METABOLIC KINETICS OF BRAIN MUSCARINIC CHOLINERGIC RECEPTORS IN NORMAL AND HYPOTHYROID MICE

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ABSTRACT

A technique for studying in vivo the production rate and turnover rate constant of mouse brain M-receptors was established. A single injection of 25 mg / kg of Benzilylcholine Mustard to living mice resulted in 90 % irreversible block of brain M-receptors. The time course of the receptor density was then monitored by ^{3}H-QNB binding assay and the production rate and turnover rate constant were calculated from the time course curve with a computer program. It was found that in normal mice the turnover rate constant was about 0.035 h^{-1} (half-life was about 20 h) and the production rate was 30—42 fmol / (h · mg protein). Parallel experiments revealed a significant slow down of the turnover of brain M-receptors in hypothyroid mice (turnover rate constant was $0.0257 \pm 0.0012\ h^{-1}$ in hypothyroid vs. $0.0356 \pm 0.0021\ h^{-1}$ in normal) while the production rate was not changed significantly. The results suggest that thyroid hormones have a regulatory action on the turnover of brain M-receptors and the elevation of brain M-receptor density together with slow down of the turnover of brain M-receptors is probably one of the important mechanisms relevant to the brain dysfunction in hypothyroidism.

Keywords: Hypothyroidism Muscarinic cholinergic receptor Benzilylcholine mustard (BCM) Metabolic kinetics of M-receptor Brain M-receptor

1 INTRODUCTION

Though it is well known that there is marked dysfunction of higher nervous system during hypothyroidism, the mechanism of such dysfunction has not been elucidated. The hormone regulation, including the effect of thyroid hormones, on brain function is not clear. A number of investigations revealed that the glucose utilization and oxygen consumption in brain are not significantly changed during hypothyroidism [1]. Recent studies [2-4] have showed that muscarinic cholinergic receptors (M-receptors) in brain are closely related to the activity of the higher nervous system. There are, however, only a few papers in literature dealing with the change of M-receptors during hypothyroidism and the results are not consistent.

Sharma *et al*[5] reported an increase of heart M-receptor density in hypothyroid rats. Patel *et al*[6] found that the M-receptor density of cerebellum but not of cerebrum was higher in the hypothyroid than in normal newborn rats. The study of Kastrup and Christensen[7] showed no change of brain and heart M-receptors in rats after two weeks' propylthiouracil treatment. In this paper we report the change of brain M-receptor density and metabolic kinetics in adult hypothyroid mice induced by long term treatment of methylthiouracil. For this purpose, a technique was established which involved the in vivo irreversible blocking of brain M-receptors by a single injection of BCM (Benzilylcholine mustard), a specific alkylating agent for M-receptors first reported by Gill and Rang[8], and the observation of the time course of M-receptor binding capacity with ^{3}H-QNB.

2 ANIMALS, MATERIALS AND METHODS

Reagents: ^{3}H-Quiniclidinyl benzilate (^{3}H-QNB, 1.55 TBq / mol) was purchased from Amersham. Methylthiouracil (MTU) and unlabelled QNB were from Sigma. BCM was synthesized by Professor Zhou Zhishan of Shanghai Medical University. Other chemical reagents were of analytical grade.

Animals: For the production of hypothyroid models, Balb/c female mice of 7—8 weeks old were randomly divided into two groups. One was given 0.03 % MTU in drinking water for 3—5 months while the other group, drinking ordinary water, served as control. Hypothyroidism was confirmed by marked declination (20—30 %) of oxygen consumption and significant decrease of plasma thyroxine ($P<0.001$). For the study of normal metabolic kinetics of M-receptors, different batches of Balb/c mice, each of same age and sex, were used.

Preparation of brain cell membrane samples: The mice were sacrificed by decapitation and their brains removed quickly. The subsequent membrane preparation and binding assay procedures were slightly modified from Yamamura and Snyder[9] and were all conducted at 0—4°C. The tissues were homogenized in ice cold 50 mmol / L phosphate buffer (with 10 μmol / L $MgCl_2$ and 0.25 mol / L sucrose, pH7.7) and centrifuged at 3500 g for 10 min. The supernatants were then centrifuged at 27000× g for 15 min and the final pellets were resuspended in reaction phosphate buffer (with 10 mmol / L $MgCl_2$ and 0.2 % Vit C, pH7.7). The final concentrations of protein were determined by micro-Lowry's method[10].

Binding assays: In saturation assays, ^{3}H-QNB (final concentrations 0.2—2.5 nmol / L) and membrane preparations (0.08—0.12 mg of protein) were incubated in reaction buffer (final volume 0.4 mL) at 37 °C for 30 min, without or with 0.1 μmol / L unlabelled QNB (for total and non-specific bindings respectively). Bound ligand was separated from free ligand by filtration onto glass fibre filters and rinsed by the aid of

a cell harvester. The dried filters were then immersed in 5 mL scintillation solution (0.4 % PPO in xylene) and measured for their radioactivities with a liquid scintillation counter. Single point binding assays were carried out in the same condition, with the concentration of ^{3}H-QNB fixed at 2.5 nmol / L.

In vivo experiments on the irreversible blocking action of BCM on brain M-receptors: Mice were sacrificed 3 h after a single intravenous injection of BCM (0.04 or 0.08 mg / mouse). Brain M-receptors were assayed by the saturation binding technique and the specific binding data obtained were treated with Scatchard Plot[11] as well as a robust curve fitting program[12] to yield the binding capacities (R_{max}) and equilibrium dissociation constants (K_D).

Dynamic binding assay of brain M-receptors: After a single subcutaneous injection of 25 mg BCM / kg of body weight, the hypothyroid and control mice were decapitated in pairs after various intervals. The time course of brain M-receptor density was then determined by the single-point ^{3}H-QNB binding assay. The mathematical model of Reed and Lane[13] was adopted to compute the kinetic parameters with slight modification. According to Reed and Lane, since the production rate of receptor molecules follows zero order kinetics and the degradation rate follows first order kinetics, the receptor density at any time after a single dose of specific irreversible blocking agent may be defined by the following equation:

$$R_t = (P_r / K)(1-e^{-Kt}) + R_o \cdot e^{-Kt} \quad (1)$$

where R_o is the minimum receptor density after the blocking agent, P_r is the production rate in fmol / (h · mg protein), K is the turnover rate constant in h^{-1} (in steady state, it equals the degradation rate constant), R_t is the receptor density in fmol / mg protein at t, which is the time after receptor density has reached its minimum value. If there are sufficient data (R_t versus t), the P_r and K can then be solved by curvilinear least square regression.

Since we need to compare the P_r's and K's of various animal groups, Eq 1 is further processed as following: when t approaches infinite, e^{-Kt} approaches zero and Eq.1 becomes $R_t = P_r / K$ which is also the receptor density at steady state, i.e., R_{max}. Thus Eq.1 may be rewritten in the following form:

$$R_{max} - R_t = (R_{max} - R_o) \cdot e^{-Kt} \quad (2)$$

Taking natural logarithms of both sides gives Eq.3:

$$\mathrm{Ln}\,(R_{max} - R_t) = \mathrm{Ln}\,(R_{max} - R_o) - K \cdot t \quad (3)$$

Since R_{max} equals P_r / K and hence $K \cdot R_{max}$ equals P_r, multiplying both sides of Eq.3 by R_{max}, yields Eq.4:

$$R_{max} \cdot \mathrm{Ln}(R_{max} - R_t) = R_{max} \cdot \mathrm{Ln}\,(R_{max} - R_o) - P_r \cdot t \quad (4)$$

Thus, K and P_r can be obtained from the slopes of regression lines of Eq.3 and Eq.4

respectively and the significance of the differences between the K's or P_r's of various groups can be tested by statistical comparison (t- or F-test) of the slopes of regression lines[14].

3 EXPERIMENTAL RESULTS

3.1 Brain M-receptor densities of normal and hypothyroid mice: By the multi-point ^{3}H-QNB binding method (MPM), satisfactory saturation curves were obtained with mouse brain preparations. Scatchard plot yielded straight lines and the Hill numbers were nearly 1. Table 1 summarizes the results of normal and hypothyroid mice. A significant elevation of R_{max} was found in hypothyroid mice when compared with normal mice by paired t-test while the K_D values were not markedly changed.

Table 1

Densities (R_{max}) and equilibrium dissociation constants (K_D) of brain M-receptors of normal adult mice and hypothyroid mice measured with ^{3}H-QNB binding assays

	MPM		SPM
	R_{max}	K_D	R_{max}
Control	1019 ± 27 (16)[a]	0.147 ± 0.016 (16)	895 ± 18 (14)
Hypothyroid	1240 ± 47 (16)[b]	0.190 ± 0.020 (16)[c]	1057 ± 59 (11)[d]

a: Mean ± SEM (number of individual) b: t-test of the two groups revealed $P < 0.001$ c: t-test showed no significant difference between the two groups d: t-test of the two groups revealed $P < 0.01$

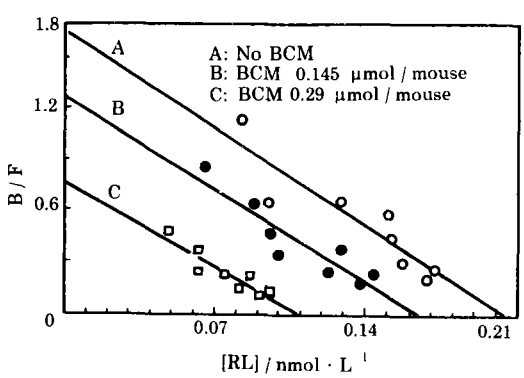

Fig.1 In vivo effect of BCM on mouse brain M-receptors

[RL] is the concentration of ^{3}H-QNB bound receptors, B/F is the concentration ratio of bound to free portions of ^{3}H-QNB

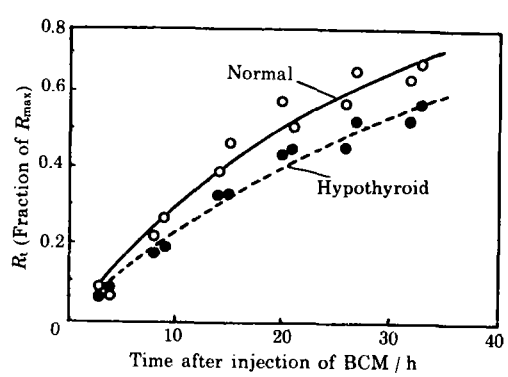

Fig.2 The time courses of mouse brain M-receptors in normal adult and hypothyroid mice after a single subcutaneous injection of 0.5 mg / mouse BCM in normal saline

R_{max} values were taken from the average of 4-5 mice not injected with BCM

The R_{max} values obtained by single point method (SPM) correlate very well with those obtained by MPM and were about 85 %—88 % of the latter. By SPM, the R_{max} of hypothyroid mice was also significantly higher than normal (Table 1).

3.2 In vivo irreversible blocking action of BCM on brain M-receptors. As shown in Fig.1, when brain M-receptors of normal mice were assayed by MPM 3 h after a single intravenous dose of BCM, the Scatchard plots were shifted to left in a dose dependent pattern without change of slope. The R_{max} values dropped markedly while K_D values were not changed. The higher the dose of BCM, the smaller the R_{max} (Table 2).

Table 2

The in vivo effect of BCM on mouse brain M-receptor density (R_{max}) and equilibrium dissociation constant (K_D) in ^{3}H-QNB binding assay

BCM dose / μmol · mouse^{-1}	0	0.145	0.290
R_{max} / fmol · (mg · prot)$^{-1}$	884	595	307
K_D / nmol · L^{-1}	0.124	0.129	0.141

3.3 The metabolic kinetics of brain M-receptors in normal mice. In each experiment, 16—18 mice of same age, same sex and close in body weights (±1 g) were given a single subcutaneous dose (25 mg / kg) of BCM at different times and decapitated at same time. The time course of brain M-receptor densities was determined by SPM. As shown in Fig.2, 3 h after BCM injection, there was about a 90 % irreversible block of brain M-receptors and then the receptor density grew gradually, reaching 3 / 4 of the original R_{max} at about 32 h after BCM injection. From the time course data, P_r and K were calculated with a computer program following Eq.3 and 4. The results of three separate experiments on normal mice were close to each other, the turnover rate constant was about 0.035 h^{-1}, $T_{1/2}$ was around 20 h and the production rates were 30—42 fmol / (h · mg protein) (Table 3).

Table 3

Kinetic parameters of brain M-receptors obtained from 3 batches of mice

Batch No.	Age / weeks	Sex	K / h^{-1}	$T_{1/2}$ / h^{-1}	P_r / fmol · (mg prot · h)$^{-1}$
1	7	Male	0.0343	20.20	33.91
2	12	Male	0.0350	19.83	35.99
3	24	Female	0.0356	19.44	42.12

Table 4

Comparison of the kinetic parameters of brain M-receptors between hypothyroid and normal mice

	K / h^{-1}	P_r / fmol · (mg prot · h)$^{-1}$	$T_{1/2}$ / h
Hypothyroid	0.0257 ± 0.0012[a]	38.42 ± 2.04	26.96 ± 1.26
Normal mice	0.0356 ± 0.0021[b]	42.15 ± 2.88[c]	19.47 ± 1.15

a: Mean ± SEM b: t-test of the two groups revealed $P<0.001$ c: t-test of the two groups revealed $P>0.1$

3.4 Change of the metabolic kinetics in hypothyroid mice. When hypothyroid mice were examined in parallel to normal mice by the above method, the time course curve of brain M-receptor densities of the former rose much slower than that of the

latter (Fig.2). The regression lines of both groups obtained by Eq.3 and Eq.4 were shown in Fig.3. The turnover rate constant K of hypothyroid mice obtained from Eq.3 was about 70 % of the K value of normal mice, t-test of the regression coefficients (slopes) revealed a very significant difference. The production rates P_r's of the two groups showed no difference (Table 4).

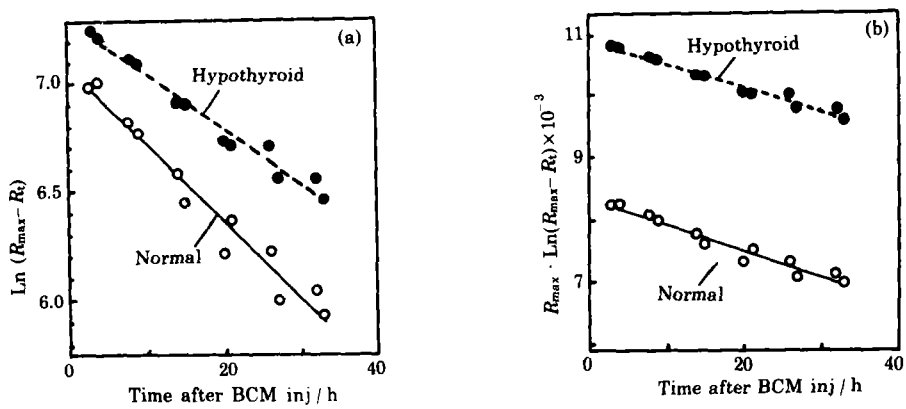

Fig.3 The linear regression results of the time courses of mouse brain M-receptor binding sites after transformation of Y-axis coordinate

(A): With $\mathrm{Ln}(R_{max}-R_t)$ as the Y-axis coordinate, the regression lines revealed a significant difference between the turnover rate constants K'_s of normal adult and hypothyroid mice (0.0356 vs. 0.0257 h^{-1}) (B): With $R_{max}\cdot \mathrm{Ln}(R_{max}-R_t)$ as the Y-axis coordinate, the regression lines showed no difference of production rates P_r's between the two groups (42.15 vs. 38.42 fmol / (mg prot · h))

4 DISCUSSION

4.1 It has been pointed out by Mahan *et al*[15] that observation of the time course of receptor density after application of an irreversible blocking agent as a tool is a relatively good method for studying the kinetics of the receptor metabolism. Because it is difficult to culture normal brain cells in vitro for sufficiently long time, the kinetics of brain M-receptor metabolism has to be studied in vivo and, as a prerequisite, it is necessary to make sure that the tool drug can irreversibly block the receptors in vivo.

Using the ^{3}H-QNB or ^{3}H-NMS binding assay, BCM and its analogue PrBCM have been proved to be irreversible blocker of M-receptors when added in vitro to incubated small intestine and heart cell membrane preparation [16, 17]. In this work we found that injection of BCM to living mice can also markedly decrease brain M-receptor density in a dose-dependent manner without changing the affinity of receptors to ^{3}H-QNB. Therefore BCM can pass through the blood brain barrier and is an efficient irreversible blocker of brain M-receptors for in vivo experiments.

4.2 It has been proved that when PrBCM was added to incubated embryonic

chicken heart to block most of the M-receptors, the subsequent gradual rise of the binding sites was not due to reactivation of blocked receptors but was due to synthesis of new receptor molecules because cycloheximide, an protein synthesis inhibitor, can eliminate the gradual rise of M-receptor density[11]. Thus, in vitro experiments using BCM or PrBCM have found that $T_{1/2}$ of guinea pig ileum M-receptors was about 12 h[8, 18] while in cultured embryonic chicken cardiac cells the M-receptor density reached the control level after 20—24 h[17]. However, no available data about the metabolic kinetics of normal brain M-receptors has been found in literature.

The results of this paper indicate that the turnover of adult mouse brain M-receptors is fairly rapid. $T_{1/2}$ obtained from three batches of Balb/c mice ranged from 19.4 to 20.2 h which are only slightly longer than the value 12 h reported for in vitro guinea pig ileum. As shown in Fig.2, at 32 h after BCM injection, the brain M-receptor density reached about 3 / 4 of R_{max} and it can be estimated that the time for complete recovery to the level of R_{max} is about 55 h. This is also only slightly longer than the value reported for cultured embryonic chicken heart. Such a rapid turnover of brain M-receptors implies a very vivid role of these receptors in the functional activity of brain.

4.3 The results of this work showed a marked increase of brain M-receptor density in hypothyroid mice. This is in agreement with the result of Sharma and Banerjee on rat heart[5] but not with the result of Kastrup and Christensen[7]. Since thyroid hormones are stored in thyroid gland and their release rate and disappearance rate are slow, it is probable that the negative result of Kastrup and Christensen was due to the short duration (2 weeks) of model production.

A recent report of Waisherg and Shainberg[19] suggested that triiodothyronine decreases the M-receptor density of incubated cardiac cells by stimulating the degradation of receptor molecules. In this work we found that the turnover rate constant of brain M-receptors was slowed down in hypothyroid mice, i.e., the fraction of existing M-receptor molecules degraded per unit of time is decreased. On the other hand, P_r, the absolute number of M-receptor molecules produced per unit of time was not changed. Consequently, the R_{max} values of M-receptors will reach a higher steady level by such a change of kinetic parameters.

4.4 Recent studies have found that several receptors, including muscarinic cholinergic, catacholaminergic and opiate receptors were involved in the activities of higher nervous system[2]. Among the various receptors, the muscarinic cholinergic receptors have attracted most attention because they have been clinically suggested to be closely related to affective disorders and geriatric mental disorders[3,4]. Although the complex function of M-receptors has not been fully clarified, two hypotheses have been suggested to explain the biological basis of these mental disorders: the cholinergic-noradrenergic imbalance hypothesis and the (hypo-)cholinergic

hypothesis, both assume a crucial role of central cholinergic mechanisms[20,21]. Therefore, the results of this work suggest that elevation of the density together with slow down of the turnover of brain M-receptors is probably one of the important mechanisms for the brain dysfunction of hypothyroid patients.

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