.2‡

* Values represent means ± SE.
† Picloram administered i.p. daily (100 mg/kg) for 7 days; controls received DMSO (vehicle) (1 ml/kg).
‡ Significantly different from control $P < 0.05$, using Behrens–Fisher test.

picloram weakly induces cytochrome P-450c to a level not detectable by SDS–PAGE, but enough to elevate ethoxyresorufin-*O*-deethylase activity. Recent study has shown that cytochrome P-450d specifically catalyses the *N*-hydroxylation of pyrolysate products Trp-P-1, Glu-P-1 and IQ [18]. *N*-hydroxylation of these products is associated with mutagen formation [18]. The activation of IQ to mutagenic species as detected by *Salmonella typhimurium* TA98 is catalysed almost exclusively by cytochrome P-450d [8, 18]. Incubation of IQ with microsomes from picloram-treated rats (Table 3) resulted in a 5-fold increase in the number of revertant colonies formed compared with controls. This suggests induction of cytochrome P-450d in microsomes from picloram-treated rats, resulting in the increased formation of IQ mutagens. When B[*a*]P (5 μg) was directly incorporated onto agar plates with a NADPH generating system and control and microsomes isolated from picloram-treated rats (25–2 mg/plate), no increased mutagenesis, associated with picloram treatment, was noted (data not shown). This result suggests that picloram does not induce cytochrome P-450c, as B[*a*]P is rather specifically activated by this isozyme.

Quantitation of spin state

Hepatic cytochrome P-450d is unique amongst other liver cytochrome P-450 isozymes in that it is predominantly a high-spin haemoprotein. Increases in cytochrome P-450d titer would therefore be consistent with increases in the proportion of native cytochrome P-450 in the high-spin state. Table 4 shows that both 3-MC- and picloram-treated microsomes have increased levels of high-spin cytochrome P-450 compared with controls as calculated from the *n*-octylamine difference spectrum. This result therefore is not inconsistent with the induction of the predominantly high-spin cytochrome P-450d.

The data presented here suggest that picloram decreases cytochrome P-450h content and induces cytochrome P-450d. The extent of cytochrome P-450d induction was not directly quantified but from the data presented appears substantial.

Aniline p-hydroxylation has been shown to be catalysed principally by cytochrome P-450d [14]. However, in a previous study, we failed to detect any increase in aniline hydroxylation after picloram treatment [1]. Several authors have noted that aniline hydroxylation is not a sensitive indicator of cytochrome P-450d induction. Ueno *et al.* [19] did not observe an increase in aniline hydroxylase activity after 3-MC induction in rats though presumably there was at least a 3–4-fold increase in cytochrome P-450d content [18]. Similarly, Seidel and Shires [20] observed low catalytic activity of a high-spin form of cytochrome P-450 (presumably identical with P-450d) toward aniline. The situation is further complicated by the effects of down regulation of constitutive enzymes in microsomal preparations. As observed here cytochrome P-450h, which is fairly active in aniline hydroxylation, was decreased by picloram administration. This decrease may have masked the induction of cytochrome P-450d-

Table 4. Effect of treatment on proportion of high-spin cytochrome present in male rat liver microsomes

Treatment	ΔA 410–500 nm / ΔA 392–500 nm	% High-spin* cytochrome P-450
Control (corn oil)	1.17 ± 0.016†	15
3-MC‡	0.79 ± 0.031§	30
Control (DMSO)	1.14 ± 0.025	17
Picloram	0.87 ± 0.013§	26

* Calculated from the *n*-octylamine difference spectrum as described by Jefcoate *et al.* [7].
† Values represent means ± SE; N = 4.
‡ 3-MC administered i.p. daily (40 mg/kg) for 3 days; controls received corn oil (2 ml/kg). Picloram administered i.p. daily (100 mg/kg) for 7 days; controls received DMSO (vehicle) 1 ml/kg.
§ Significantly different from respective control $P < 0.05$.

mediated aniline hydroxylase activity. Thus, the failure to observe aniline hydroxylase induction in picloram-treated microsomes does not exclude induction of cytochrome P-450d.

Studies by Thomas *et al.* [21] suggest that cytochrome P-450c and P450d are under co-ordinate control. Thus, inducers of cytochrome P-450c would be expected to induce cytochrome P-450d and vice versa. Recent work, however [22], has shown that 4-aminoazobenzene derivatives appear to induce cytochrome P-450d without increasing cytochrome P-450c content. From the present studies it would appear that picloram induction is similarly associated with the induction of cytochrome P-450d but not P-450c. It is interesting then to consider the literature on compounds structurally related to picloram. Hexachlorobenzene (HCB) and pentachlorophenol (PCP) are chlorinated aromatic systems that are structurally similar to picloram and both compounds have been shown to induce ethoxyresorufin-*O*-deethylase activity in rats [23]. A recent publication [24] showed that when HCB-induced microsomes were subjected to SDS–PAGE a band of MW 52,000 was induced, not MW 56,000 as would be expected if cytochrome P-450c was increased. These results suggest that, like picloram, HCB may be a cytochrome P-450d inducer and further, that chlorinated monocyclics such as PCP induce ethoxyresorufin-*O*-deethylase activity by inducing cytochrome P-450d rather than P-450c.

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