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Studies on rat liver cytochrome P-450s involved in the metabolism of antipyrine: phenobarbital- and 3-methylcholanthrene-inducible isozymes possessing 4-hydroxylase activity

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Antipyrine (AP)* plasma half-life or clearance has been used widely to assess the drug metabolizing activity in man [1, 2], although the ability to metabolize AP is not always correlated with that of other drugs [3, 4]. AP is metabolized to 4-hydroxy [5], *N*-desmethyl [6], 3-hydroxymethyl [7], 3-carboxy [8], 3,4-dihydrodihydroxy [9] and 4,4′-dihydroxy [10] derivatives in mammals (Fig. 1).

Several workers have already suggested that these metabolic pathways are catalyzed by different forms of cytochrome P-450 [11–16]. This, however, was introduced mainly by *in vivo* studies showing different magnitudes of enhancement of the above metabolic reactions by pretreatment with typical inducers such as phenobarbital (PB) and 3-methylcholanthrene (MC). In the present study, the role of cytochrome P-450s induced by pretreatment with PB and MC in the 4-hydroxylation and 3-methyl hydroxylation of AP was investigated using liver microsomes and purified cytochrome P-450 isozymes from inducer-pretreated rats.

Materials and methods

Chemicals. AP was obtained from a commercial source. 4-Hydroxy-AP and 3-hydroxymethyl-3-nor-AP were isolated from urine of rats treated with AP in our laboratory [7]. All other reagents used were from the sources described elsewhere [17, 18] or of the highest quality commercially available.

Purification of enzymes. Major forms of cytochrome P-450, which are induced with PB and MC, were partially purified from livers of male Sprague–Dawley rats (150–250 g) by the method of Guengerich and Martin [19] with some modifications. The fraction designated as the B_2 fraction by the above workers was further purified as follows. In the case of PB-pretreated animals, this fraction was found to contain two distinct forms of cytochrome P-450 (P-450-SD-I and -II). After dialysis of the B_2 fraction against 20 mM potassium phosphate (pH 7.25) containing 20% (v/v) glycerol, the above isozymes were separated by a carboxymethyl Sephadex C-50 (Pharmacia Fine Chemicals) column chromatography using the same buffer as a effluent. While almost all of the P-450-SD-I was adsorbed, P-450-SD-II passed through the column. Purified P-450-SD-I was then obtained by eluting the column with 150 mM potassium phosphate (pH 7.25) containing 20% (v/v) glycerol. On the other hand, P-450-SD-II was dialyzed against 5 mM potassium phosphate containing 20% (v/v) glycerol, and purified by rechromatography with carboxymethyl Sephadex C-50 column using the dialyzing buffer as an effluent. Purification of MC-inducible cytochrome P-450 (P-450-SD-III) from the B_2 fraction was carried out by the method described previously [20] using hydroxylapatite column. The specific contents of P-450-SD-I, -II and -III were shown to be 14.1, 10.0 and 15.1 nmole P-450/mg protein, respectively.

NADPH-cytochrome P-450 reductase (34.3 μmole cytochrome *c* reduced/min/mg protein) was isolated from PB-treated rats by the method of Guengerich and Martin [19] with some modifications. One unit of the reductase was defined as activity of 1 μmole cytochrome *c* reduced/min/mg protein.

Determination of hydroxylase activity for antipyrine. Assay of AP 4-hydroxylase and 3-methyl hydroxylase in liver microsomes was conducted in the incubation mixture consisting of 20 μmole of AP, microsomes containing about 4 mg protein, 0.66 μmole of NADP, 16 μmole of glucose-6-phosphate, 0.2 units of glucose-6-phosphate dehydrogenase, 12 μmole of magnesium chloride and 50 mM of Tris–HCl (pH 7.4) to make a final volume of 2 ml. After incubation for 12.5 min at 37°, the reaction was terminated by ice-cooling, and *p*-phenetidine (1.6 μg), an internal standard, was added to the solution. The incubation mix-

* Abbreviations used: AP, antipyrine; PB, phenobarbital; MC, 3-methylcholanthrene.

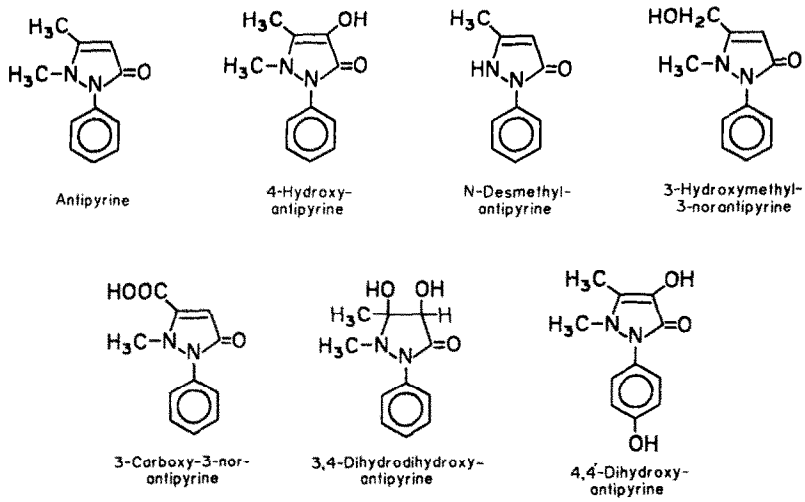

Fig. 1. Chemical structures of antipyrine and its metabolites.

ture was first extracted with 20 ml of *n*-pentane–dichloromethane (3:1, v/v) and then with 20 ml of ethyl acetate. Both organic phases were combined and evaporated to dryness under a stream of N_2 at a temperature below 35°. The residue was treated with diazomethane, and the resulting products were dissolved in 0.5 ml of methanol. A portion (10–30 μl) of this solution was applied to a Hitachi 638-30 high-pressure liquid chromatograph equipped with an ultraviolet absorption detector. The instrument was fitted with a cartridge compression unit (Z module, Waters Assoc.) which was inserted by a 0.8 × 10 cm reversed phase column, NOVA-PAK Cartridge C_{18} (Waters Associates). The chromatograph was operated to analyze 4-hydroxy-AP (as 4-methoxy derivative) and 3-hydroxymethyl-3-nor-AP by the method of Kahn *et al.* [21] with minor modifications.

AP metabolism in the reconstituted system containing purified cytochrome P-450 was measured in the incubation mixture consisting of 10 μmole of AP, 0.2 nmole of cytochrome P-450, 1.5 units of NADPH–cytochrome P-450 reductase, 15 μg of dilauroyl phosphatidyl choline, 0.33 μmole of NADP, 8 μmole of glucose-6-phosphate, 0.1 units of glucose-6-phosphate dehydrogenase, 6 μmole of magnesium chloride and 50 mM of Tris–HCl (pH 7.4) to make a final volume of 1 ml. The incubation was carried out for 12.5 min at 37° and the reaction mixture was extracted with 10 ml each of *n*-pentane–dichloromethane (3:1, v/v) and ethyl acetate. The subsequent procedure was performed in an identical manner with the microsomal study.

Results and discussion

As shown in Table 1, pretreatment of rats with PB and MC afforded a significant increase of AP 4-hydroxylase activity of liver microsomes compared with the respective

Table 1. Catalytic activity of microsomes and purified cytochrome P-450 from phenobarbital- and 3-methylcholanthrene-treated rats on the metabolism of antipyrine

Preparation	Pretreatment	Activity (nmole metabolite formed/min/mg protein)	
		4-Hydroxyantipyrine	3-Hydroxymethyl-3-norantipyrine
Microsomes	Saline	0.74 ± 0.17	3.22 ± 0.27
Microsomes	Phenobarbital	3.98 ± 0.94*	2.79 ± 0.16
	Phenobarbital / Saline	5.38	0.86
Microsomes	Corn oil	0.50 ± 0.06	3.29 ± 0.40
Microsomes	3-Methylcholanthrene	0.85 ± 0.17*	1.45 ± 0.26*
	3-Methylcholanthrene / Corn oil	1.70	0.44
P-450-SD-I	Phenobarbital	60.0 (4.26)†	N.D.
P-450-SD-II	Phenobarbital	47.4 (4.74)†	N.D.
P-450-SD-III	3-Methylcholanthrene	10.9 (0.72)†	N.D.

Phenobarbital (saline solution) was administered i.p. at a dose of 80 mg/kg/day for four days in microsomal study, or at a dose of 100 mg/kg/day during three consecutive days for purification of cytochrome P-450. 3-Methylcholanthrene (corn oil solution) was administered i.p. at a dose of 20 mg/kg/day for four days.

Results are the mean ± S.E. of four rats (microsomal study) or the mean of three determinations (study in the reconstituted system containing purified cytochrome P-450).

* Significantly different ($P < 0.05$) from the control.

† Activity as nmole metabolite formed/min/nmole P-450 are shown in parentheses.

N.D.: Not detected.

controls. In this case, the effect of PB (5.4-fold) was much greater than that of MC (1.7-fold). In contrast to AP 4-hydroxylase, AP 3-methyl hydroxylase activity was not enhanced by both inducers, but was suppressed significantly when the animals were treated with MC (Table 1). This finding strongly suggested that different forms of cytochrome P-450 are involved in the two hydroxylations described above, and only the isozyme(s) possessing 4-hydroxylase activity is inducible by PB- and MC-pretreatment. Kahn *et al.* [22] recently examined the inductive effect of PB on the *in vitro* metabolism of AP in a similar manner with the present study, and reported that AP 4-hydroxylase was induced 2.3-fold over the control value but AP 3-methyl hydroxylase was not affected by PB-treatment.

In order to confirm the above suggestion, three forms of cytochrome P-450 induced with PB (P-450-SD-I and -II) and MC (P-450-SD-III) were purified, and their activities of the hydroxylations of AP were assessed in the reconstituted system. As indicated in Table 1, all three forms of cytochrome P-450 showed some activities of AP 4-hydroxylase. The activities of two of the isozymes isolated from PB-treated rats were much higher than that from MC-treated rats. This was in good agreement with the inducibility of AP 4-hydroxylase in microsomes by PB- and MC-pretreatment. On the other hand, any activity for 3-methyl hydroxylase of AP was not detected with the three enzymes (Table 1), also supporting the results of microsomal study in which this reaction was not enhanced by both inducers. Thus, we provided evidence for the first time that a high regioselectivity exists in the cytochrome P-450-catalyzed hydroxylation of AP.

Among three forms of cytochrome P-450, P-450-SD-I and -II exhibited high activity for *N*-demethylation of benzphetamine (87.1 nmole/min/nmole P-450), and 3-hydroxylation of benzo[*a*]pyrene (1.02 nmole/min/nmole P-450) and *O*-deethylation of 7-ethoxycoumarin (80.3 nmole/min/nmole P-450), respectively. However, P-450-SD-II did not show such strong activity for these substrates. Based on this and a variety of other features such as molecular weight and spectral and immunochemical nature (data not shown), it was concluded that P-450-SD-I, -II and -III correspond to the samples of Guengerich [23], PB-B, PB-D and βNF-B, respectively.

In summary, our observation showed that: (1) microsomal AP 4-hydroxylase is induced with PB (5.4-fold) and MC (1.7-fold), while AP 3-methyl hydroxylase is not enhanced at all; (2) major forms of cytochrome P-450 induced with PB and MC can function as AP 4-hydroxylase but not 3-methyl hydroxylase; and (3) the isozymes from PB-treated rats have much higher activity than that from MC-treated animals in 4-hydroxylation of AP. The catalytic nature of purified cytochrome P-450s toward metabolism of AP well explained the inducibility of AP hydroxylase with PB and MC.

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