Reversal of α -Methyltyrosine-Induced Hypoactivity by 6-(*R*)-5,6,7,8-Tetrahydro-L-Erythrobiopterin (*R*-THBP) in Mice

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ABSTRACT—The effects of peripheral administration of 6-(*R*)-5,6,7,8-tetrahydro-L-erythrobiopterin dihydrochloride (*R*-THBP), a natural cofactor for tyrosine and tryptophan hydroxylases, were investigated in mice treated with a competitive inhibitor of tyrosine hydroxylase, α -methyltyrosine (α -MT). A subcutaneous dose of 250 mg/kg of α -MT decreased markedly both ambulatory activity and cerebral contents of norepinephrine, dopamine and their metabolites in mice. An intraperitoneal dose of 100 mg/kg of *R*-THBP, which did not alter ambulatory activities in normal mice, improved the hypoactivity in α -MT-treated mice. Moreover, *R*-THBP at intraperitoneal doses of 60 and 100 mg/kg inhibited the impairment of cerebral catecholamine metabolism induced by α -MT in mice. We suggest that the reversal of the α -MT effects by *R*-THBP might be due to reactivation of tyrosine hydroxylase in the central nervous system.

6-(*R*)-5,6,7,8-Tetrahydro-L-erythrobiopterin dihydrochloride (*R*-THBP) is a natural cofactor for tyrosine and tryptophan hydroxylases (1–4), the initial and rate-limiting enzymes in monoamine biosynthesis (5). Intracerebroventricular injection of *R*-THBP into rats increased the accumulation of 3,4-dihydroxyphenylalanine (DOPA) and 5-hydroxytryptophan (5-HTP) after inhibition of aromatic L-amino acid decarboxylase and caused an increase in dopamine (DA) and serotonin (5-HT) metabolites in the brain (6). However, it was reported that an intraperitoneal injection of THBP does not change the levels of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in rat brain (7).

Similarly, *R*-THBP peripherally administered had no effect on CSF levels of HVA, 3-methoxy-4-phenylethyleneglycol (MHPG) and 5-HIAA in the rhesus monkey (8). Thus, the role of this cofactor in biogenic amine synthesis *in vivo* under normal conditions remains unclear.

We investigated a possible role of the synthetic cofactor *R*-THBP in determining behavioral activities of animals with normal or abnormal cerebral monoamine synthesizing capacity. Tyrosine hydroxylase activity in the central nervous system was inhibited by α -methyltyrosine *in vivo*. Our data indicate that peripherally-administered *R*-THBP would improve centrally mediated behavioral changes in mice.

MATERIALS AND METHODS

Animals

Male ddY mice were purchased at 4 weeks of age from Shizuoka Laboratory Animal Center (Shizuoka, Japan). Five mice per cage ($23.5 \times 15.5 \times 11.0$ cm) were housed and maintained under controlled conditions of temperature ($23 \pm 1^\circ\text{C}$), humidity ($55 \pm 5\%$) and lighting (dark period, 19:00–07:00 hr) with free access to food and water. The experiment was conducted when the mice were 5 weeks of age and weighed 25 to 35 g.

Ambulatory activity

The 482 mice were divided into 53 groups, each group consisting of more than 6 mice. The mouse's ambulatory activity was automatically recorded with a tilting-type ambulo-meter (ACTY-303, Bio Medica, Ltd., Osaka, Japan), consisting of a plastic activity cage (20 cm in diameter, 30 cm in height). The ambulatory activities of 10 mice were measured individually at the same time, using 10 ambulo-meters.

R-THBP (synthesized at Suntory) was administered intraperitoneally immediately after a subcutaneous injection of 250 mg/kg of *dl*- α -methyltyrosine metylester hydrochloride (α -MT; Aldrich Chemical, Milwaukee, MO), which is sufficient dose to inhibit tyrosine hydroxylase activity (9). Ambulatory activity for 1 hr as an index of exploratory activity of different mice was measured at 1, 2, 3, 4, 5, 6, 7 and 8 hr after the drug administration; that is, different groups were used only once for each time point without habituation. Drugs were always administered at 10:00 hr. R-THBP and α -MT were dissolved in 0.1 M phosphate buffer solution containing 6 mM *l*-ascorbic acid and 3 mM *l*-cysteine (pH 7.4) and 0.9% physiological saline, respectively.

Biochemical assay

Mice were used in groups of 6–10 animals each. Each animal was decapitated and the head was frozen in liquid nitrogen. The whole brain, except for the cerebellum, was rapidly

excised. The tissue samples were weighed and homogenized in 4 ml 0.1 N perchloric acid containing 4 $\mu\text{g/ml}$ dihydrobenzylamine (internal standard). The samples were centrifuged at 12000 rpm for 15 min at 4°C . The supernatant obtained was used to determine the contents of norepinephrine (NE), DA, DOPAC, HVA, 5-HT and 5-HIAA by high-performance liquid chromatography (HPLC) with electrochemical detection (LCEC, Bioanalytical Systems, USA). The conditions for LCEC were as follows: mobile phase, 0.1 M citrate buffer (pH 2.72) containing 0.65 mM sodium octyl sulfate, 0.5 mM EDTA 2Na, 0.6 ml/l diethylamine and 4% acetonitrile; applied potential, +750 mV vs. Ag/AgCl; Biophase ODS column (5 μm in pore size, 250×4.6 mm); flow rate and temperature, 1 ml/min and 35°C , respectively. The mobile phase was previously filtered through a 0.45- μm membrane filter and degassed.

Statistics

The results are presented as the means $\pm$ S.E.M. The statistical evaluations of ambulatory activity and cerebral contents of monoamines were carried out by the Mann-Whitney *U*-test and Student's *t*-test, respectively. A *P* value less than 0.05 was considered significant.

RESULTS

Effect on α -MT-induced hypoactivity

An intraperitoneal dose of 100 mg/kg of R-THBP itself produced no significant changes as compared with the vehicle-treated group in the ambulatory activity of mice up to 9 hr after the administration (Fig. 1). A subcutaneous dose of 250 mg/kg of α -MT began to reduce ambulatory activity from 2 hr after the administration, and the decrease became significant and more remarkable from 4 hr to 9 hr after the administration (Fig. 2).

R-THBP at the dose of 30 mg/kg had no effect on the α -MT-induced hypoactivity, but at 100 mg/kg, tended to inhibit it from 4 hr to 8 hr after the administration. A significant effect of R-THBP appeared at 8–9 hr after

Fig. 1. Effect of R-THBP on ambulatory activity in normal mice. R-THBP or vehicle (0.1 M phosphate buffer containing 6 mM *L*-ascorbic acid and 3 mM *L*-cysteine) was administered intraperitoneally immediately after subcutaneous injection of saline. Ambulatory activity for 1 hr was measured at 1, 2, 3, 4, 5, 6, 7 and 8 hr after the administration. Each value represents the mean $\pm$ S.E.M. of 6 to 15 mice. $\square$: saline (s.c.) + vehicle (i.p.), ▨ : saline (s.c.) + R-THBP, 100 mg/kg (i.p.).

Fig. 2. Time course of changes in the effect of R-THBP on α -methyltyrosine (α -MT)-induced hypoactivity in mice. R-THBP or vehicle was administered intraperitoneally immediately after subcutaneous injection of 250 mg/kg of α -MT. Ambulatory activity for 1 hr was measured at 1, 2, 3, 4, 5, 6, 7 and 8 hr after the administration. Each value represents the mean $\pm$ S.E.M. of 6 to 28 mice. * and **: significantly different from the α -MT-treated group at $P < 0.05$ and $P < 0.01$, respectively (Mann-Whitney *U*-test). $\square$: saline (s.c.) + vehicle (i.p.), $\blacksquare$: α -MT (s.c.) + vehicle (i.p.), ▨ : α -MT (s.c.) + R-THBP, 30 mg/kg (i.p.), ▩ : α -MT (s.c.) + R-THBP, 100 mg/kg (i.p.).

the administration; that is, R-THBP almost completely reversed α -MT-induced hypoactivity. Secondly, the dose-related effects of R-THBP were studied on α -MT-induced hypoactivity at the time (8–9 hr after the administration) of the significant behavioral effect. As shown in Fig. 3, R-THBP at an intraperitoneal dose of 60 mg/kg tended to improve α -MT-induced hypoactivity. At an increased dose of 100 mg/kg, the R-THBP effect became more marked and significant.

Effect on α -MT-induced decrease in cerebral contents of monoamines

A biochemical assay was carried out at the time (8 hr after the administration) of the significant behavioral effect of R-THBP. A subcutaneous dose of 250 mg/kg of α -MT significantly reduced the cerebral contents of NE, DA, DOPAC and HVA as compared with the vehicle-treated group at 8 hr after the administration (Fig. 4 and Table 1). An intraperi-

toneal dose of 30 mg/kg of R-THBP slightly improved the α -MT-induced decrease in the contents of catecholamines and their metabolites, but the effect was not significant as compared with the α -MT-treated group. At a dose of 60 mg/kg, R-THBP significantly improved the impairment of NE and DOPAC levels by α -MT (Fig. 4); and when the dose was increased to 100 mg/kg, there was a significant and more marked improvement in NE, DA, DOPAC and HVA levels (Table 1). Neither α -MT alone nor R-THBP combined with α -MT had any effects on 5-HT metabolism (data not shown).

DISCUSSION

The present study demonstrated that (i) R-THBP, a natural cofactor for tyrosine and tryptophan hydroxylases, improves not only the α -MT-induced hypoactivity of mice but also the decreases in the cerebral contents of

Fig. 3. Dose-related effects of R-THBP on α -MT-induced hypoactivity in mice. R-THBP or vehicle was administered intraperitoneally immediately after subcutaneous injection of 250 mg/kg of α -MT. Each value expresses the mean ambulatory activity counts for 1 hr from 8 hr to 9 hr after the drug administration ($n = 6-28$). **: significantly different from the α -MT-treated group at $P < 0.01$ (Mann-Whitney U -test).

Fig. 4. Effect of R-THBP on the levels of norepinephrine, dopamine and their metabolites in whole brain except the cerebellum of α -MT-treated mice. R-THBP or vehicle was administered intraperitoneally immediately after subcutaneous injection of 250 mg/kg of α -MT. Cerebral contents of catecholamine and their metabolites were measured 8 hr after the drug administration. Each value represents the mean $\pm$ S.E.M. of 6 to 10 mice. * and **: significantly different from the α -MT-treated group at $P < 0.05$ and $P < 0.01$, respectively (Student's *t*-test). $\square$: saline (s.c.) + vehicle (i.p.), $\blacksquare$: α -MT (s.c.) + vehicle (i.p.), $\boxtimes$: α -MT (s.c.) + R-THBP, 30 mg/kg (i.p.), $\boxplus$: α -MT (s.c.) + R-THBP, 60 mg/kg (i.p.).

Table 1. Improving effect of R-THBP on the impairment of catecholamine metabolism in whole brain, except for the cerebellum, of α -MT-treated mice

Groups	n	Contents (μ g/g tissue)			
		NE	DA	DOPAC	HVA
Saline + Vehicle	10	0.4946 $\pm$ 0.0178 ^{a)} **	1770.8 $\pm$ 47.7**	134.6 $\pm$ 5.2**	176.1 $\pm$ 5.2**
α -MT + Vehicle	10	0.1940 $\pm$ 0.0200	651.6 $\pm$ 116.3	28.2 $\pm$ 5.6	55.9 $\pm$ 6.8
α -MT + R-THBP, 100 mg/kg	10	0.2976 $\pm$ 0.0217**	1091.1 $\pm$ 81.4**	60.3 $\pm$ 8.4**	84.1 $\pm$ 7.0**

R-THBP or vehicle was administered intraperitoneally immediately after subcutaneous injection of 250 mg/kg of α -MT. Cerebral contents of catecholamine and their metabolites were measured at 8 hr after the drug administration. ^{a)}: Each value represents the mean $\pm$ S.E.M. of 10 mice. **: significantly different from the α -MT-treated group at $P < 0.01$ (Student's *t*-test).

catecholamines and their metabolites, and (ii) R-THBP, at the dose at which it inhibits α -MT-induced hypoactivity, does not alter the ambulatory activity of normal mice.

α -MT is a competitive inhibitor of tyrosine hydroxylase (5, 10), the enzyme that catalyzes the synthesis of DOPA from tyrosine. Inhibition of tyrosine hydroxylase by α -MT is well-established to result in a reduction in the rate of norepinephrine and dopamine synthesis in the central nervous system. We also confirmed that catecholamine metabolism is impaired, but indoleamine metabolism is not. The α -MT-induced hypoactivity such as sedation or reduction in motor activity results from impaired central catecholaminergic neuron activities (11). In the present study, R-THBP was shown to cause both a significant reversal of α -MT-induced hypoactivity and the improving effects on impaired central catecholamine levels. Therefore, the reversal of the α -MT effect by R-THBP may result from reactivation of tyrosine hydroxylase through its action as a cofactor for tyrosine hydroxylase. R-THBP required much longer latency (at least 4 hr) to reverse the α -MT effect. This finding agrees with observations in mice that an intraperitoneal administration of 300 mg/kg of R-THBP begins to exhibit toxic symptoms such as ptosis, mild tremor or staggering gait from 11 hr after the injection (12), and that onset of action of peripherally-administered R-THBP is much delayed (at least 2–5 hr) in improving the scopolamine-induced memory impairment (12) and enhancing the methamphetamine-induced hyperactivity without altering the spontaneous locomotor activity of normal mice (13). However, R-THBP may require much longer latency to restore the α -MT effect, since the dose of α -MT used in the present study inhibits markedly tyrosine hydroxylase activity in the rat brain (9).

The cytosolic levels of R-THBP are generally considered to be subsaturating for tyrosine hydroxylase and tryptophan hydroxylase in the monoaminergic neurons of normal brain (14, 15). However, the contents of monoamine neurotransmitters and their metabolites in rat

striatum and hippocampus are not increased after systemic administration of THBP (7), and the CSF levels of HVA, MHPG and 5-HIAA in rhesus monkey, are not changed following an intravenous injection of R-THBP (8). On the other hand, there are some clinical lines of evidence that exogenously administered R-THBP enhances monoamine synthesis in abnormal or pathological brains with decreased levels of endogenous R-THBP or in Parkinson's disease (16) and frequently improves the characteristic symptoms of Parkinson's disease (17, 18) and endogenous depression (17, 19). The present results with R-THBP which restored the previously compromised activity of tyrosine hydroxylase in mice are compatible with these clinical findings. Thus, the reactivation of compromised tyrosine hydroxylase activities by R-THBP may be able to exert beneficial effects on abnormal and pathological brain.

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