Increased Densities of Binding Sites for the Peripheral-Type Benzodiazepine Receptor Ligand [³H]PK 11195 in Congenital Ornithine Transcarbamylase–Deficient Sparse Fur Mouse

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ABSTRACT. Peripheral-type (mitochondrial) benzodiaze-pine receptors (PTBR) were studied in the brain and pe-ripheral organs (kidney, liver, and testis) of normal male mice (CD-1/Y) and the congenitally hyperammonemic sparse fur (spf/Y) mouse. Radioligand binding assays were performed with [³H]PK 11195, a ligand with high selectiv-ity and affinity for PTBR. Densities (maximal number of binding sites) of [³H]PK 11195 binding sites were greatest in kidney, followed by liver, testis, and brain. Densities of [³H]PK 11195 binding sites were significantly increased in all tissues of spf mice compared with control animals. In view of the localization of PTBR on the outer mitochondrial membrane, changes in PTBR in spf mouse tissues may modulate the altered mitochondrial function and oxidative metabolism, in brain and peripheral tissues, in congenital OTC deficiency. The positron emission tomography ligand ¹¹C-PK 11195 could find an application in the assessment of end organ dysfunction in this disorder. (*Pediatr Res* 34: 777–780, 1993)

Abbreviations

OTC, ornithine transcarbamylase
PTBR, peripheral-type benzodiazepine receptor
DBI, diazepam-binding inhibitor
Bmax, maximal number of binding sites

OTC deficiency is the most commonly encountered congenital hyperammonemic syndrome in children. In newborn males, OTC deficiency is manifested by hyperammonemic coma that often leads to death, and in those who recover from coma, mental retardation and cerebral palsy are observed. Older children may have milder phenotypes of OTC deficiency resulting in episodic hyperammonemia, with mental retardation and be-havioral abnormalities during adolescence or adulthood (1). Re-cently, many cases of adult-onset OTC deficiency have also been reported (1, 2).

The neurologic syndrome associated with OTC deficiency, characterized by seizures and mental retardation, is generally considered to be the direct consequence of ammonia neurotox-icity (3). However, the precise nature of the pathophysiologic mechanisms responsible for the neurologic syndrome in OTC

deficiency remains to be elucidated. Studies of the cerebral consequences of congenital OTC deficiency have been facilitated by the availability of an appropriate animal model, the sparse fur (spf) mouse. The spf mouse has an X-chromosomal defect of ornithine transcarbamylase (4, 5) resulting in 2- to 5-fold eleva-tions of blood ammonia (6, 7).

PTBR are present in brain as well as in a number of peripheral tissues including kidney, heart, testis, and other endocrine glands (8). In all cases, PTBR are found to be associated with the outer mitochondrial membrane (9, 10), leading to the suggestion that these receptors may be modulators of intermediary metabolism (11). Radioligands such as [³H]PK 11195 and [³H]Ro 5-4864 bind to PTBR with a high degree of selectivity (8). PTBR are pharmacologically and biochemically distinct from the central-type (γ-aminobutyric acid–related) benzodiazepine receptors. Although the precise physiologic function of these receptors is still unclear, a role in the regulation of cell growth and differen-tiation has been suggested (12). In peripheral tissues, PTBR are associated with diverse functions. For example, in testis and other endocrine organs they are coupled to steroidogenesis (13). In kidney, they appear to play a role in natriuresis (14). In brain, PTBR are preferentially localized in astrocytes (11, 15), and there is evidence to suggest that alterations of PTBR and their endog-enous ligands play a role in the astrocytic response to chronic liver disease, and, in this way, may contribute to the pathogenesis of hepatic encephalopathy. In favor of this contention, increased densities of binding sites for [³H]PK 11195 were reported in several brain regions (16, 17) and peripheral tissues (17) of portacaval shunted rats as well as in autopsied brain samples from patients with chronic liver disease who died in hepatic coma (18). It was also reported that the concentrations of endog-enous ligands for these receptors, such as the neuropeptides DBI and octadecaneuropeptide, are elevated in hyperammonemic states resulting from liver dysfunction (19, 20).

To further elucidate the role played by PTBR in hyperam-monemic states, the present study was undertaken to evaluate [³H]PK 11195 binding to the membranes of cerebral cortex, cerebellum, brain stem, liver, kidney, and testis of congenitally hyperammonemic sparse fur (spf/Y) mice. Results of the study indicate that OTC deficiency and the ensuing hyperammonemia result in a generalized increase in densities of PTBR in both brain and peripheral tissues.

MATERIALS AND METHODS

Chemicals. [³H]PK 11195 (sp act 76.9 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Nonradioactive PK 11195 was a gift from Dr. R. Michaud, Rhone-Poulenc Santé, Gennevillers, France. All other chemicals used were of analytical

grade and were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals. Adult male sparse fur (spf/Y) mice, aged 12–15 wk, were used in the study. The parent stock for the colony of spf mutant mice was originally supplied by Dr. L. B. Russel of the Oakridge National Laboratories, Oakridge, TN (4). In the spf/Y mouse colony used in this study, hepatic OTC activity was 10 to 15% of control values (control values ranged from 62 to 72 μmol of citrulline produced/mg of protein/h); serum ammonia and glutamine levels were elevated 2- and 5-fold, respectively (7, 21, 22). The spf/Y mice used presently were obtained by crossing spf/spf homozygous females with normal +/Y males (CD-1/Y strain supplied by Canadian Breeding Farms, St. Constant, Quebec, Canada). All male progeny, being spf/Y, were separated by simple sexing. CD-1/Y strain male mice were used for normal control studies. Animals were housed under 12 h light/dark cycles with free access to water and food (Purina Mouse Chow). All animals were maintained and experimental procedures performed according to the guidelines of the Canadian Council of Animal Care.

Preparation of membranes. CD-1/Y and hyperammonemic spf/Y mice were killed by decapitation; brain, kidney, liver, and testis were quickly dissected out, rinsed with ice-cold saline, frozen immediately on dry ice, and stored at −70°C. On the day of membrane preparation, thawed tissues were homogenized in 20 vol of 40 mM Tris-HCl buffer (pH 7.4) using a Brinkman Polytron (setting 10, 15 s). The homogenates were centrifuged at 38 000 × g for 15 min, and the pellets were washed twice, by rehomogenization in ice-cold buffer and repeated centrifugation at 38 000 × g for 15 min. Final pellets were resuspended in 40 mM Tris-HCl buffer and were frozen at −70°C until the day of the assay.

On the day of the assay, membrane preparations were thawed and washed twice with Tris-HCl buffer (40 mM, pH 7.4) by homogenization and centrifugation at 38 000 × g for 15 min. The final pellet was suspended in 20 vol of Tris-HCl buffer and protein content was adjusted to 1 mg/mL before use in binding assays. Protein content of the membrane preparations was measured by the method of Lowry *et al.* (23).

Binding assay for [³H]PK 11195. [³H]PK 11195 binding assays were performed in triplicate using slight modifications of previously published procedures (18, 24). Equilibrium binding assays were performed in a final volume of 250 μL containing 0.25 to 16 nM [³H]PK 11195 and 40 μg (liver, kidney, and testis) or 100 μg (cerebral cortex, cerebellum, and brain stem) of protein equivalent of membranes in 40 mM Tris-HCl buffer (pH 7.4). After 2 h of incubation at 4°C, binding was terminated by the addition of 4 mL of ice-cold Tris-HCl buffer and rapid filtration under negative pressure through Whatman GF/B glass microfiber filters placed in a Millipore filter manifold. The tubes were rinsed with 4 mL of ice-cold buffer; filters were washed twice with 3 mL of ice-cold buffer and transferred to scintillation vials. Filters were air-dried overnight at room temperature and 10 mL of Ready Protein Liquid scintillation cocktail (Beckman Instruments Inc., Fullerton, CA) was added. Radioactivity was determined in a Beckman LS-1800 liquid scintillation spectrometer. Nonspecific binding was determined in parallel assays performed in the presence of 1 μM nonradioactive PK 11195. Specific binding was the difference between total binding and nonspecific binding. Nonspecific binding was approximately 10–25% of the total binding at different ligand concentrations. Data from the binding assays was analyzed using an iterative curve-fitting procedure to a single rectangular hyperbola (18). Scatchard analysis was performed by first order nonlinear regression analysis of the data from saturation isotherms. Bmax and k_d values were calculated from these plots.

The Tris-HCl buffer used for membrane preparation and binding assay was prepared fresh and filtered twice (once immediately after preparation and once just before the assay)

through 0.45-μm Millipore filters. This care was taken to avoid artifacts due to microbial contamination (25, 26).

Statistics. Statistical analysis was performed by unpaired two-tailed *t* test.

RESULTS

Initial experiments showed that [³H]PK 11195 binding was linear with respect to protein concentration in preparations of cerebral cortex (25 to 200 μg) and peripheral tissues (10 to 100 μg). In all experiments performed, specific binding was observed to be 75–90% of the total binding at different substrate concentrations.

Figure 1 shows representative saturation isotherms and their corresponding Scatchard plots for the specific binding of [³H]PK 11195 to membranes of cerebral cortex of control and spf mice. A saturable specific binding with a high affinity to a single site of receptors was observed in all tissues.

The average Bmax and equilibrium k_d calculated from the Scatchard plots generated from the independent experiments are presented in Table 1. In both control and spf mice, the maximal density of binding sites (as expressed by Bmax values) and equilibrium k_d were greatest in kidney, followed by liver, brain, and testis.

Compared with normal mice, the number of specific binding sites for [³H]PK 11195 were significantly increased in all tissues from spf mice. However, marked tissue differences were observed. In liver and kidney preparations, [³H]PK 11195 specific binding was increased by 67% in spf mice compared with controls. Bmax values for [³H]PK 11195 binding were increased by 50% (testis), 27% (cerebral cortex), 38% (cerebellum), and 102% (brain stem) in spf mice. There were no statistically significant
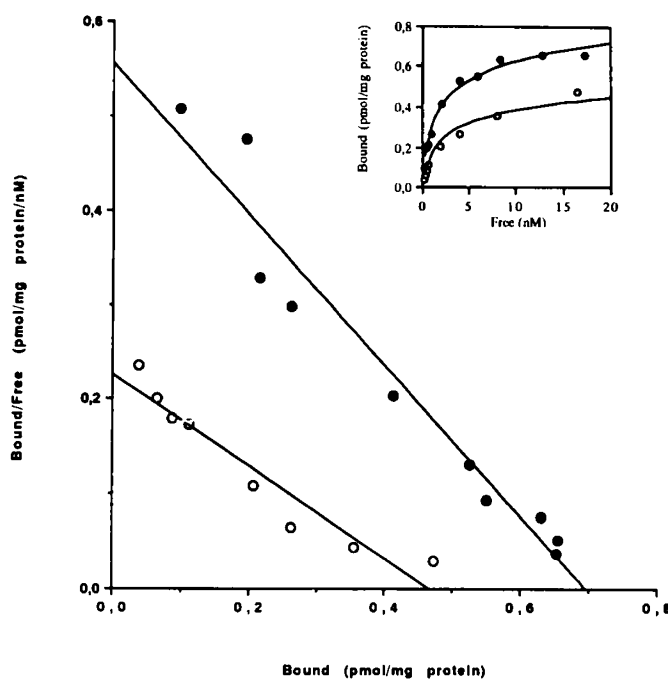

Bound (pmol/mg protein)

Fig. 1. Representative Scatchard plots and their corresponding saturation isotherms (*inset*) of [³H]PK 11195 specific binding to the membranes of cerebral cortex of normal CD-1/Y (O) and hyperammonemic spf/Y (●) mice. Specific binding is the difference between total binding and nonspecific binding (in the presence of 1 μM nonradioactive PK 11195). Nonspecific binding was, in all cases, less than 20% of the total binding at all ligand concentrations. The plots shown here are from one representative experiment. Each value is a mean of triplicate determinations. The time of incubation was 2 h at 4°C. One hundred μg of protein equivalent of membranes were used in a total reaction volume of 250 μL.

Table 1. *Affinities and densities of [^{3}H]PK11195 binding sites in various tissues of control and spf mice*[*]

	Control (CD-1/Y)	Mean r^2 values (Scatchard)	Hyperammonemic (spf/Y)	% increase over control	Mean r^2 values (Scatchard)
Bmax (pmol/mg protein)					
Brain					
Cerebral cortex	0.59 ± 0.05 (5)	0.86	0.75 ± 0.04 (6)	+27[†]	0.93
Cerebellum	0.48 ± 0.03 (5)	0.86	0.67 ± 0.05 (5)	+38[†]	0.96
Brain stem	0.25 ± 0.03 (5)	0.91	0.50 ± 0.02 (5)	+102[†]	0.93
Kidney	25.35 ± 1.85 (5)	0.88	42.33 ± 6.43 (6)	+67[†]	0.82
Testis	3.85 ± 0.33 (5)	0.84	5.79 ± 0.54 (5)	+50[†]	0.93
Liver	17.88 ± 0.98 (5)	0.93	29.95 ± 3.56 (5)	+68[†]	0.95
K_d (nM)					
Brain					
Cerebral cortex	1.86 ± 0.20 (5)		2.22 ± 0.70 (6)	+19	
Cerebellum	3.61 ± 0.07 (5)		2.46 ± 0.04 (5)	+32	
Brain stem	1.56 ± 0.16 (5)		2.40 ± 0.30 (5)	+58[†]	
Kidney	5.64 ± 0.51 (5)		9.76 ± 1.36 (6)	+73[†]	
Testis	2.16 ± 0.30 (5)		1.92 ± 0.35 (5)	−11	
Liver	4.22 ± 0.98 (5)		4.84 ± 0.65 (5)	+14	

[*] Each value is the mean ± SD of triplicate determinations; the number of experiments are indicated in parentheses.

[†] $p < 0.05$ compared with control values (t test).

differences in the k_d values in any tissue studied with the exception of kidney and brainstem, where the k_d value of spf mouse was increased by 73% and 58%, respectively (Table 1).

DISCUSSION

Results of the present study reveal increased densities of binding sites for the PTBR ligand [^{3}H]PK 11195 in brain and peripheral tissues of the spf mouse. PK 11195 is a highly selective ligand for PTBR having low affinity for the benzodiazepine recognition site associated with the γ-aminobutyric acid$_A$ (GABA$_A$) receptor (27). PTBR are preferentially associated with astrocytes, where they are present in high densities on the outer mitochondrial membrane (11). Congenital OTC deficiency is characterized neuropathologically by Alzheimer type II astrocytosis (28). Alzheimer type II astrocytosis is also encountered in brain in chronic hyperammonemia resulting from the feeding of ammonia resins to rats after portacaval anastomosis (29) and in rats made hyperammonemic after urease treatment (30) or methionine sulfoximine administration (31), as well as in the brains of cirrhotic patients who died in hepatic coma (32) and in patients with congenital hyperammonemias (33). Furthermore, exposure of cultured astrocytes to millimolar concentrations of ammonia results in Alzheimer type II alterations (34). The Alzheimer type II astrocyte is characterized by mitochondrial proliferation (29). The localization of PTBR on the outer mitochondrial membrane, together with the consistent finding of increased densities of these binding sites in hyperammonemic syndromes, strongly suggests that these sites mediate the astrocytic response to hyperammonemia. In favor of such a possibility, increased densities of PTBR have been reported in the brains of experimental hyperammonemic animals (16, 17) and in the brains of humans (18) with chronic liver disease. Mitochondrial proliferation and swelling have consistently been described in astrocytes after portacaval anastomosis (35, 36). Furthermore, exposure of glioma cells in culture to PTBR ligands results in proliferation and swelling of mitochondria (37). The association of PTBR with the outer mitochondrial membrane coupled with the findings of the present study of alterations of these receptors in spf mouse tissues suggests that these receptors or their endogenous ligands could play a role in the alterations of energy metabolism described in the brains of these animals (7); exposure of kidney mitochondrial preparations to PTBR ligands, including PK 11195, results in significant alterations of mitochondrial respiratory control (38). Similar to chronic human OTC deficiency, spf male mice frequently exhibit only mild hyperammonemia but survive relatively well on a normal diet. One explanation for

this resilience of spf mice is the adaptation of hepatic mitochondria in these animals. Livers from spf mice contain 30–40% increases of mitochondrial protein per gram wet weight compared with age- and weight-matched controls (39). Because PTBR appear to modulate mitochondrial proliferative processes, it is tempting to speculate that increased densities of these receptors observed in the present study in liver preparations from spf mice could be a reflection of the hepatic mitochondrial proliferative response to hyperammonemia in these animals.

Alterations of PTBR and of mitochondrial function in congenital hyperammonemias could result from modifications of endogenous ligands for these receptors. In hyperammonemic states associated with chronic liver failure, increased concentrations of neuropeptides with micromolar affinities of PTBR have been reported. For example, in one report, increased cerebrospinal fluid levels of DBI were described in cirrhotic patients with hepatic encephalopathy (19) and increased immunostaining for octadecaneuropeptide, a fragmentation product of DBI, and itself an endogenous ligand for PTBR, was described in the brains of rats made hyperammonemic after portacaval anastomosis (20). However, no studies to date have addressed the issue of endogenous PTBR ligands in the congenital hyperammonemias.

Although mitochondrial changes offer a feasible explanation for PTBR increases in spf mouse tissues, other mechanisms could also be involved. PTBR modifications have been described in response to stress and alterations of feeding, as well as hormonal changes (40). Although there is no evidence to suggest that such changes occur in the spf mouse, their possible importance cannot be definitively excluded. The findings in the present study of increased binding sites for [^{3}H]PK 11195, coupled with previous reports of increased binding sites for this ligand in brain and peripheral tissues in hyperammonemic syndromes associated with chronic liver dysfunction, suggest that a circulating factor (in all likelihood, ammonia) with high permeability for the blood-brain barrier and for cell membranes is responsible for the observed changes. Whether ammonia *per se* or one of its metabolites is the trigger for the sequence of events resulting in PTBR changes in hyperammonemic syndromes must be determined by additional studies.

It is premature to speculate on possible implications of the findings of increased PTBR for the potential treatment and management of congenital hyperammonemias. It remains to be established whether similar alterations of PTBR are a feature of OTC deficiency in humans. In the meantime, the positron emission tomography ligand ^{11}C-PK 11195, presently in use in humans for the imaging of glial tumors (41), could, in the light of the findings of increased densities of [^{3}H]PK 11195 binding sites

in the present study, find an application in the assessment of end organ dysfunction in congenital hyperammonemic syndromes.

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