Biochemical Pharmacology, Vol. 30, No. 10, pp. 1142–1145, 1981.
Printed in Great Britain.

0006-2952/81/101142-04 $02.00/0

Effects of chronic administration of pentobarbital or morphine on the brain microsomal cytochrome P-450 system

(*Received* 4 *August* 1980; *accepted* 24 *November* 1980)

The phenomenon of induction of hepatic drug-metabolizing enzymes by barbiturates in various laboratory animals has been well documented [1–4]. In contrast to barbiturates, morphine has been reported to have species and sex-dependent hepatic microsomal activity [5–11]. The rat appears unique in exhibiting a sex-dependent morphine metabolism, since sex dependency has not been seen in the mouse, guinea pig, cat or dog [11–13]. It has been demonstrated, in addition, that morphine significantly inhibits the microsomal metabolism of ethylmorphine in both sexes of mice [14].

In spite of the interest in studying microsomal cytochrome(s) P-450 of brain, only a few reports concerning the properties of the brain microsomal electron transport components have appeared in the literature [15–17]. Two main circumstances are responsible for this lack of knowledge with regard to brain microsomes. First, the microsomal fractions obtained by differential centrifugation methods are almost always contaminated by hemoglobin. Second, the quantities of monooxygenase components in brain microsomes are very small compared to those in hepatic microsomes. Recently, a spectrophotometric method for assaying cytochrome(s) P-450 in pulmonary microsomes that are contaminated with large amounts of hemoglobin and methemoglobin was developed by Johannesen and De Pierre [18]. Using this method, we have undertaken this study to measure mouse brain microsomal cytochrome(s) P-450 levels in pentobarbital- or morphine-tolerant and non-tolerant mice.

Male ICR mice from the Charles River Breeding Laboratories (Wilmington, MA), weighing 15–30 g, were used. Mice were s.c. implanted with 75-mg pentobarbital pellets (acid form) or 75-mg morphine pellets (base form) for 3 days [19–21]. Animals of the control group were implanted with placebo pellets. Forty-five to sixty mice were used in each group.

The following procedures were used to prepare brain and hepatic microsomes. Freshly removed whole brains, excluding the olfactory bulbs, were rinsed three times with icc-cold 0.32 M sucrose–0.10 M Tris buffer (pH 7.4) to remove contaminating blood. The brains were homogenized with 9 vol. of the Tris buffer in a Potter–Elvehjem glass homogenizer with a hand-driven glass pestle (five passes). Three livers were pooled and chopped for rinsing. The chopped livers were homogenized with 3 vol. of 1.15% KCl solution in a Potter–Elvehjem glass homogenizer. Cellular debris, nuclei, and mitochondria were removed from the homogenate by centrifugation at 12,000 *g* (Sorvall RC2-B refrigerated centrifuge) for 20 min. The mitochondrial supernatant fraction was centrifuged at 100,000 *g* (Beckman L5-65 Ultracentrifuge) for 60 min. The resulting microsomal pellet was stored at −80° until assayed. It was then resuspended in 0.10 M Tris buffer (pH 7.4) at the time of assay. The final protein concentration of the resuspension was adjusted to 15–20 mg of brain microsomal protein/ml.

Hepatic microsomal cytochrome P-450 was quantified according to the method of Omura and Sato [22]. NADPH–cytochrome *c* reductase and NADPH dehydrogenase activities were determined according to the methods of Williams and Kamin [23] and Gillette *et al.* [24] respectively. Brain microsomal cytochrome P-450 was measured by the modified method of Johannesen and De Pierre [18]. Brain microsomal protein concentration was adjusted to 0.8 to 2 mg/ml for assay of brain microsomal cytochrome P-450(s) in the presence of ascorbate (250 μM) and phenazine ethosulfate (2.5 μM). Carbon monoxide was bubbled into the microsomal suspension for 1 min. The suspension was then divided between two cuvettes; the auto baseline was maintained throughout the spectral measurements. A few grains of dithionite were added to the sample cuvette and the spectrum was recorded using a Varian Cary 219 spectrophotometer at 24 ± 1°. An extinction coefficient of 91 mM^{-1} cm^{-1} was used for the calculation of the microsomal cytochrome(s) P-450 quantities.

In an attempt to increase our accuracy, the mean of the absorption at 450 nm minus the absorption at 460 nm and the absorption at 450 nm minus the absorption at 440 nm was used for calculation. This was done because for some unexplained reason the absorption sometimes increased steadily from 460 to 490 nm, as reported previously by Johannesen and De Pierre [18]. Using these assay conditions, a linear relation between brain microsomal protein concentration and cytochrome(s) P-450 content with good reproducibility, was obtained.

In another experiment, incorporation of [^{3}H]leucine into the brain microsomal fraction was studied. Mice were treated with 18.5 nmoles/kg (1.33 mCi/kg) of L-[4,5-^{3}H]leucine (sp. act. 72 Ci/mmole, Amersham/Searle, Arlington Heights, IL), i.p., and a 75-mg pentobarbital or morphine pellet (implanted concurrently) 48 hr prior to the mice being killed. This schedule was based on our previous experiment which indicated that a significant increase of protein synthesis occurred in the whole brains of animals that had been concurrently implanted with a pentobarbital pellet and given [^{3}H]leucine for 48 or 72 hr [25].

Animals in the control group received similar treatment except that each was implanted with a placebo pellet.

Five brains were pooled and rinsed with ice-cold 0.32 M sucrose–0.10 M Tris buffer (pH 7.4). They were homogenized with 9 vol. of the Tris buffer using a Potter–Elvehjem homogenizer. Cellular debris and nuclei (P_1) were removed from the homogenate by centrifugation at 1,000 *g* for 10 min. The P_1 pellets were washed with the Tris buffer and centrifuged again at 1000 *g* for 10 min. The 1000 *g* supernatant fractions from washings were centrifuged for 20 min at 12,000 *g* to sediment mitochondria. The mitochondrial supernatant fraction was centrifuged for 60 min at 100,000 *g*. The resulting microsomal fraction pellets were resuspended using a Potter–Elvehjem homogenizer. Aliquots of microsomal homogenate were added to 10 ml of PCS II (Amersham/Searle), and radioactivity was counted using a Searle Mark II 6847 liquid scintillation counter with a counting efficiency of 33 per cent. Protein was measured using the method of Lowry *et al.* [26]. All data were subjected to statistical analyses, and the statistical significance was determined by Student's *t*-test (two-tailed).

When carbon monoxide served as a ligand, spectral analysis of the mouse brain microsomal fraction revealed

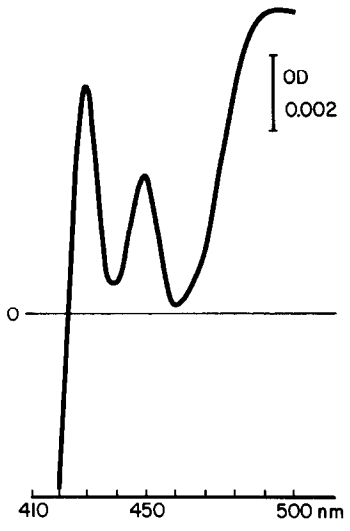

Fig. 1. Measurement of cytochrome(s) P-450 in brain microsomes. The difference spectrum of brain microsomal cytochrome(s) P-450 was redrawn from data obtained as described in the text. The microsomal protein concentration was 1.7 mg/ml.

two prominent peaks (Fig. 1). The larger peak, with maximum absorption at 430 nm, resulted from the presence of cytochrome b_5 [15]. The smaller one, presumably associated with one or more microsomal hemoproteins, peaked at 450 nm.

As shown in Table 1, the levels of brain microsomal cytochrome P-450 in the control group were only 2.04 per cent of the hepatic microsomal cytochrome(s) P-450 contents. After 3-day continuous treatment with pentobarbital, hepatic microsomal cytochrome P-450 levels increased significantly, compared to those of the placebo implanted group. The content of brain microsomal cytochrome(s) P-450, however, did not change with 3-day pentobarbital treatment. While the level of hepatic microsomal cytochrome P-450 of the morphine 3-day-implanted group was decreased significantly, the brain microsomal cytochrome P-450 content was not altered by morphine.

As shown in Table 2, the activity of brain microsomal NADPH dehydrogenase was 16.2 per cent of that of liver in placebo control mice. Hepatic microsomal NADPH dehydrogenase activities were increased significantly in the pentobarbital group and were decreased significantly in the morphine group. Brain microsomal NADPH dehydrogenase activities, however, were not affected by the 3-day treatment with pentobarbital or morphine.

The activity of brain microsomal NADPH–cytochrome *c* reductase was 4.99 per cent of that in liver (Table 2). The activity of hepatic microsomal NADPH–cytochrome *c* reductase was also increased significantly after 3 days of continuous treatment with pentobarbital; the brain microsomal enzyme activities, however, were not altered in pentobarbital-treated mice. Morphine administration did not affect either the hepatic or the brain activity.

The effect of 3 days of continuous treatment with pentobarbital or morphine on [^{3}H]leucine incorporation by brain microsomal fractions is shown in Table 3. In spite of the treatment, leucine incorporation into brain microsomal fractions was not changed significantly.

The present data demonstrate that mouse brain microsomal cytochrome(s) P-450 can be measured spectrophotometrically by applying the method of Johannesen and De Pierre [18]. The values obtained in this study agree closely with the ratio of rat brain to liver microsomal cytochrome P-450 content reported by Sasame *et al.* [16] and Marietta *et al.* [17]. A difference between the levels of brain and liver microsomal cytochrome(s) P-450, however, has been noted in the literature [16, 17]. Thus, our results may be indicative of species variations in cytochrome(s) P-450 contents in liver and brain. Marietta *et al.* [17] reported hepatic microsomal cytochrome(s) P-450 contents in the rat of 35 pmoles/mg protein in brain and 1186 pmoles/mg protein in liver. In our mouse experiments, lower levels of microsomal cytochrome P-450 were calculated, being, respectively, 31 and 44 per cent in brain and liver, compared to the values (above) reported in the rat [17].

In agreement with earlier studies [2, 27], pentobarbital administration caused increases in cytochrome P-450 levels and in the activities of NADPH dehydrogenase and NADPH–cytochrome *c* reductase, in the hepatic microsomal fraction. Morphine had the opposite effect on hepatic cytochrome P-450 levels and NADPH dehydrogenase activity. These results support our previous observations that pentobarbital metabolism in the liver is accelerated in pentobarbital-tolerant mice [28] and is inhibited in morphine-tolerant animals [14, 29]. On the other hand, 3-day continuous administration of pentobarbital or morphine had no effect on these variables in brain microsomal preparations. Our results also reveal that 3-day continuous treatment with pentobarbital or morphine did not affect microsomal protein synthesis, as shown by [^{3}H]leucine incorporation into the mouse brain microsomal fraction. It has been demonstrated that various inducers affect multiple forms of cytochrome P-450 in liver microsomes [30]. Although multiple forms of cytochrome P-450 may exist in brain microsomes, they do not appear to be affected by

Table 1. Effects of 3-day continuous treatment with pentobarbital or morphine on hepatic and brain microsomal cytochrome(s) P-450 levels*

	Cytochrome P-450†					
	Liver			Brain		
Treatment	(pmoles/mg protein)	(nmoles/g tissue)	N	(pmoles/mg protein)	(nmoles/g tissue)	N
Placebo	525 ± 39	23.9 ± 1.18	8	10.7 ± 0.2	0.108 ± 0.01	17
Pentobarbital	867 ± 39‡	37.1 ± 0.67‡	8	10.9 ± 0.3	0.112 ± 0.01	12
Morphine	367 ± 29§	14.9 ± 0.15‡	8	9.7 ± 0.4	0.093 ± 0.01	9

* Each hepatic sample was from three pooled livers. Each brain sample was from five pooled brains. Values are means ± S.E.M.; N = number of samples.

† A molar extinction coefficient of 91 mM^{-1} cm^{-1} [22] was used for the calculations.

‡ $P < 0.001$, compared to placebo implanted group.

§ $P < 0.01$, compared to placebo implanted group.

Table 2. Effects of 3-day continuous treatment with pentobarbital or morphine on hepatic and brain microsomal NADPH dehydrogenase and NADPH–cytochrome *c* reductase activities*

Treatment	NADPH dehydrogenase† [nmoles·(mg protein)$^{-1}$·min^{-1}]				NADPH–cytochrome *c* reductase‡ [nmoles·(mg protein)$^{-1}$·min^{-1}]			
	Liver	N	Brain	N	Liver	N	Brain	N
Placebo	4.14 ± 0.17	8	0.67 ± 0.06	11	63.9 ± 1.0	5	3.01 ± 0.18	16
Pentobarbital	5.54 ± 0.33‖	8	0.66 ± 0.02	13	101.2 ± 1.2§	5	2.88 ± 0.10	13
Morphine	3.44 ± 0.19¶	8	0.65 ± 0.05	13	67.1 ± 1.7	5	2.28 ± 0.33	16

* Each sample was prepared as described in the text. Values are means ± S.E.M.; N = number of samples.

† A molar extinction coefficient of 6.2 mM^{-1} cm^{-1} [24] was used for the calculations.

‡ A molar extinction coefficient of 18.5 mM^{-1} cm^{-1} [23] was used for the calculations.

§ $P < 0.001$, compared to placebo group.

‖ $P < 0.01$, compared to placebo group.

¶ $P < 0.02$, compared to placebo group.

Table 3. Effects of 3-day continuous treatment with pentobarbital or morphine on [^{3}H]leucine incorporation into brain microsomal fraction*

Treatment	[^{3}H]Leucine incorporation (fmoles/mg protein)	N
Placebo	45.3 ± 3.5	8
Pentobarbital	43.3 ± 3.3	4
Morphine	40.6 ± 1.8	4

* Each brain sample was from five pooled brains. Values are means ± S.E.M.; N = number of samples.

continuous 3-day treatment with pentobarbital or morphine. Thus, our results characterize the brain microsomal cytochrome(s) P-450 system as possibly being quantitatively and qualitatively different from that of liver. It appears that these data directly substantiate the role of functional tolerance in the brain and nullify the role of dispositional tolerance to pentobarbital or morphine within the CNS [31].

In summary, male ICR mice were rendered tolerant to either pentobarbital or morphine by pellet implantation methods. Brain microsomal cytochrome(s) P-450 content, NADPH dehydrogenase activity, NADPH–cytochrome *c* reductase activity, and protein synthesis were determined in "tolerant" and naive animals. Brain cytochrome(s) P-450 content, NADPH dehydrogenase activity, NADPH–cytochrome *c* reductase activity and [^{3}H]leucine incorporation into protein of the brain microsomal fraction were not altered by 3-day continuous treatment with pentobarbital or morphine. In contrast to brain microsomal enzyme activities, hepatic microsomal variables were altered significantly after pentobarbital or morphine treatment. These data suggest that the brain microsomal cytochrome P-450 system may be significantly different from the hepatic system with respect to its participation in the development of dispositional tolerance to pentobarbital. As far as morphine is concerned, it appears that there is no relation between development of tolerance to morphine and changes in the hepatic and brain microsomal cytochrome P-450 systems.

Acknowledgements—These studies were supported by Grants DA-01403 and DA-01310 from the National Institute on Drug Abuse. We thank Miss Jeanne Smith and Mr. Marland Dulaney, Jr. for their assistance.

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