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Effects of chronic prenatal, neonatal and adult exposure to barbiturates on mitochondrial benzodiazepine receptors in mouse testis

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Abstract—In the present study we investigated the effect of chronic exposure to phenobarbital, administered to mice during the prenatal or neonatal period, as well as to adult mice, on mitochondrial benzodiazepine receptors in the testis. Three modes of treatment were investigated: (1) offspring of pregnant mice receiving food containing 3 g/kg phenobarbital until gestational day 18 were killed at 22 or 50 days of age and assayed for receptor binding (prenatal group); (2) offspring of untreated mice were injected subcutaneously once daily with 50 mg/kg phenobarbital on days 2–21 of age and killed at 22 or 50 days of age (neonatal group); (3) adult mice were injected subcutaneously once daily for 3 weeks with 50 or 100 mg/kg phenobarbital (adult group). Prenatal or neonatal exposure to phenobarbital did not alter the testicular weight in all groups (except for the neonatally exposed group killed at 22 days of age), or the mitochondrial benzodiazepine receptor binding characteristics. However, the maximal number of these receptors in the testes of mice in the adult group receiving 100 mg/kg phenobarbital was significantly increased (42%, $P < 0.05$), compared to controls. The administration of 50 mg/kg phenobarbital to the adult group also induced an increase (27%, non-significant) in testicular mitochondrial benzodiazepine receptors. Phenobarbital administration did not affect the receptor affinity values or the weight of the testis. It is unclear whether these receptor alterations due to chronic phenobarbital exposure of adult mice reflect functional changes in the testis.

Key words: testis; phenobarbital; mitochondrial benzodiazepine receptor; PK 11195

Women during pregnancy, as well as neonates, may be treated repeatedly with barbiturates for epilepsy or sleep disturbances or when undergoing a variety of general-anesthetic procedures. Numerous studies have indicated the pre- and postnatal exposure to barbiturates can cause gross changes in the central nervous system and in the morphology of peripheral organs [1–3]. These changes are accompanied by alterations in hormone and receptor levels and can affect behavioral paradigms [2, 4–6].

Barbiturates exert their action by interaction with the supramolecular complex of the GABA*/central BZ receptor, which regulates chloride ion channel influx into the postsynaptic neuron [7]. Central BZ receptors are coupled to the GABA receptors on the neuron surface and mediate the anxiolytic, hypnotic and anticonvulsant effects of BZs. Chronic treatment with barbiturates decreases central BZ receptor densities in adult mouse brain and diminishes stimulation of BZ binding induced by GABA and phenobarbital in primary chick embryo cultures *in vitro* [8]. Administration of phenobarbital to pregnant rats during the last half of pregnancy reduces the number of central BZ receptors in the brains of their offspring [9].

In addition to central BZ receptors, another type of BZ receptor, the peripheral type, are present in most peripheral organs, but also, at low concentrations, in the brain [10]. These receptors differ from the central receptors in many aspects, including localization within the cell, affinity to ligands and function. Peripheral BZ receptors are localized on the mitochondrial outer membrane [11] and bind with high affinity the BZ Ro 5-4864 and the isoquinoline carboxamide derivative PK 11195 [12], ligands which are not bound by central-type receptors. Because of their subcellular localization, they have been termed mitochondrial BZ receptors, suggesting a role in cellular energy turnover [13], which is manifested especially during active cellular differential and proliferative processes [14]. A striking feature of mitochondrial BZ receptors is their localization in steroidogenic tissues, in particular the adrenal cortex and testis, where they play a major role in steroidogenesis [15].

The aim of the present study was to investigate whether exposure to barbiturates during the pre- and neonatal periods, as well as during adulthood, would induce changes in mitochondrial BZ receptors in the mouse testis, an organ which completes its maturation only during puberty. Alterations in these receptors might shed light on the dynamic stages of growth and development of this organ.

Materials and Methods

Chemicals. [^{3}H]PK 11195 (74.3 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). Unlabeled PK 11195 was kindly donated by Dr Anne Bouvier (Rhône-Poulenc Santé, Vitry sur Seine, France). Lumax was obtained from Lumac (Schaesberg, The Netherlands). All other chemicals were purchased from commercial sources.

Prenatal administration. The study was approved by the Animal Care and Use Committee of the Hebrew University-Hadassah Medical School. Heterogeneous stock (HS/Ibg) mice were used. Four females and one male were housed in each mating cage and maintained under standard laboratory conditions of 24° and a 12-hr light/12-hr dark cycle. Female mice were checked daily at 0800 hr for insemination, as evidenced by a vaginal plug. Females with plugs were separated from the males and housed with other pregnant females (gestational day 1). On gestational day 9 the females were placed in individual cages. Treated females then received milled mouse food containing 3 g/kg phenobarbital in acid form, which was the only food source, and water, both available *ad lib*. Control animals received milled food and water. Drug administration continued until gestational day 18. One group of treated and control offspring were killed by decapitation at 22 days of age, and a second group were killed at 50 days of age. Their testes were removed, cleaned of adjacent tissues and stored at −70° until use.

* Abbreviations: GABA, γ-aminobutyric acid, BZ, benzodiazepine; 6β-OH, 6β-hydroxylase; 2α-OH, 2α-hydroxylase.

Table 1. Effects of pre- and neonatal administration of phenobarbital (PHB) on mitochondrial BZ receptors in the testis

Study	Type of treatment	N	Age at receptor assay (days)	K_d (nM)	B_{max} (fmol/mg protein)	Weight of testes (mg)
I	Control	7	22	2.4 ± 0.3	1156 ± 89	43 ± 3
	Prenatal PHB	6	22	2.1 ± 0.4	1133 ± 74	44 ± 5
II	Control	7	50	1.7 ± 0.6	1294 ± 104	141 ± 8
	Prenatal PHB	6	50	2.2 ± 0.2	1203 ± 76	158 ± 3
III	Control	7	22	2.8 ± 0.5	1036 ± 95	47 ± 3
	Neonatal PHB	6	22	1.5 ± 0.4	948 ± 63	38 ± 3*
IV	Control	7	50	1.7 ± 0.2	1113 ± 112	176 ± 9
	Neonatal PHB	6	50	2.0 ± 0.2	1244 ± 105	155 ± 6*

Offspring of pregnant mice receiving PHB in food (3 g/kg) until gestational day 18 were assayed for receptor binding at 22 and 50 days of age (prenatal group, studies I and II). Offspring of untreated mice were treated with PHB (50 mg/kg, subcutaneously) on days 2–21 of age and assayed for receptor binding at 22 or 50 days of age (neonatal group, studies III and IV).

Results are expressed as means ± SEM.

* $P < 0.05$ vs corresponding control.

Neonatal administration. Adult female mice were housed with males until pregnancy became apparent by visual observation. At this time the females were removed to individual cages. After delivery, pups in each litter were divided into barbiturate-treated and untreated control groups. Treated pups received a daily subcutaneous injection of 50 mg/kg sodium phenobarbital in distilled water (10 mL vehicle/kg mouse) on days 2–21 of age. Control pups received injections of vehicle only. One group of treated and control pups were killed by decapitation at 22 days of age; a second group were maintained with their mothers, received no further treatment and were killed at 50 days of age. Their testes were removed and stored at −70° until assayed.

Adult administration. Adult male mice received a daily subcutaneous injection of either 50 mg/kg or 100 mg/kg sodium phenobarbital in distilled water (10 mL/kg mouse) for 3 weeks. Control mice were injected with vehicle. Their testes were removed and stored at −70° until assayed.

Membrane preparation. Prior to the binding assay, tissues were thawed and homogenized in 20 mL of 50 mM Tris–HCl buffer, pH 7.4, at 4° and centrifuged at 49,000 *g* for 15 min. The supernatants were discarded and the pellets resuspended in 200 volumes of Tris–HCl buffer and used for binding studies.

Binding assay. Membrane homogenates (400 μL) were incubated with 25 μL [^{3}H]PK 11195 (0.2–6 nM final concentration) in the absence (total binding) or presence (non-specific binding) of 10^{-5} M (final concentration) of unlabeled PK 11195. After incubation for 60 min at 4°, samples were filtered under vacuum over Whatman GF/B filters and washed three times with 5 mL of ice-cold Tris–HCl buffer. Filters were placed in vials; 4 mL of xylene-Lumax (3:1) scintillation fluid were added, and radioactivity was counted after an 8-hr equilibration period. Protein concentrations were measured by the method of Lowry *et al.* [16], using bovine serum albumin as the standard.

Statistical analysis of the data was performed using the Student's *t*-test and ANOVA. All results are expressed as means ± SEM.

Results

As shown in Table 1, there was a small, insignificant increase in the weight of the testes of mice in the prenatally phenobarbital-exposed group killed at 50 days of age. In the neonatally phenobarbital-exposed group killed at 22 days of age, there was a decrease in the weight of the testes (19%, $P < 0.05$) vs the control groups. In addition, we noted differences between the weight of control testes of the prenatal and neonatal offspring groups. Nevertheless, alterations in testicular weight were not accompanied by changes in mitochondrial BZ receptor binding affinities (equilibrium dissociation constant, K_d) or densities (maximal number of binding sites, B_{max}). Administration of phenobarbital for 3 weeks to adult mice did not affect testicular weight (Table 2). However, it induced a significant

Table 2. Effects of phenobarbital (PHB) administration to adult mice on mitochondrial BZ receptors in the testis

	N	Weight of testes (mg)	K_d (nM)	B_{max} (fmol/mg protein)
Control	12	159 ± 6	1.7 ± 0.1	1036 ± 61
PHB, 50 mg/kg	6	147 ± 7	2.3 ± 0.3	1312 ± 173
PHB, 100 mg/kg	8	162 ± 4	2.4 ± 0.2	1475 ± 156*

Adult mice were injected subcutaneously with 50 or 100 mg/kg PHB once daily for 3 weeks.

Results are expressed as means ± SEM.

* $P < 0.05$ vs control.

elevation in B_{max} values of the mitochondrial BZ receptors in the testes of 100 mg/kg phenobarbital-treated groups (42%, $F(2,23) = 4.057$, $P < 0.05$) vs the control group. The administration of 50 mg/kg phenobarbital to adult mice also induced an increase (27%, non-significant) in testicular mitochondrial BZ receptors. The alterations in B_{max} values were not accompanied by changes in K_d values (Table 2).

Discussion

Previous investigation has shown that prenatal exposure to barbiturates results in reproductive dysfunction in male rats [3]. In humans, the risk of congenital malformations such as congenital heart disease, cleft lip with or without cleft palate, and microcephaly is more common in children of epileptic women taking barbiturates than in the general population [17]. Additionally, the incidence of genital abnormalities such as hypospadis, undescended testis and inguinal hernia was also higher compared to a control population.

Phenobarbital also interferes with the thyroid system [18] and can induce metabolic degradation of thyroxine [19]. Treating neonatal mice with barbiturates abolished peak serum thyroxine levels, which normally appear at the end of the second postnatal week, without significantly affecting non-peak levels at other ages [2]. Ultrastructural damage has been observed in the brain of adult mice even long after discontinuation of barbiturate treatment [2]; the damage is expressed from gestational day 14 to at least gestational day 50 and affects mostly mitochondria, but its distribution is uneven and dispersed among populations of neurons apparently unaffected by treatment. The ultrastructural abnormalities caused by barbiturates in many aspects resemble the effects of early relative hypothyroidism [20]. In a previous study we found that mitochondrial BZ receptors in the rat testis are sensitive to thyroxine [21]. The present study shows that the transient barbiturate-induced change in neonatal profile thyroxine levels does not affect mitochondrial BZ receptor characteristics in the testis.

Autoradiographic studies in rat testis have revealed a high density of mitochodrial BZ receptors in the interstitial tissue, where Leydig cells, which produce testosterone, are present. Lower concentrations have been found in the epithelium of the seminiferous tubules, where sperm cells and Sertoli cells predominate [22]. Chronic administration of diazepam, an agent active at both the central- and mitochondrial-type BZ receptors, to the human male increases blood testosterone levels [23]. A stimulatory effect on the testicular production of testosterone has also been observed in *in vitro* studies [24, 25]. These latter observations strengthen the involvement of BZ receptors in male hormone steroidogenesis (for review, see Ref. 15).

Phenobarbital also affects testosterone metabolism by induction of liver microsomal cytochrome P450 [26]. This drug does not significantly alter the endogenous plasma testosterone concentration, but increases urinary excretion of testosterone [27]. The increased metabolism of testosterone may affect mitochondrial BZ receptors in the testis, which are sensitive to male sex hormones. We have found that chronic administration of testosterone to adult male rats lowers mitochondrial BZ receptor density in the testis [28]. It could be that increased metabolism of testosterone produces an opposite effect on mitochondrial BZ receptors in the testis, thus explaining the observed increase in testicular mitochondrial BZ receptors following chronic exposure of adult mice to barbiturates. Since the activity of testosterone-oxidizing enzymes (6β-OH and 2α-OH) is very low in the fetus [between 1–2% (6β-OH) and 40% (2α-OH) of adult activity] [29], testosterone metabolism is less affected and mitochondrial BZ receptors in the testis do not change by exposure to phenobarbital during prenatal and neonatal life.

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