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DISTRIBUTION OF CYTOCHROME P450 ACTIVITIES TOWARDS ALKOXYRESORUFIN DERIVATIVES IN RAT BRAIN REGIONS, SUBCELLULAR FRACTIONS AND ISOLATED CEREBRAL MICROVESSELS*

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Abstract—The regional and subcellular distributions of rat brain cytochrome P450 and cytochrome P450-dependent activities were examined. Cytochrome P450 was found to be mainly localized in mitochondria in all the six cerebral regions studied. The activities of the isoforms mostly implicated in drug metabolism, cytochromes P450 b and c, were measured by the dealkylation of two alkoxyresorufins, that are sensitive probe substrates for these isoforms. These activities have been measured in microsomal and mitochondrial fractions obtained from six different regions in male rat brains, as well as in microvessels. Resorufin derivatives dealkylation specific activities were higher in brain microsomal fractions than in hepatic ones in all the six regions examined when results were expressed per cytochrome P450 content. These brain microsomal specific activities were also higher than in mitochondrial fractions. Olfactory bulbs showed the highest cytochrome P450 content and activities in both microsomal and mitochondrial fractions. A sex-linked difference in cytochrome P450-dependent activities was also found. After an *in vivo* inducing pretreatment of rats, only 3-methylcholanthrene induced ethoxyresorufin *O*-deethylase activity, in the three preparations studied. These results provided (i) direct evidence that cytochromes P450 b and c isoforms are active in brain microsomal fractions, with regional and sex-linked differences, and (ii) the first demonstration of cytochrome P450-dependent activities in isolated rat brain microvessels.

Despite their relatively low content in the brain tissue, cytochromes P450 (P450§) have been identified as functional enzymes, allowing the central nervous system to metabolize a variety of substrates of both exogenous and endogenous origin (for a review, see Ref. 1). The possible cerebral metabolism of exogenous molecules includes desulfuration of parathion to paraoxon [2], N-demethylation of morphine [3], hydroxylation of amphetamine [4], arylhydrocarbons [5, 6] and cannabinol derivatives [7]. Several studies provide evidence that brain P450 catalysing xenobiotic metabolism is located in the microsomal fraction [8–10]. However, cerebral P450 is mostly located in mitochondria [11, 12] where it catalyses the biotransformation of both endogenous substrates like cholesterol [13] and exogenous aryl hydrocarbon [14, 15]. The presence of P450 has been also demonstrated in endothelial cells of brain capillaries forming the blood–brain barrier [16].

Some tentative identifications of P450 isoforms present in brain regions, neuronal cultures or subcellular fractions have been done by immunological and immunohistochemical techniques, using polyclonal and/or monoclonal antibodies raised against rat liver isoforms [17–20]. On the first hand, immunoinhibition studies showed that polyclonal antibodies raised against microsomal hepatic P450 b inhibited the O-deethylation of ethoxycoumarin, a substrate of several hepatic P450 isoforms, in a rat whole brain microsomal fraction [17, 21]. So immunocytochemical studies indicated the presence of immunoresponsive proteins, and suggested a heterogenous repartition, but, on the other hand, did not give information about the ability of these proteins to catalyse xenobiotic biotransformation. Earlier work of our group showed that 7-ethoxyresorufin *O*-deethylase activity (EROD) was detectable in both rat brain mitochondria and microsomes, and was weakly inducible by 3-methylcholanthrene (3MC), confirming the presence of a cerebral P450 c [15].

In the present study, several alkoxyresorufin (or alkoxyphenoxazone) derivatives have been used as substrates in order to characterize the main cytochrome P450 isoforms involved in cerebral drug metabolism. 7-Ethoxyresorufin and 7-pentoxyresorufin are in the liver specific substrates for the major P450 isozymes induced by 3MC and by phenobarbital (PB) [22], i.e. P450 c and P450 b, respectively, the isoenzymes known to be expressed by CYP1A1 and CYP2B1 genes [23], whereas 7-benzoxyresorufin is a substrate for both isoenzymes

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§ Abbreviations used: BROD, 7-benzoxyresorufin *O*-dealkylase activity; EROD, 7-ethoxyresorufin *O*-deethylase activity; PROD, 7-pentoxyresorufin *O*-dealkylase activity; P450, cytochromes P450; PB, phenobarbital; 3MC, 3-methylcholanthrene.

[22]. The results reported here show that alkoxyresorufin activities were present in rat brain microsomes and mitochondria as well as in isolated microvessels. We report also the regional distribution of these activities, some sex-related differences and their response to an inducing treatment.

MATERIALS AND METHODS

Chemicals. 7-Benzoxy- and 7-pentoxyresorufin were purchased from Boehringer (Mannheim, F.R.G.) and were purified by thin-layer chromatography. 7-Ethoxyresorufin was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). All other reagents were of the highest purity commercially available.

Animals. Adult male, weighing 200–250 g, and female Sprague–Dawley rats, weighing 160–200 g, obtained from Iffa-Credo (St Germain-sur-l'Arbresle, France) were used throughout. The animals were housed for 5 days before the experiments in plastic cages with hardwood bedding in a room maintained at 22° with a 12 hr light/dark cycle. Standard rat chow and tap water were available *ad lib.*

Treatment of animals. For induction studies, rats were injected i.p. either with PB in 0.9% NaCl (60 mg/kg daily for 10 days) or with 3MC dissolved in corn oil (50 mg/kg for 3 days). Control rats received the same volume (0.5 mL) of saline or corn oil.

Tissue preparations. The animals were killed by decapitation, and the brains (cortices and cerebellum) were rapidly removed and chilled in the ice-cold 0.3 M sucrose, 10 mM Tris–HCl, 0.5 mM K-EDTA, pH 7.4 isolation medium. For regional distribution studies, brains were cut in six regions, olfactory bulbs, cerebral cortices, midbrain, hypothalamus, medulla oblongata and cerebellum as described in Ref. 24.

Brain non-synaptic mitochondria and microsomes were prepared after a gentle manual homogenization of the tissue, preventing the disruption of mitochondria [25]. The microvessels fraction was prepared from pooled rat brains (cortices and cerebellum) according to the method of Mrsulja *et al.* [26], slightly modified [16]. As the preparation of cerebral subcellular fractions of convenient purity was rendered difficult by the heterogeneous cellular composition of the brain, we checked that the cross-contamination of cerebral fractions remained at a low level by marker enzymes activities measurements, as described previously [12]. Liver microsomes were prepared as described in Ref. 15.

Cytochrome P450 measurements. The cytochrome P450 content was determined by the second derivative spectrophotometric method described in Ref. 25 in the cerebral fraction and by the method in Ref. 27 in the liver.

Protein concentration. The protein content was determined according to Lowry *et al.* [28] using bovine serum albumin as a standard.

Enzymatic assays. The measurements of cytochrome P450-dependent activities were carried out using 7-benzoxy-, 7-pentoxy- or 7-ethoxyresorufin as substrates. 7-Alkoxyresorufin *O*-dealkylase activities were measured by a sensitive fluorimetric procedure (detection limit: 0.01 pmol/min mg protein), using a Hitachi F-2000 spectrofluorimeter. Cerebral subcellular fractions (20–100 μg proteins) were poured into the fluorimeter microcuvette containing 2.265 mL of 50 mM Hepes, 15 mM $MgCl_2$, 0.1 mM K-EDTA buffer, pH 7.5, and 5 μL of 600 μM 7-alkoxyresorufin dissolved in DMSO. The reaction was started by the addition of 5 μL of 50 mM NADPH or NADH, and was run at 37° for 20 min. The initial rate was linear for at least 30 min. The fluorimeter was calibrated by addition of standard solutions of resorufin, the fluorescence being recorded at 584 nm with a 559 nm excitation wavelength for 7-benzoxyresorufin *O*-dealkylase activity (BROD) and 7-pentoxyresorufin *O*-dealkylase activity (PROD) and at 584 nm with a 537 nm excitation wavelength for EROD.

Enzymatic activities were corrected from non-enzymatic formation of resorufin resulting from the direct interaction of cofactors with the substrates and protein interferences with the signal by blank assays run with all components and boiled cerebral preparations.

Statistical tests. Differences between two groups were assessed by the non-parametric Mann–Whitney test. Analysis of variance was performed if more than two different results were to be compared.

RESULTS

Regional subcellular distribution of cerebral P450 content

The cytochrome P450 amounts measured in both microsomal and mitochondrial fractions isolated from male rat brain were shown in Table 1. P450 content varied between 3 to 5 pmol/mg protein in microsomal fractions and between 25 to 50 pmol/mg protein in mitochondria, depending on the region studied. P450 levels were always higher in mitochondria than in microsomes in all regions examined, with a ratio mitochondrial fraction to microsomes ranging between 5.4 to 11.2. The highest P450 amounts were observed in both fractions isolated from olfactory bulbs. These results confirm and complete previous data reported by our group, showing a mean ratio of 10 to 12 in the rat brain [12, 25].

Regional subcellular distribution of P450-dependent activities

Although P450 content was largely higher in mitochondrial than in microsomal fractions, BROD was lower in mitochondria than in microsomes for all regions studied (range from 2.5 to 11 pmol/min . mg protein in microsomes, and from 0.15 to 1.5 in mitochondria). The distribution of EROD and PROD between both subcellular fractions was heterogenous, depending on the region studied (Table 2A).

When these activities were expressed per P450 content in the fraction, the values obtained were always higher in microsomes than in mitochondria (4- to 80-fold, depending on the region studied and the substrates used). Moreover, in these conditions, BROD was 6 to 21 times higher in brain microsomes than in liver microsomes whatever the brain region examined (Table 2B).

Table 1. P450 content of cerebral microsomal and mitochondrial fractions isolated from six different cerebral regions in male rats

Regions	P450 content (pmol/mg protein)		Ratio 2/1
	Microsomes (1)	Mitochondria (2)	
Olfactory bulbs	4.33 ± 1.22	48.53 ± 6.77*	11.20
Cerebellum	3.13 ± 0.45	31.15 ± 4.49	9.95
Cortex	4.20 ± 0.43	36.44 ± 4.07	8.67
Hypothalamus	3.63 ± 0.62	25.45 ± 9.00	7.01
Medulla oblongata	4.70 ± 0.29	25.36 ± 8.77	5.40
Midbrain	4.66 ± 0.53	27.10 ± 4.68	5.81

Means ± SD, obtained from at least three values measured on the fractions obtained from pooled brain regions.

* $P < 0.02$ as compared to the mean mitochondrial P450 content of the six brain regions by analysis of variances (test of Kruskal and Wallis).

A heterogenous regional repartition of the three activities measured was also observed. The highest microsomal BROD was found in the olfactory bulbs and the lowest in the cerebellum, but only the olfactory bulbs showed a higher BROD when results were related to P450 content. Olfactory bulbs showed the highest mitochondrial BROD and cortex the lowest, but only hypothalamus, cerebral cortex and midbrain showed a lower BROD when results were expressed per mg of P450 content (Table 2).

PROD showed the highest values in microsomes isolated from both olfactory bulbs, and cerebellum, but this activity was not detectable in hypothalamus and midbrain and the other regions showed intermediate activities. No EROD was detected in hypothalamus and medulla oblongata, whereas other regions showed statistically different activities. Mitochondrial PROD distribution was very heterogenous in the six regions studied: the highest activity was observed in olfactory bulbs (0.6 pmol/min.mg protein) and the lowest in hypothalamus and cortex (0.06 pmol/min.mg protein). These differences were less important when activities were expressed per P450 content, but in this case the specific activities were very low. Mitochondria isolated from olfactory bulbs and cerebellum contained the highest EROD (Table 2A and B).

Depending on the originating region, the ratio of EROD to PROD activities varied between 2.2 and 8.2 in mitochondria and between 0.9 and 4.6 in microsomes, suggesting a heterogenous distribution of the P450 isoforms involved in these activities.

Sex-related differences

Several factors, including sex, have been found to alter P450-dependent activities. For instance, sex-related differences have been reported in several liver microsomal O-dealkylation activities [29, 30]. Thus assays were run in order to determine whether rat brain P450-dependent activities are associated with sex differences. No significant difference was observed between male and female total brain P450 contents (data not shown). BROD, PROD and EROD in microsomes and mitochondria isolated from male and female rat brains are given in Table 3. No changes were observed in the three 7-alkoxyresorufin *O*-dealkylase activities in mitochondrial fractions. Conversely, sex-linked differences appeared in microsomal PROD and EROD. The highest cerebral activities recorded were PROD in female microsomes and EROD in male microsomes when results were expressed either per mg of protein or per pmole of P450.

Effects of an inducing treatment

The modifications produced by an *in vivo* chronic treatment of rats with PB or 3MC on P450 content and 7-alkoxyresorufin *O*-dealkylase activities in rat brain subcellular fractions are shown in Table 4. In mitochondrial and microsomal fractions, we observed a significant 2- and 3-fold increase of EROD after 3MC administration, confirming previous results [15]. No other P450-dependent activity was significantly altered by PB or by 3MC treatments.

P450 in isolated brain microvessels

The P450 content of isolated microvessel homogenate was 16 pmol/mg protein, in agreement with previous measurements showing a higher P450 content in brain microvessels as compared to rat brain homogenate [16]. No differences in total P450 content were observed between rat male and female brain preparations (not shown). Table 3 shows that neither male nor female microvessels contained any detectable EROD, whereas a significant sex-linked difference appeared with BROD, that was higher in female microvessels. No significant increase of total P450 (not shown) neither PROD and BROD was observed after either PB or 3MC treatment, but EROD became detectable after a chronic treatment with 3MC (Table 4).

DISCUSSION

The presence of cytochromes P450 in nervous tissue is now well recognized, as both the functional activity and the presence of the protein in the whole brain or in subcellular preparations has been demonstrated by several groups. However, even if its predominant presence in brain mitochondrial fraction was reported by several authors [11, 12, 25, 31], its subcellular repartition has been questioned, due probably to methodological differences [19]. The

Table 2. Benzoxy- (BROD), pentoxy- (PROD) and ethoxyresorufin *O*-dealkylase activities (EROD) in microsomes and mitochondria isolated from six different regions from male rat brain

	Microsomes			Mitochondria		
	BROD	PROD	EROD	BROD	PROD	EROD
(A) Activities (pmol/min . mg protein)						
Brain regions						
Olfactory bulbs	11.05 ± 3.39*	0.46 ± 0.21	0.40 ± 0.01	1.54 ± 0.34	0.60 ± 0.08	1.41 ± 0.20†
Cerebellum	2.50 ± 0.19*	0.23 ± 0.08	0.44 ± 0.02	0.93 ± 0.07	0.33 ± 0.04	1.37 ± 0.13
Cortex	3.51 ± 0.19	0.16 ± 0.04	0.74 ± 0.03*	0.15 ± 0.02	0.05 ± 0.02	0.41 ± 0.05
Hypothalamus	4.35 ± 1.37	ND	ND	0.27 ± 0.04	0.06 ± 0.01	0.49 ± 0.06
Medulla oblongata	4.26 ± 0.46	0.19 ± 0.08	ND	0.46 ± 0.07	0.14 ± 0.04	0.39 ± 0.04
Midbrain	3.27 ± 0.29	ND	0.76 ± 0.17*	0.41 ± 0.01	0.15 ± 0.01	0.34 ± 0.03
Liver	80.23 ± 21.97	68.58 ± 11.07	59.79 ± 5.45			
(B) Activities (pmol/min . pmol cytochrome P450)						
Brain regions						
Olfactory bulbs	2.552 ± 0.780*	0.106 ± 0.048	0.093 ± 0.018	0.031 ± 0.007	0.012 ± 0.002	0.029 ± 0.004
Cerebellum	0.798 ± 0.060*	0.073 ± 0.025	0.140 ± 0.007	0.029 ± 0.002	0.010 ± 0.001	0.043 ± 0.003*
Cortex	0.840 ± 0.050	0.038 ± 0.009	0.175 ± 0.007*	0.004 ± 0.001	0.001 ± 0.001	0.011 ± 0.001
Hypothalamus	1.200 ± 0.380	ND	ND	0.011 ± 0.002	0.002 ± 0.001	0.019 ± 0.002
Medulla oblongata	0.906 ± 0.098	0.040 ± 0.017	ND	0.018 ± 0.003	0.006 ± 0.002	0.015 ± 0.002
Midbrain	0.701 ± 0.062	ND	0.163 ± 0.037*	0.015 ± 0.001	0.006 ± 0.005	0.012 ± 0.001
Liver	0.12 ± 0.03	0.10 ± 0.02	0.09 ± 0.01			

Means ± SD were obtained from at least three values measured on the same pools as in Fig. 1.
ND, not detectable.
* $P < 0.05$, † $P < 0.01$ as compared, for each activity considered, to the mean activity of the six brain regions in each subcellular fraction by analysis of variances (test of Kruskal and Wallis).

Table 3. Sex-linked differences in brain cytochromes P450-dependent activities

Fractions	BROD	PROD (pmol/min . mg protein)	EROD
Microsomes			
Male	2.58 ± 0.22	0.17 ± 0.06	0.55 ± 0.03*
Female	3.08 ± 0.69	0.32 ± 0.09*	0.29 ± 0.04
Mitochondria			
Male	0.70 ± 0.14	0.26 ± 0.09	0.68 ± 0.02
Female	0.85 ± 0.17	0.31 ± 0.07	0.59 ± 0.15
Microvessels			
Male	0.79 ± 0.07	0.47 ± 0.08	ND
Female	1.23 ± 0.13*	0.49 ± 0.06	ND

Means ± SD were obtained from at least three values measured on different pools.
ND, not detectable.
* $P < 0.05$, Mann–Whitney test.

present results show clearly that the cytochrome P450 content of rat brain mitochondrial fractions was always at least five times higher than in microsomes in all cerebral regions examined. This peculiar distribution corresponds to that found in steroidogenic organs like adrenals [32], in accordance with the ability of brain mitochondria to metabolize cholesterol to pregenolone [13]. Moreover, the activities of brain P450 towards exogenous substrates are generally higher in microsomes than in mitochondria, especially if the activities are expressed towards P450 content (Table 2A and B). These results suggest that brain mitochondrial P450s are mainly involved in endogenous metabolism [13], but it must be remembered that the cytochromes P450 originating from both fractions are able to metabolize xenobiotics [6, 14, 15, 31, this work].

After subcellular fractionation of pools obtained from different brain regions, the characterization of functional P450 isoforms was carried out using the specific substrates 7-pentoxy- and 7-ethoxyresorufin, respectively, metabolized in the liver by P450 b and P450 c, and 7-benzoxyresorufin, a substrate for both isoenzymes [22, 33]. The use of these substrates allowed the characterization of P450 b- and c-like activities in the rat brain, where the purification of P450 isoforms is technically very difficult due to the relatively low content of enzyme [12, 34]. Using exactly the same experimental methods for the measurement of P450-dependent activities, we found that BROD from control rat brain microsomes, if related to the P450 content, was higher than in control liver microsomes, whereas PROD and EROD showed the same order of activity in olfactory bulbs, cerebellum and cortex microsomes than in the liver (Table 2). In these same conditions, the mitochondrial activities are very low, suggesting that P450 b and c are preferentially microsomal isoforms of P450 in the brain.

We observed also that both subcellular P450 content and P450-linked activities are the highest in olfactory bulbs. This is partly in accordance with a study on the regional activity of NADH-dependent mitochondrial aryl hydrocabon hydroxylase, in which Das *et al.* [14] reported that olfactory lobes showed an activity at least 60% higher than in any other brain regions having a similar enzyme activity. These results could be explained in part by the fact that rodents are animals having an important use of their olfactory capacities, and by the probable involvement of cytochromes P450 in several brain functions. However, in the present study, the other regions also showed strong differences. A heterogenous repartition has also been reported in several works using immunocytochemical techniques for P450 identification.

As microsomes and mitochondria may contain variable proportions of enzymatically inactive yet immunoresponsive isoforms, it was useful to measure the specific activities of these isoforms in order to complete immunocytological results. In the present work, we found that PROD was high in microsomes obtained from olfactory bulbs and cerebellum, showed intermediate levels in cortex and medulla oblongata, but was undetectable in hypothalamus and midbrain. On the other hand, EROD was the highest in cortex and midbrain microsomes, medium in cerebellum and olfactory bulbs, whereas no significant activities were recorded in microsomes isolated from hypothalamus and medulla oblongata. There are some discrepancies between the immunologic responses reported by several authors, due probably to the use of different methodologies. For instance, Kapitulnik *et al.* [18] described by immunohistochemical localization in rat brain an immunoreactivity toward P450 c, but were unable to find an immunoreactivity towards P450 b. Conversely, by the use of immunoinhibition methods Warner and co-workers [19, 21] reported a recognition of brain P450 by polyclonal antibodies raised against both b and c liver isoforms. Nevertheless, these different methodological approaches do not explain why we have observed the highest levels of activities in olfactory bulbs, whereas the hypothalamus showed undetectable levels of EROD and PROD in *microsomes* only, and Kapitulnik *et al.* [18] reported high levels of immunoreactivity against immunoglobulin-G to P450 c in hypothalamus.

When the cerebral activities of P450 isoforms were compared in male and female, both PROD and

Table 4. Effects of an *in vivo* treatment with phenobarbital (PB) or 3-methylcholanthrene (3MC) on P450-linked activities in the rat brain

Fraction	BROD	PROD (pmol/min . mg protein)	EROD
Microsomes			
Control	2.12 ± 0.11	0.19 ± 0.02	0.48 ± 0.02
PB	2.32 ± 0.51	0.23 ± 0.05	0.89 ± 0.11
3MC	2.38 ± 0.39	0.17 ± 0.02	1.44 ± 0.50*
Mitochondria			
Control	0.45 ± 0.07	0.20 ± 0.05	0.30 ± 0.07
PB	0.51 ± 0.11	0.26 ± 0.08	0.34 ± 0.08
3MC	0.43 ± 0.05	0.16 ± 0.03	0.63 ± 0.04*
Microvessels			
Control	0.63 ± 0.03	0.48 ± 0.06	ND
PB	0.85 ± 0.27	0.76 ± 0.26	ND
3MC	0.76 ± 0.03	0.40 ± 0.01	0.24 ± 0.02*

Means ± SD were obtained from at least three values measured on different pools.
ND, not detectable.
* $P < 0.05$ as compared to control values by the Mann–Whitney test.

EROD are markedly sex-dependent metabolic pathways in microsomes, whereas BROD is sex-dependent in microvessels. As the total amount of P450 present in male rat brain microsomes was not different to that in female, this suggests that the specific catalytic properties differ between male and female brain preparations. Accordingly, EROD was higher in male brain microsomes than in female ones, whereas PROD was higher in female microsomes as compared to male. Brain mitochondrial activities showed no sex differences. Some sex differences in P450-linked catalytic properties have been previously observed in liver microsomes of rats [29], indicating that particular species of P450 are involved in exhibiting the sex differences. However, unlike the situation in the brain, PROD was found to be lower in female microsomes as compared to male liver activities [30].

Previous reports suggested that rat brain P450-dependent monooxygenase activities are few, if not inducible, in experimental conditions that produce a high increase of activities of liver enzyme [2, 9, 15]. In the present work, we observed an increase only for EROD after a 3MC *in vivo* treatment in both brain microsomal and mitochondrial fractions as previously shown [15], as well as in isolated microvessels. PB seemed to be an inefficient inducer of these brain P450-linked activities, suggesting that the expression of cerebral isoforms involved in these biotransformations, especially P450 b, is not regulated by mechanisms like those demonstrated in the liver.

The presence of P450 was confirmed in isolated microvessels, and the present results provide the first evidence that high cytochrome P450-dependent benzoxy- and pentoxyresorufin *O*-dealkylase activities occur in brain capillaries. Thus, their possible role in an enzymatic blood–brain barrier for the protection of the central nervous system against chemical aggressions is confirmed. Conversely, EROD was undetectable in this preparation isolated from control brains, but appeared after a 3MC treatment. These results suggest that P450 contained by brain endothelial cells possess different regulation mechanisms than the brain enzymes.

Although rat brain contains lower amounts of P450 than the liver, several P450-dependent catalytic pathways are efficient in both cerebral microsomal and mitochondrial fractions. Sex-related differences have also been demonstrated, suggesting the presence in rat brain of male- and/or female-specific isoforms. Even if the functional role of cerebral P450 is poorly understood, the results presented here show clearly that the central nervous system possess a significant capability to metabolize xenobiotics.

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