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IDENTIFICATION OF THE CYANOPREGNENOLONE-INDUCIBLE FORM OF HEPATIC CYTOCHROME P-450 AS A CATALYST OF ALDRIN EPOXIDATION

SAMMYE L. NEWMAN and PHILIP S. GUZELIAN*
Division of Clinical Toxicology and Environmental Medicine, Medical College of Virginia, Richmond, VA 23298, U.S.A.

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Abstract—In light of recent suggestions that hepatic microsomal aldrin expoxidation activity selectively reflects the phenobarbital (PB)-inducible form(s) of cytochrome P-450 (P-450$_{PB}$), we tested the effect of pregnenolone-16α-carbonitrile (PCN), a synthetic steroid that induces P-450$_{PCN}$, a form of the cytochrome biochemically and immunochemically distinguishable from P-450$_{PB}$. In hepatic microsomes prepared from rats receiving PB, 3-methylcholanthrene (3-MC), or PCN, the latter compound produced a greater increase in aldrin epoxidation activity relative to control than did PB, whereas 3-MC decreased enzyme activity. Moreover, the aldrin epoxidation activity in microsomes prepared from PCN- or PB-pretreated rats was selectively inhibited by form-specific antibodies directed against P-450$_{PCN}$ or P-450$_{PB}$, respectively, whereas anti-P-450$_{MC}$ antibodies gave no inhibition with microsomes prepared from induced or control animals. We conclude that P-450$_{PCN}$, P-450$_{PB}$, and probably other cytochromes P-450 catalyze aldrin epoxidation, precluding use of this enzyme as a specific marker of a single form of the cytochrome.

There is conclusive evidence that the hepatic microsomal monooxygenases known collectively as cytochrome P-450 represent a family of structurally distinct isoenzymes capable of oxidizing numerous environmental or endogenous substrates. Although tests of catalytic activity with model substrates have classically been employed to attempt to differentiate one form of cytochrome P-450 from another, it is clear that purified cytochrome P-450 isoenzymes exhibit overlapping substrate specificities (reviewed in Ref. 1). Hence, a given xenobiotic oxidizing activity, even when tested in the presence of "diagnostic inhibitors" [2], may reflect the combined effects of several distinct molecular forms of cytochrome P-450. Nevertheless, in theory there might be substrates that are specifically and exclusively oxidized by a single form of cytochrome P-450. Indeed, it has been suggested that the conversion of aldrin to its epoxide derivative, dieldrin, is catalyzed selectively by P-450$_{PB}$†, the major form of cytochrome P-450 in the liver of adult rats treated with phenobarbital (PB) [3–6]. Thus, aldrin epoxidation is absent from fetal microsomes, is inducible only by PB, and is blocked by inhibitors of P-450$_{PB}$-supported catalytic activity. However, aldrin monooxygenase is reported to be inducible by steroids in cultures of hepatoma cells [7] or fetal hepatocytes [5], systems wherein cytochrome P-450 is not inducible by PB. Moreover, the ontogenic appearance of aldrin epoxidation in rat liver parallels that of ethylmorphine demethylase [3], a monooxygenase activity preferentially induced by the steroid, pregnenolone-16α-carbonitrile (PCN) [8]. This inconsistency prompted us to test the hypothesis that aldrin epoxidation is catalyzed by P-450$_{PCN}$, a form of hepatic cytochrome P-450 predominant in PCN-treated rats that can be distinguished from P-450$_{PB}$ by biochemical and immunochemical criteria [8]. Furthermore, P-450$_{PCN}$ is inducible by steroids in hepatocyte cultures [9]. Relying on form-specific antibodies to P-450$_{PB}$ and P-450$_{PCN}$, we now demonstrate that aldrin epoxidation is catalyzed by at least these two forms of cytochrome P-450.

MATERIALS AND METHODS

Materials. Male (175–200 g) and female (75–100 g) Sprague–Dawley rats were purchased from Flow Laboratories (Dublin, VA) and were housed in wire-bottomed cages with free access to chow and water. Aldrin and dieldrin standards were obtained from Supelco, Inc. (Belefonte, PA). All other chemicals and reagents were of the highest purity commercially available.

Analytical techniques. Microsomes were prepared as described [8] from the livers of male (180–200 g) or female (75–100 g) rats prepreated by intraperitoneal injections of PB (100 mg/kg in saline daily for 4 days), 3-methylcholanthrene (3-MC) (20 mg/kg in corn oil daily for 3 days), PCN (75 mg/kg in water given orally every 12 hr for five doses), or their respective vehicle-treated controls. Protein content was determined colorimetrically [10]. Aldrin epoxidation by microsomes *in vitro* was measured as the

* Address all correspondence to: Dr Philip S. Guzelian, Division of Clinical Toxicology and Environmental Medicine, Medical College of Virginia, Box 267, MCV Station, Richmond, VA 23298, U.S.A.

† Abbreviations: PB, phenobarbital; PCN, pregnenolone-16α-carbonitrile; 3-MC, 3-methylcholanthrene. P-450$_{PB}$, P-450$_{PCN}$, and P-450$_{MC}$ refer to the major forms of cytochrome P-450 purified from rat liver microsomes prepared from animals treated with PB, PCN, and 3-MC respectively.

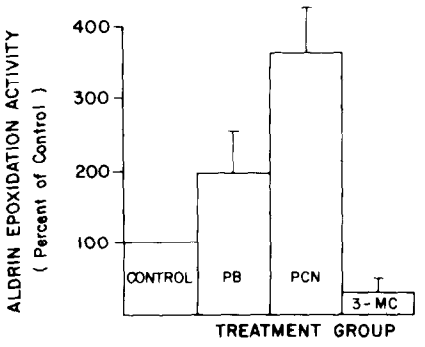

Fig. 1. Effect of cytochrome P-450 inducers on liver microsomal aldrin epoxidation activity. Rats were treated with PB (males), 3-MC (males), or PCN (females), or the respective vehicles; liver microsomes were prepared, and conversion of aldrin to dieldrin *in vitro* was measured by gas chromatography (see Materials and Methods). Results were calculated as nmoles of dieldrin formed per min per mg of microsomal protein and are expressed as the percent (mean ± S.D., N = 4 or 5) of respective control values which were 3.82 ± 0.83 (N = 4) and 0.71 ± 0.19 (N = 3) for males and females respectively.

rate of formation of dieldrin determined by gas chromatography with the use of authentic dieldrin standards to monitor recovery [3]. In some experiments, IgG prepared from the sera of non-immune goats or goats immunized against purified cytochromes P-450 (provided by Dr. N. A. Elshourbagy) [8] (saturating amounts up to 15 mg IgG/nmole cytochrome P-450 based on preliminary experiments) was incubated with microsomes for 5 min at 37° before addition of the other components of the reaction mixture. The dieldrin was quantified by measuring peak heights in comparison to a standard curve and correcting for recovery (average 85%) with the use of dieldrin standards (four concentrations per experiment).

RESULTS AND DISCUSSION

We measured rates of dieldrin formation by liver microsomes prepared from rats treated with inducers and found that PB increased catalytic activity (by 2-fold), whereas 3-MC decreased microsomal aldrin epoxidation (by 70%) as compared to the rates in microsomes prepared from control animals (Fig. 1). These observations are in agreement with those reported by Wolff *et al.* [3]. However, the highest increase in aldrin epoxidation was achieved using microsomes prepared from animals treated with PCN, a cytochrome P-450 inducer not tested by Wolff *et al.* (Fig. 1). This finding suggests that aldrin epoxidation is not exclusively supported by P-450$_{PB}$, but may also be catalyzed by P-450$_{PCN}$.

It might be argued that the increase in aldrin expoxidation activity in PCN-treated livers resulted from an incidental induction of P-450$_{PB}$ by PCN. One might test this hypothesis by comparing in a reconstituted system the catalytic activities of purified P-450$_{PB}$ and P-450$_{PCN}$ but unfortunately the latter purified hemoprotein is inactive in such a system [8]. Therefore, we tested the inhibitory effects of form-specific anti-cytochrome P-450 antibodies on aldrin epoxidation in microsomes from "induced" animals (Table 1). We chose to use antibodies rather than certain "diagnostic inhibitors" such as SKF-525A or 7,8-benzoflavone which have been used in the past to differentiate between the various forms of cytochrome P-450 [4] because the specificity of such inhibitors for isolated forms of cytochrome P-450 has not yet been established. In contrast, anti-P-450$_{PB}$ IgG and anti-P-450$_{PCN}$ IgG react specifically with P-450$_{PB}$ and P-450$_{PCN}$, respectively, with no cross-reactivity as judged by Ouchterlony double diffusion analysis [8] or by immunoprecipitation tests [9, 11]. The results revealed that anti-P-450$_{PCN}$ IgG significantly inhibited aldrin expoxidation activity in liver microsomes derived from rats treated with each of the three inducers tested, but not in microsomes from control animals (Table 1). The extent of inhibition of aldrin epoxidation by anti-P-450$_{PCN}$ IgG was comparable to its inhibition of ethylmorphine demethylase activity [8]. Significant inhibition by anti-P-450$_{PB}$ IgG was only detected in microsomes from PB-treated animals. These data are in line with

Table 1. Effect of anti-cytochrome P-450 antibodies on aldrin expoxidation by rat liver microsomes*

Source of liver microsomes		Aldrin epoxidation (% of control value with non-immune IgG)		
Treatment	Gender	Anti-P-450$_{PCN}$	Anti-P-450$_{PB}$	Anti-P-450$_{MC}$
Vehicle	Female	114	98 ± 104	145
Vehicle	Male	115 ± 19	103 ± 21	141 ± 20
PCN	Female	56 ± 14†	97 ± 8	132 ± 8†
PCN	Male	87 ± 12	94 ± 12	120 ± 16
PB	Male	87 ± 8†	33 ± 7†	118 ± 8
3-MC	Male	59 ± 6†	75 ± 12	112 ± 8

* Aldrin epoxidation was measured in liver microsomes prepared from rats receiving the indicated treatments (see Materials and Methods). For each microsomal preparation, the inhibitory effect of adding form-specific anti-cytochrome P-450 IgG was compared to that of a control incubation containing an equal amount of non-immune IgG. With the exception of the untreated female, all results represent means ± S.D. for three to five animals.

† Values achieving statistical significance ($P < 0.05$ by Student's *t*-test).

the conclusion that aldrin epoxidation is actively supported by P-450$_{PCN}$ as well as by P-450$_{PB}$. The lack of inhibition by the three antibodies on activity in control microsomes implies the involvement of other basal forms of cytochrome P-450 as well. Moreover, the possibility of sex-related differences in such postulated basal forms may be inferred from the levels of aldrin epoxidation in untreated animals (Fig. 1).

Immunoquantitation of cytochromes P-450 has revealed that (a) microsomes from untreated rats contain low or undetectable amounts of P-450$_{PCN}$ and P-450$_{PB}$ [12–15] and that (b) PB treatment induces not only P-450$_{PB}$, but P-450$_{PCN}$ as well [15, 16]. This would explain (a) our failure to find inhibition of aldrin epoxidation in control microsomes by anti-P-450$_{PCN}$ IgG or anti-P-450$_{PB}$ IgG, and (b) the inhibition of enzyme activity in PB microsomes by anti-P-450$_{PCN}$ IgG. However, the inhibition of activity by anti-P-450$_{PCN}$ IgG in microsomes from 3-MC-treated animals remains unexplained since 3-MC does not prominently induce P-450$_{PCN}$ [15]. A possible clue in this regard is that anti-P-450$_{MC}$ IgG actually increased aldrin epoxidation activity in all of the types of microsomal samples tested (Table 1). It is possible that P-450$_{MC}$ in 3-MC microsomes inhibits aldrin epoxidation in some manner—for example, by converting aldrin to an inhibitory metabolite—(Fig. 1), and that the low, residual activity reflects the presence of small amounts of other cytochromes P-450 including P-450$_{PCN}$. Indeed, a recent statistical analysis of the time course of induction of various murine monooxygenase activities suggested that aldrin epoxidation depends on the coordinated activities of several forms of cytochrome P-450 [17]. We conclude that the presence of aldrin epoxidation activity need not indicate the presence of P-450$_{PB}$, and that reliance on tests of catalytic activity as the sole means of characterizing cytochromes P-450 may lead to erroneous interpretations.

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