Rat brain cytochromes P-450: catalytic, immunochemical properties and inducibility of multiple forms

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Cytochrome P-450 (P-450) and associated mono-oxygenase activities were estimated in male and female rat brain microsomes. The P-450 concentration in male rat brain was one-tenth the corresponding hepatic levels, which is considerably higher than earlier reports. A distinct sex-related difference was observed in the levels of total P-450 and mono-oxygenase activities known to be mediated by P-450$_{b,e}$; the female brain levels were 60% of those in the males. Immunoinhibition and immunoblot studies using antisera to P-450$_{b,e}$ and P-450$_{c,d}$ indicated the presence of multiple forms of P-450, immunologically similar to P-450$_{b,e}$, P-450$_c$ and P-450$_d$ in the rat brain. Prior treatment with phenobarbital resulted in two-fold increase of total P-450 and selective induction of aminopyrine *N*-demethylase (APD) and morphine *N*-demethylase (MND) activities. Administration of 3-methylcholanthrene, selectively induced the levels of ethoxycoumarin *O*-deethylase (ECD) and arylhydrocarbon hydroxylase, although the levels of total P-450 were not increased. 3-Methylcholanthrene induction was also accompanied by a shift in the absorption maximum of the reduced carbon monoxide difference spectrum from 452 to 448 nm. Immunocytochemical localization using antibodies to P-450$_{b,e}$ indicated the presence of P-450 predominantly in the neuronal cell bodies and to a lesser extent in the fibre tracts in cerebral cortex, cerebellum, thalamus, hypothalamus, hippocampus and brainstem. These studies indicate that the brain contains significant amounts of P-450, which exists in multiple forms and can be selectively induced by prior exposure to phenobarbital or 3-methylcholanthrene.

Cytochrome P-450 (P-450) containing mono-oxygenases is involved in the biotransformation of foreign compounds (drugs and environmental toxins) and endogenous compounds (steroids, eicosanoids etc.). Generally, xenobiotic metabolism mediated through P-450 leads to the formation of hydrophillic, non-toxic metabolites, that are easily excreted from the body. However, there are several instances wherein an inert, non-toxic compound is converted to a reactive, toxic metabolite[5].

Although liver is the primary organ involved in P-450 mediated metabolism, recent advances in toxicology have demonstrated the importance of extrahepatic P-450 in target organ toxicity. Cerebral P-450 could have a multifunctional role in the metabolism of steroids and local pharmacological modulation of drugs acting on the central nervous system. Further, considering the limited regenerative capability of the CNS, bioactivation of xenobiotics in situ within the brain could have far reaching consequences in causing neoplastic transformation or disruption of neuronal function[10].

Recent studies have implicated a causative role for environmental toxins in the etiopathogenesis of certain neurodegenerative disorders, and the vulnerability of the CNS to damage through toxicants[2,7,17]. In view of this,

bioactivation and detoxification of environmental toxins by cerebral P-450 may be important.

The presence of P-450 in rat brain microsomes was demonstrated and reported to be only 3–3.6% of the hepatic levels[16]. The P-450-associated mono-oxygenases in the brain have also been reported to be present in low concentration[3,6,8,11,19].

Recent studies from our laboratory have demonstrated that although the total P-450 levels in the mouse brain were very low (~4% of hepatic levels), the concentration of certain P-450-associated mono-oxygenase activity was considerably higher in mouse brain as compared to the corresponding hepatic levels[13]. Determination of P-450-associated mono-oxygenase activity in brain and liver from human autopsy samples also revealed that the specific activity of morphine *N*-demethylase in the brain was higher than the hepatic levels in the same individual[14].

We now report investigations on rat brain P-450, the presence of multiple forms of P-450, their inducibility and immunological similarity to rat liver P-450.

Wistar rats (3 months old) from the stock colony of the institute were used for all experiments. Brain and liver microsomes were prepared from 10 rats for each experi-

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ment and the purity was ascertained as described[12].

P-450 levels were measured by the method of Matsubara[9], with certain modifications[4]. NADPH cytochrome c reductase, 7-ethoxycoumarin O-deethylase (ECD), benzo(a)pyrene hydroxylase (BPH), aminopyrine N-demethylase (APD) and morphine N-demethylase (MND) activities were estimated as described[4]. Protein was determined by the dye-binding method[1].

Two forms of hepatic P-450 were purified to apparent homogeneity from phenobarbital (P-450$_{b,e}$) and 3-methyl-cholanthrene (P-450$_c$) treated rats[4]. The specific contents were 13 nmoles of P-450/mg protein for each of the purified forms. Antibodies were raised in rabbits to both the purified forms of P-450. No cross-reactivity was observed between P-450$_{b,e}$ and P-450$_c$. However, the antisera raised to P-450$_c$ exhibited immunological cross-reactivity to P-450$_d$ and hence this antisera is referred to as anti-P-450$_{c,d}$. SDS-polyacrylamide gel electrophoresis of microsomal proteins was carried out and the proteins were transferred to nitrocellulose sheets and immuno-stained as described[13].

In order to determine the induction of cerebral P-450, phenobarbital (80 mg/kg b. wt.) was given i.p. for 10 days and rats were sacrificed 24 h after the last dose. 3-Methylcholanthrene (3-MC), (45 mg/kg b. wt.) was administered s.c. for 4 days and rats were sacrificed 24 h after the last dose and the microsomes were prepared from brain and liver as described earlier[12]. Rat brain sections were processed for immunocytochemistry using antisera to P-450$_{b,e}$[14].

The content of P-450 and associated mono-oxygenase activities in rat brain and liver microsomes is given in Table I. The male rat brain P-450 content was one-tenth of the corresponding hepatic levels. A distinct sex-related difference was observed; the female rat brain P-450 levels were significantly lower than the male brain levels. This difference was also reflected in the activities of APD and MND; the concentration of the enzyme in the female rat brain was only 49 and 51%, respectively, of the levels in male rat brain. Further, the specific activity of these enzymes was significantly higher in the male rat brain as compared to the hepatic levels. ECD and BPH activities did not exhibit any sex-related difference and their concentration in the brain was significantly lower than the hepatic levels. NADPH cytochrome c reductase level in the rat brain was 30% of the hepatic levels. Treatment with phenobarbital for 10 days resulted in a two-fold induction of total P-450 as compared to untreated controls (Table I). The sex-related difference was significant in phenobarbital-induced animals also. Following treatment with phenobarbital, both APD and MND activities were induced, while ECD activity was not significantly different from the controls. The NADPH

TABLE I

Cytochrome P-450 and associated mono-oxygenase activities in rat brain and liver microsomes

Activities are represented as (a) nmoles of cytochrome P-450/mg protein, (b) nmoles of product formed/mg protein/min and reductase (NADPH cytochrome c reductase) is expressed as nmoles of cytochrome c reduced/mg protein/min. Values are means ± S.D. of 3 experiments. ND, not determined.

	Brain		Liver	
	Male	Female	Male	Female
P-450[a]				
Control	0.09±0.005	0.05±0.004	0.86±0.02	0.63±0.03
PB	0.17±0.01*	0.10±0.01*	2.12±0.05*	1.39±0.04*
3-MC	0.09±0.01	ND	1.00±0.14	ND
BPH[b]				
Control	0.01±0.001	0.01±0.002	1.78±0.02	1.61±0.05
3-MC	0.02±0.003*	ND	4.01±0.52*	ND
ECD[b]				
Control	1.5±0.3	1.6±0.4	6.5±1.2	5.4±1.3
PB	1.17±0.25	1.11±0.19	5.2±0.19	5.3±0.5
3-MC	3.5±0.42*	ND	12.75±1.06*	ND
APD[b]				
Control	174.3±19.8	85.3±24.9	107.3±17.8	81.1±14.1
PB	374.1±37.5*	188.2±31.9*	317.8±20.1*	223.2±27.3*
3-MC	150.3±9.2	ND	120.2±2.4	ND
MND[b]				
Control	128.7±9.6	66.2±10.9	74.1±8.7	55.9±4.3
PB	244.0±29.0*	124.7±12.6*	217.9±25.9*	159.6±12.6*
Reductase				
Control	21.3±2.4	23.3±2.8	74.6±22.0	68.6±2.9
PB	57.4±4.6*	55.9±8.6*	265.0±16.97*	269.0±52.3*

* Indicates values significantly different ($P < 0.001$) from respective untreated control.

cytochrome c reductase activity was also induced in the brain following phenobarbital treatment. Administration of the condensed aromatic hydrocarbon, 3-MC, did not result in the elevation of total P-450 levels in the male rat brain, although the ECD and BPH activities were selectively and significantly increased (233 and 182%, respectively). No sex-related difference was seen (data not shown). The absorbance maximum of the reduced carbon monoxide difference spectrum of brain microsomes from control rats was observed at 452 nm. Following treatment with phenobarbital, the absorption maximum shifted to 450 nm, while 3-MC treatment resulted in the shift to 448 nm. These results indicate the preferential induction of specific forms of P-450. The activity of brain P-450-mediated mono-oxygenases was dependent on the presence of oxygen and NADPH, and was significantly inhibited by carbon monoxide and specific inhibitors of P-450, namely, piperonyl butoxide and SKF 525A (data not shown).

SDS-PAGE of rat brain and liver microsomal proteins, followed by immunoblotting with antisera to P-450$_{b,e}$, revealed the presence of a single protein band in both
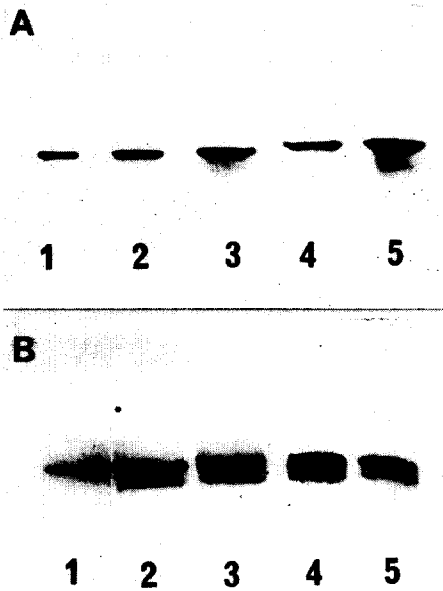
A

B

Fig. 1. Immunoblots of rat brain and liver microsomes following staining with antisera to (A): P-450$_{b,e}$ and (B): P-450$_{c,d}$. A: the lanes contained (1) purified P-450$_{b,e}$ antigen [4 μg], (2) female rat brain microsomes [42 μg], (3) female rat liver microsomes [7.5 μg], (4) male rat brain microsomes [34 μg] and (5) male rat liver microsomes [7.5 μg]. B: the different lanes contained (1) purified P-450$_c$ antigen [5 μg], (2) untreated rat brain microsomes [80 μg], (3) 3-MC treated male rat brain microsomes [55 μg], (4) 3-MC treated male rat liver microsomes [10 μg] and (5) untreated male rat liver microsomes (25 μg]. The protein concentration is given in parentheses.

brain and liver microsomes. This band co-migrated with purified P-450$_{b,e}$ (Fig. 1A). Addition of 100 μl of antisera resulted in significant inhibition of APD and MND (85% and 61%, respectively) activity as compared to microsomes treated with preimmune serum. However, ECD activity was only partially inhibited (29%) by the same antisera (data not shown). Immunoblot analysis of rat brain and liver microsomes using the antisera to P-450$_{c,d}$ is depicted in Fig. 1B. Purified P-450$_c$ exhibited a single band. In the liver and brain microsomes two bands, corresponding to P-450$_c$ (which co-migrated with purified P-450$_c$) and P-450$_d$, were observed. Pretreatment with 3-MC resulted in the induction of these forms in the brain
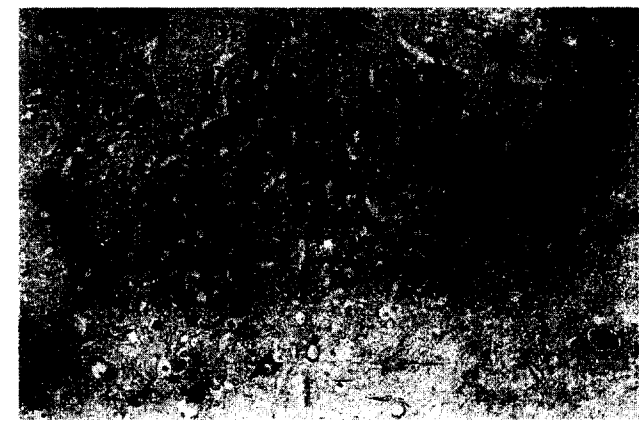
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Fig. 2. Immunocytochemical localization of P-450 in the male rat brain using antisera to rat liver P-450$_{b,e}$. A: immunoreactive neurons of the cerebral cortex. Note the reduced staining of the lower layers of the cortex. (×122). B: immunoreactive neuronal cell bodies in the thalamus and posterior hypothalamus (dark arrow). Note the relative paucity of staining in the white matter (×49). C: higher magnification of the area marked by arrow in (B), showing immunoreactive neuronal somata of the posterior hypothalamus (×182). D: strong immunostaining of the large neurons and processes of the mesencephalic trigeminal nucleus. There is lack of staining of the adjacent small neurons (×160).

and liver microsomes (Fig. 1B). The activity of ECD was almost completely inhibited (83.5%) by the antisera to P-450$_{c,d}$, while APD activity was inhibited only partially (49%) by the same antisera (data not shown).

Immunohistochemical localization of P-450 using antibodies to P-450$_{b,e}$ revealed robust staining of the neuronal cell bodies; the staining of the white matter was considerably less. The staining of the white matter was better discernable in the compactly arranged fibre tracts (Fig. 2). The neuronal somata of the cerebral cortex (Fig. 2A), the thalamus (Fig. 1B), the hypothalamus (Fig. 2B and 2C), and the brainstem (Fig. 2D) revealed significant immunoreactivity. In the cerebellum, the vermis showed variable staining of the Purkinje cells and glial elements. In the brainstem, the neurons of the mesencephalic trigeminal nucleus (Fig. 2D), the large reticular neurons and some of the cranial nerve nuclei revealed strong immunoreactivity. The neuronal somata of the spinal grey horn cells also showed variable staining.

The P-450 levels in the rat brain have been reported to be 3–3.6% of the hepatic levels (36 picomoles of P-450/mg protein)[16]. However, using stringent conditions for isolation of microsomes, much higher levels of P-450 (~85 pmoles/mg protein) were detected. In an earlier report[19], partial purification of rat brain homogenate has been carried out to determine P-450 levels from the reduced carbon monoxide difference spectrum. However, since the brain mitochondrial P-450 content is reported to be significantly high[18] (and as observed by us), estimation of P-450 in total brain homogenate would include contribution from both microsomal and mitochondrial P-450.

Although cerebral P-450 level was determined to be only one-tenth of the hepatic level, APD and MND activities in the male brain were significantly higher than in the male liver (Table I). Thus, the brain may possess considerable metabolizing capability for certain substrates. A distinct sex-related difference was observed in P-450 levels and APD and MND activities (Table I). In the rat liver, it has been demonstrated that sex-related differences exist in the constitutive forms of P-450$_b$ and P-450$_e$[20].

Although the APD and MND activities were higher in the brain as compared to liver, ECD activity was only 23% of hepatic activity. The BPH activity in brain was particularly low, being only 0.56% of the corresponding hepatic levels. No sex-related difference was observed in cerebral ECD and BPH activities, which are induced by 3-MC treatment. 3-MC inducible forms of P-450 in rat liver, namely, P-450$_c$ and P-450$_d$ do not exhibit a sex-related difference[5].

The presence of multiple forms of P-450 in rat brain is demonstrated by the selective inhibition of mono-oxygenase activity by antisera to different forms of rat liver P-450. Thus, the brain APD activity is inhibited to a greater extent by the antisera to P-450$_{b,e}$, while ECD activity is inhibited almost completely by the antisera to P-450$_{c,d}$. These results seem to indicate that the APD and ECD activities are mediated by different forms of P-450 in the rat brain. This is further substantiated by the immunoblot experiments, where multiple forms were detectable in the brain. The presence of multiple forms of P-450 was also demonstratable by the differential induction of various mono-oxygenase activities by phenobarbital and 3-MC. While ECD and BPH activities were induced by 3-MC treatment, APD and MND activities were induced following administration of phenobarbital (Table I). The present results demonstrate the presence of multiple forms of P-450 in the rat brain which are immunologically similar to the rat liver P-450$_{b,e}$ and P-450$_c$ and P-450$_d$.

Immunohistochemical studies using antibodies to P-450$_{b,e}$ revealed the localization of the enzyme, essentially in the neuronal cell bodies and to a lesser extent in the white matter. The intense staining of the large reticular neurons in the brainstem, revealed a pattern of staining similar to that seen in the human brain[14]. The immunohistochemical localization of NADPH cytochrome P-450 reductase, an essential component of the P-450 mono-oxygenase system, in brain[15], has revealed the co-localization of the P-450 and NADPH cytochrome P-450 reductase.

The present study demonstrates that the rat brain does indeed contain significant amounts of P-450 and high concentration of certain P-450 mediated mono-oxygenases. P-450 exists in multiple forms in the rat brain which are selectively induced and localized predominantly in the neurons. The induction of selective mono-oxygenase activity in the brain by phenobarbital would have far reaching implications in pharmacological modulation of drugs in combination drug therapy, since phenobarbital is a commonly used anti-convulsant. Further, the induction of certain cerebral P-450-mediated mono-oxygenases by 3-methylcholanthrene indicates that continued exposure to environmental pollutants could result in increased bioactivation or detoxification of environmental toxins and drugs which are metabolized by this form of P-450.

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