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High activity of cytochrome *P*-450-linked aminopyrine *N*-demethylase in mouse brain microsomes, and associated sex-related difference

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The presence of cytochrome *P*-450 and associated mono-oxygenase activities was examined in brain microsomes from male and female mice. Although the cytochrome *P*-450 level in male mouse brain was very low as compared with mouse liver, the aminopyrine *N*-demethylase and morphine *N*-demethylase specific activities in male mouse brain were much higher than those observed in mouse liver. Ethoxycoumarin *O*-de-ethylase and aniline hydroxylase activities were, however, not detected in mouse brain. Sex-related differences were observed in both the cytochrome *P*-450 levels and aminopyrine *N*-demethylase activity in mouse brain, the levels of both being higher in male mouse brain as compared with female mouse brain. Aminopyrine *N*-demethylase activity in mouse brain microsomes was dependent on the presence of oxygen and NADPH and could be inhibited by piperonyl butoxide, *N*-octyl imidazole and carbon monoxide. Antiserum raised to the phenobarbital-inducible form of rat liver cytochrome *P*-450 [*P*-450$_{(b+e)}$] inhibited mouse brain aminopyrine *N*-demethylase activity by around 80%. Mouse brain microsomal protein exhibited cross-reactivity against this antiserum when examined by Ouchterlony double diffusion and immunoblotting. The present results indicate the presence of a phenobarbital-inducible form of cytochrome *P*-450 (or a form of cytochrome *P*-450 that is similar immunologically) in mouse brain microsomes, which is associated with a sex-related difference.

INTRODUCTION

Cytochrome *P*-450 (referred to as '*P*-450') and associated mono-oxygenase activities play an important role in the metabolism of endogenous compounds and xenobiotics. Although the liver is the primary organ involved in the metabolism of foreign compounds, recent interest has centred upon the capability of extra-hepatic organs to metabolize xenobiotics (Gram *et al.*, 1986). The presence of *P*-450 in the brain could play an important role in the pharmacokinetic modulation of psychoactive drugs in the central nervous system. Further, since the brain has limited regenerative capability, bioactivation of xenobiotics to reactive metabolites by cerebral *P*-450 may be particularly hazardous. Studies on the properties of cerebral *P*-450 have been scanty due to the instability of the cerebral *P*-450, very low levels of detectable activity and the contamination of the microsomal fractions with haemoglobin (Mensil *et al.*, 1984).

In rat brain, *P*-450 and associated mono-oxygenases have been detected in low amounts compared with levels in rat liver (Sasame *et al.*, 1977; Marietta *et al.*, 1979; Guengerich & Mason, 1979; Das *et al.*, 1981; Srivatsava *et al.*, 1983; Mensil *et al.*, 1985; Walther *et al.*, 1986). However, in all the above studies, the detectable mono-oxygenase activity was much lower than that observed in the liver. Mouse brain microsomes were reported to contain even lower amounts of *P*-450 than rat brain (Nabeshima *et al.*, 1981).

In the present study, *P*-450-linked mono-oxygenases were estimated in brain microsomes from male and female mice, and compared with the respective hepatic levels. We report the presence of a high concentration of aminopyrine *N*-demethylase activity in mouse brain and an associated sex-related difference. The immunological similarity of the mouse brain *P*-450 with the phenobarbital-inducible form of rat liver *P*-450 [*P*-450$_{(b+e)}$] is also described.

MATERIALS AND METHODS

Male and female Swiss Albino mice (3 months old), obtained from the stock colony of the Institute, were used in all the experiments. Animals had access to pelleted diet (Hindustan Lever Ltd., Bombay, India) and water *ad libitum*. Prior to decapitation, animals were anaesthetized with ether and their brains were perfused via the left ventricle with Tris-buffered saline (10 ml). Brains were quickly removed, rinsed in perfusion buffer, blotted and weighed. The tissue was then homogenized in 10 vol. of 0.1 M-Tris containing 0.1 mM-dithiothreitol, 0.1 mM-phenylmethanesulphonyl fluoride, 0.2 mM-EDTA, 1.15% (w/v) potassium chloride and 20% (v/v) glycerol (pH 7.4). The homogenate was centrifuged at 17000 *g* in a refrigerated centrifuge (4 °C) for 30 min. The supernatant was decanted and the pellet was homogenized in 5 vol. of the Tris/glycerol buffer and centrifuged again at 17000 *g* for 30 min. The supernatants were pooled and centrifuged at 100000 *g* (4 °C) for 1 h. The pellet was resuspended in the buffer, homogenized gently and centrifuged at 100000 *g* (4 °C) for 1 h. The washed pellet was suspended in a small volume of Tris/glycerol buffer, aliquoted, stored in liquid N_2 and used within 1 week of

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preparation. Microsomes were prepared from brains from 20 mice, pooled for each experiment. Livers were also perfused during transcardial perfusion. The right central lobes of the livers were also removed, pooled and used for the preparation of microsomes. The purity of the brain microsomal fraction was examined by electron microscopy, and the 100000 *g* pellet was found to be highly enriched with microsomal vesicles and contained less than 3% contamination with mitochondria. Assay of monoamine oxidase activity (Krajl, 1965) in mouse brain microsomes revealed the activity to be only 0.26% of that present in the mitochondrial fraction. Mitochondrial activity was 3.54 nmol/min per mg of protein.

Cytochrome *P*-450 levels were measured by the method of Matsubara (Matsubara *et al.*, 1976). NADPH–cytochrome *c* reductase (Phillips & Langdon, 1962), aniline hydroxylase (Imai *et al.*, 1966), 7-ethoxycoumarin *O*-de-ethylase (Greenlee & Poland, 1978) and total protein using bovine serum albumin (Fraction V, Sigma Chemical Co.) as standard (Bradford, 1976) were measured as described. Aminopyrine *N*-demethylase and morphine *N*-demethylase activities were estimated according to the procedure of Werringloer (1978). Incubations were carried out in 0.05 M-Tris buffer (pH 7.5) containing 20 mM-magnesium chloride, 1.8 mM-aminopyrine and 10 μg of microsomal protein in a total volume of 1 ml. After preincubation for 3 min at 37 °C, the reaction was initiated by the addition of NADPH to a final concentration of 3.1 mM. After 10 min of incubation at 37 °C, the reaction was stopped by addition of 0.5 ml of 10% (w/v) trichloroacetic acid. The formaldehyde formed was estimated in the supernatant following centrifugation, using NASH reagent as described (Werringloer, 1978). Blank tubes containing boiled microsomes, no substrate or no NADPH were run simultaneously and these blank values, which were similar in all cases, were subtracted from the experimental values. All analyses were performed in duplicate for each batch of microsomes and values given are means of at least three experiments. In experiments with inhibitors of *P*-450, blanks containing inhibitors but no NADPH were also run.

Hepatic *P*-450 was purified from phenobarbital-pretreated rats to apparent homogeneity (Guengerich & Martin, 1980). The specific content was 13 nmol of *P*-450/mg of protein. Antibodies were raised to *P*-450 (Kamataki *et al.*, 1976) in rabbits, and antiserum was collected and stored at −70 °C. Ouchterlony double diffusion was performed on agar-coated plates as described (Ouchterlony, 1962). There was a clear immunoprecipitation band between purified *P*-450 from phenobarbital-pretreated rat liver and the antiserum raised to *P*-450. No precipitin bands were observed with preimmune serum. Ouchterlony double diffusion was performed using mouse brain microsomes and the antiserum to *P*-450. The assay of mouse brain microsomal mono-oxygenase activity was carried out in the presence of antiserum raised to *P*-450 in certain experiments. In these experiments, control tubes contained equal quantities of preimmune serum. Microsomes were preincubated with sera (both preimmune and immune) for 30 min at 37 °C prior to carrying out the assay. SDS/polyacrylamide-gel electrophoresis of microsomal proteins was carried out as described (Laemmli, 1970). Proteins were transferred from the gel to nitrocellulose sheets using the procedure of Towbin *et al.* (1979). A current setting of 200 mA was used for the transfer. Subsequent to the transfer, the proteins on the nitrocellulose sheets were immunostained with antiserum to *P*-450 as described (Guengerich *et al.*, 1982).

RESULTS

The microsomal aminopyrine *N*-demethylase activity in mouse brain and liver is given in Table 1. A sex-related difference was observed; the male mouse brain had much higher activity than the female mouse brain. No such difference was observed in the liver. Further, the specific activity of aminopyrine *N*-demethylase in male mouse

Table 1. Cytochrome *P*-450-linked mono-oxygenases and NADPH–cytochrome *c* reductase in male and female mouse hepatic and cerebral microsomes

Values are means ± S.D. of at least three separate experiments, with three different batches of microsomes.

Enzyme	Brain		Liver	
	Male	Female	Male	Female
Aminopyrine *N*-demethylase (nmol of formaldehyde formed/min per mg of protein)	173.75 ± 5.00	91.25 ± 3.75	97.5 ± 10.00	98.75 ± 8.75
Morphine *N*-demethylase (nmol of formaldehyde/min per mg of protein)	40.52 ± 3.95	41.29 ± 2.99	23.37 ± 1.89	21.40 ± 3.07
7-Ethoxycoumarin *O*-de-ethylase (nmol of 7-hydroxycoumarin formed/min per mg of protein)	ND*	ND*	7.33 ± 1.12	6.51 ± 1.70
Aniline hydroxylase (nmol of *p*-aminophenol formed/min per mg of protein)	ND†	ND†	32.11 ± 1.34	29.52 ± 0.55
Cytochrome *P*-450 (nmol/mg of protein)	0.043 ± 0.01	0.016 ± 0.005	0.92 ± 0.06	0.90 ± 0.04
NADPH–cytochrome *c* reductase (nmol of cytochrome *c* reduced/min per mg of protein)	23.79 ± 4.47	23.23 ± 4.97	78.84 ± 8.91	90.43 ± 26.12

* Minimal detectable activity was 0.05 nmol of product formed/min per mg of protein.
† Minimal detectable activity was 0.01 nmol of product formed/min per mg of protein.

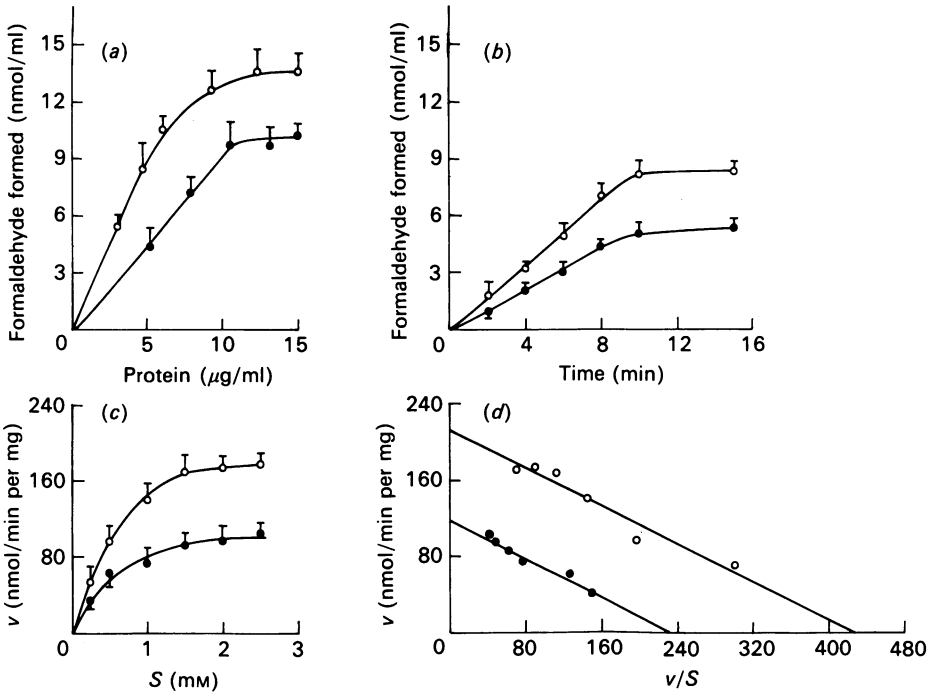

Fig. 1. Effects of (*a*) microsomal protein concentration, (*b*) incubation time and (*c*, *d*) substrate concentration on brain microsomal aminopyrine *N*-demethylase activity

○, Male mouse brain activity; ●, female mouse brain activity. Values are expressed as means ± S.D. ($n = 3$, different batches of microsomes). (*a*) Microsomal protein was incubated for 10 min; aminopyrine concentration was 1.8 mM. (*b*) Microsomal incubations were carried out with 5 and 6 μg of male and female mouse brain microsomal protein respectively per ml. Aminopyrine concentration was 1.8 mM. (*c*) Microsomal incubations were performed for 5 min. Protein concentrations were 5 and 6 μg of male and female mouse brain microsomal protein respectively per ml. *v* represents the velocity, expressed as nmol of formaldehyde formed/min per mg of protein, and *S* represents aminopyrine concentrations in mM. (*d*) Eadie–Hofstee plot of data from (*c*). K_m values for male and female brain aminopyrine *N*-demethylase were 0.49 mM and 0.52 mM, respectively and $V_{max.}$ values were 215 and 120 nmol of formaldehyde formed/min per mg of protein respectively.

brain was much higher than that observed in liver. A similar sex difference was also observed in total cerebral *P*-450, which in the male brain was only 4.6% of the hepatic level. Ethoxycoumarin *O*-de-ethylase and aniline hydroxylase activities were not detectable in cerebral microsomes, although they were detectable in the liver. In both male and female mouse brain, the activity of morphine *N*-demethylase was higher than that observed in liver; but no sex-related differences were detected. The level of NADPH–cytochrome *c* reductase in mouse brain was 27% of that present in liver (Table 1).

The effect of protein concentration on male and female brain microsomal aminopyrine *N*-demethylase activity is shown in Fig. 1(*a*). The enzyme activity was linear up to a protein concentration of 10 μg/ml of incubation medium. The enzyme activity was also linear up to 10 min of incubation time, and did not increase thereafter (Fig. 1*b*). The dependence of the enzyme activity on substrate concentration is shown in Fig. 1(*c*) and the Eadie–Hofstee plot of these data is depicted in Fig. 1(*d*). The $V_{max.}$ of aminopyrine *N*-demethylase activity was calculated as 215 and 120 nmol of product formed/min per mg of protein in male and female mouse brain microsomes respectively. The respective K_m values were 0.49 and 0.52 mM, as calculated from the Eadie–Hofstee plot (Fig. 1*d*). The sex-related difference in cerebral aminopyrine *N*-demethylase activity was observed consistently.

Piperonyl butoxide at a concentration of 0.5 mM inhibited microsomal aminopyrine *N*-demethylase in male mouse brain by 45%. Addition of *N*-octyl imidazole (1 mM), another inhibitor of *P*-450, resulted in 50% inhibition of aminopyrine *N*-demethylase. When incubations were carried out in an atmosphere of 100% carbon monoxide, a greater loss of activity (82%) was observed (Table 2). The enzyme activity was dependent

Table 2. Effect of inhibitors on aminopyrine *N*-demethylase activity in male mouse brain microsomes

All incubations were carried out at 37 °C. Control incubations were carried out in an atmosphere of air, blanks containing no substrate or no NADPH were run and their values subtracted from experimental values. The control value for male mouse brain aminopyrine *N*-demethylase activity was 177.7 ± 8.2 nmol of formaldehyde formed/min per mg of protein. Values are means ± S.D. of three analyses on the same batch of microsomes.

Treatment	Activity (% of control)
Control	100
100% O_2	136.50 ± 6.12
100% CO	17.60 ± 9.14
100% N_2	36.28 ± 12.03
N-Octyl imidazole (1 mM)	50.12 ± 3.50
Piperonyl butoxide (0.5 mM)	54.70 ± 2.50

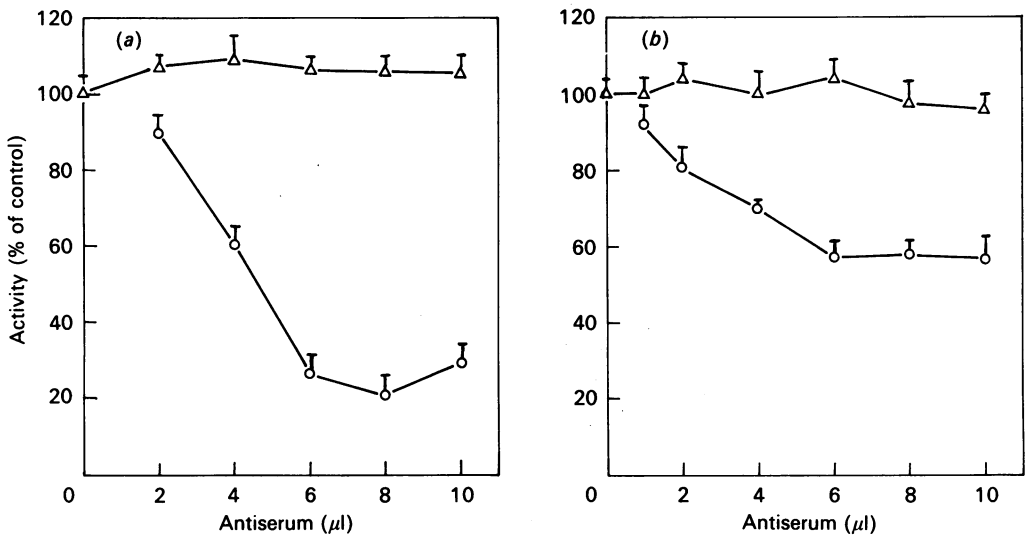

Fig. 2. Inhibition of mouse brain (*a*) aminopyrine *N*-demethylase and (*b*) morphine *N*-demethylase activity by antiserum to a phenobarbital-inducible form of rat liver *P*-450

Microsomes were preincubated with preimmune (△) or immune (○) serum for 30 min at 37 °C prior to addition of substrate and NADPH. Microsomal incubations contained 5 μg of protein/ml and were incubated with substrate for 10 min. Values are means ± S.D. from three analyses on same batch of microsomes.

on the presence of oxygen and NADPH; a marked enhancement in activity occurred when incubations were carried out in a 100% oxygen atmosphere.

Addition of antiserum to phenobarbital-inducible rat liver *P*-450 inhibited mouse brain aminopyrine *N*-demethylase in a dose-dependent manner, maximal inhibition being 80% (Fig. 2*a*). No inhibition was observed when pre-immune serum was added. Morphine *N*-demethylase was inhibited to a lesser extent (45%) by the same antiserum (Fig. 2*b*). Ouchterlony double diffusion of mouse brain microsomes and antiserum to rat liver phenobarbital-inducible *P*-450 showed a clear immunoprecipitin line (Fig. 3). No precipitin lines were observed with preimmune serum. SDS/polyacrylamide-gel electrophoresis of mouse brain microsomes revealed several protein bands of approx. M_r 50000 after Coomassie Blue staining (results not shown). Following SDS/polyacrylamide-gel electrophoresis, the proteins were transferred electrophoretically to nitrocellulose membranes and immunostained with antiserum to phenobarbital-inducible rat liver *P*-450. A single protein band (corresponding to a similar band in mouse liver microsomes) was revealed by the antiserum in both male and female brain microsomes (Figs. 4*a* and 4*b*), which co-migrated with purified $P\text{-}450_{(b+e)}$.

DISCUSSION

The specific activities of aminopyrine *N*-demethylase and morphine *N*-demethylase in male mouse brain were

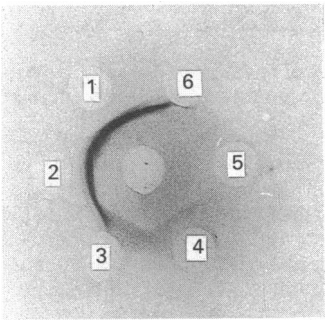

Fig. 3. Ouchterlony immunodiffusion of male mouse brain microsomes and antiserum to phenobarbital-inducible rat liver *P*-450

The central well contained 86 μg of microsomal protein and the outer wells contained antiserum diluted in the following concentrations: 1, undiluted; 2, 2 times; 3, 4 times; 4, 8 times; 5, 16 times; 6, 32 times. The gel was incubated at 4 °C for 48 h, washed and stained with Coomassie Blue.

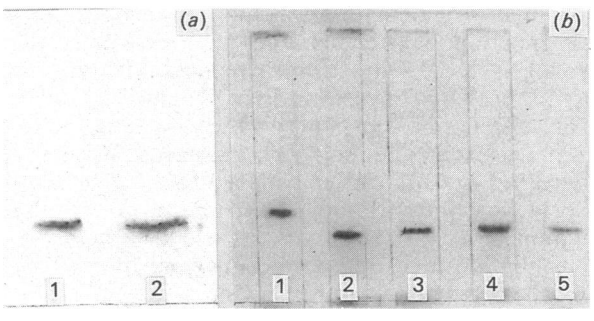

Fig. 4. Immunoblots of purified cytochrome $P\text{-}450_{(b+e)}$ and mouse brain and liver microsomes after staining with antiserum to phenobarbital-inducible rat liver *P*-450

(*a*) Lane 1, purified $P\text{-}450_{(b+e)}$ antigen (5 μg/lane); lane 2, male brain microsomes (38 μg/lane). (*b*) Lane 1, marker protein (M_r 66000); lane 2, male liver microsomes (32 μg/lane); lane 3, male brain microsomes (43 μg/lane); lane 4, female liver microsomes (33 μg/lane); lane 5, female brain microsomes (47 μg/lane).

higher than those observed in the mouse liver. However, ethoxycoumarin *O*-de-ethylase and aniline hydroxylase activities were not detectable in mouse brain microsomes, indicating that certain isoenzymes of *P*-450 probably predominate in mouse brain. Further, the presence of high specific activities of aminopyrine and morphine *N*-demethylase in mouse brain indicates that the brain may possess considerable metabolizing capability against certain substrates. Distinct sex-related differences were observed in aminopyrine *N*-demethylase activity, which could be immunochemically inhibited by the antibody to phenobarbital-inducible *P*-450. In rat liver, similar sex-related differences are known in phenobarbital-inducible *P*-450 and associated mono-oxygenase activities (Kato & Gillette, 1965; Kato & Onada, 1970; Thomas *et al.*, 1981). More recently it has been demonstrated that sex-related differences exist in the constitutive forms of *P*-450$_b$ and *P*-450$_e$ in rat liver, and these differences are enhanced following administration of phenobarbital (Yamazoe *et al.*, 1987). The phenobarbital-inducible *P*-450$_b$ has very high *N*-demethylase activity towards aminopyrine and benzphetamine (Guengerich, 1987); aminopyrine is *N*-demethylated to a lesser extent by other isoenzymes of *P*-450. Further, it has also been demonstrated that pretreatment of mice with phenytoin causes an induction of benzphetamine *N*-demethylase activity in mouse brain and liver (Volk *et al.*, 1988). Thus, the immunochemical inhibition, Ouchterlony double diffusion, and Western immunoblot data support the presence of *P*-450$_{(b+e)}$ or a closely related form in mouse brain. The presence of this form in mouse seems to be linked with the sex-related difference.

Although the level of *P*-450 in the brain is much lower than that in liver, certain associated mono-oxygenase activities are very high in the brain. This indicates that the brain may possess considerable xenobiotic metabolizing capability for certain substrates. This would have far-reaching consequences, as exposure to environmental toxins has been implicated in the pathogenesis of certain degenerative neurological disorders (Calne *et al.*, 1986). The functional role of cerebral *P*-450 has not been well understood. The sex difference observed in mouse brain *P*-450 may serve as a useful model to evaluate the role of *P*-450 in brain.

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