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The Efflux Transport of Choline through Blood-Brain Barrier is Inhibited by Alzheimer's Disease Therapeutics

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Abstract – In the present study, we examined the effects of several therapeutics of Alzheimer's disease, such as donepezil hydrochloride, tacrine and α-phenyl-*n*-*tert*-butyl nitrone (PBN) on choline efflux from brain to circulating blood. The brain-to-blood efflux of [^{3}H]choline in rats was significantly inhibited by tacrine and PBN. Also the [^{3}H]choline efflux was reduced by tacrine and donepezil hydrochloride in the TR-BBB cells, *in vitro* the blood-brain barrier (BBB) model. These results suggest that these drugs may influence choline efflux transport from brain to blood and regulate the choline level in brain resulting in the increase of acetylcholine synthesis.

Keywords: Blood-brain barrier, Choline transport, Alzheimer's disease therapeutics, *in vivo* brain efflux index method, TR-BBB cells

INTRODUCTION

The blood-brain barrier (BBB) is comprised by tight junctions of the brain capillary endothelial cells, which are highly restrictive for the entry of drugs used in the treatment of CNS disorder from the blood to the brain parenchyma cells (Cornford, 1985; Pardridge, 1988). Actually, many clinical developments of CNS-acting drugs have to be stopped because of poor permeability across the BBB (Ohtsuki and Terasaki, 2007). In the brain, various transporters exist on the endothelial cell membrane and carry out the transport of specific endogenous compounds as well as many pharmacological agents to the brain (Spector, 1989). For example, L-DOPA, delivered into the brain through the amino acid transporter at the BBB, showed the therapeutic effect at Parkinson's disease in the brain (Smith, 1993). Similarly, the BBB choline transporter has been proposed as a drug delivery vectors for various amine compounds. Basic amine drug, eperisone, competitively inhibited choline uptake in rats (Kang *et al*., 1990), and the derivatives of lobeline and isoarecolone could enter into the brain via the BBB choline transporter (Metting *et al.*, 1998). Therefore, it is necessary to understand the relationship between various transporters and CNS drugs at the BBB, and this knowledge can be applied to clinical development of CNS drugs.

Tacrine is the first drug approved specifically for the treatment of Alzheimer's disease (AD). It has amino acridine ring, and was proved to improve memory, language, praxis and activities of daily life (Grason, 1996; Knapp et al., 1994). Donepezil hydrochloride is a piperidine-based reversible cholinesterase inhibitor that exhibits high specificity for centrally active acetylcholinesterase approved the treatment of AD (Rho and Lipson, 1997). In addition, α-phenyl-*n*-*tert*-butyl nitrone (PBN), a nitrone-based spin trapping agent has been proposed as a therapeutic agent for stroke (Knecht and Mason, 1993; Zhao *et al*., 1994). The brain distribution of these drugs was relatively high (MaNally et al., 1996; Matsui *et al*., 1999), but the transport mechanism has not yet been known. Especially, cholinesterase inhibitors such as tacrine and donepezil hydrochloride represent the standard therapeutic approach for the treatment of AD, and acetylcholine level in the brain has been considered to be one of important thesis as maintaining the brain function. Choline is a precursor of acetylcholine, which has to be supplied from blood circulation through the choline transporter at the BBB to maintain brain acetylcholine level, because it is synthesized minimally in the brain (Blusztajn and Wurtman, 1983; Wurtman, 1992). In the previous study, we found that tacrine, donepezil hydrochlolide and PBN inhibited choline uptake at the BBB suggesting the possibility of these drugs being transported into the brain through the choline transporter (Kang *et al*., 2005). Our

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study concerns the effects of tacrine, donepezil hydrochloride, and PBN on choline efflux to understand more about the relationship between choline transporter and these drugs used for the treatment of AD or stroke.

MATERIALS AND METHODS

Materials

[Methlyl-^{3}H]Choline ([^{3}H]choline, 86.0 Ci/mmol) and [carboxyl-^{14}C]inulin ([^{14}C]inulin, 1.92 mCi/g) was purchased from NEN Life Sciences (Boston, MA, USA). α-Phenyl-n-tert-butyl-nitrone (PBN) was obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin, USA). Tacrine, 9-amino-1, 2, 3, 4-tetrahydroacridine hydrochloride, was provided from Jeil Co. (Seoul, Korea). Donepezil hydrochloride, (±)-2-[(1-benzylpiperidin-4-yl) methyl]-5, 6-dimethoxyindan-1-one monohydrochloride, was provided from Daewoong Co. (Seoul, Korea). Ketamine hydrochloride was obtained under help from Yuhan Co. (Seoul, Korea) used an anesthetics. Hionic-fluor and Soluene-350 were purchased from Packard Instruments (Meriden, CT, USA). All other chemicals were of reagent grade and available commercially.

Brain efflux index (BEI) study

In vivo brain efflux experiments were performed as described previously (Kakee et al., 1996). Male Sprague-Dawley rats (Samtaco, Osan, Korea) weighing 230-270 g were anesthetized with an intramuscular injection of a mixture of ketamine (100 mg/kg) and xylazine (2 mg/kg) and their heads were fixed in a stereotaxic frame (Stoelting Co., Wood Dale, IL, USA). After exposing the skull, a 1.0 mm hole was made in the skull, 0.20 mm anterior and 5.5 mm lateral to the bregma using a dental drill (Eicom Co., Tokyo, Japan). Then, a 0.50 μL of an extracellular fluid (ECF) buffer containing [^{3}H]choline (80 nCi) and [^{14}C]inulin (4 nCi) in the presence or absence of drugs was administered to rat brain over 1 min via a 5.0 μL microsyringe (Hamilton, Reno, NE, USA) fitted with a needle (100 μm i.d., 350 μm o.d.; Natsume, Tokyo, Japan) which was inserted into the Par2 region through a hole to a depth of 4.5 mm. The ECF buffer (pH 7.4) was composed of 122 mM NaCl, 25 mM $NaHCO_3$, 10 mM D-glucose, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, and 10 mM HEPES. At appropriate time, the brain was removed, and ipsilateral (left) cerebrum was isolated. After weighing each of these, tissue samples were dissolved in 3.0 mL of Soluene-350 at 60°C for 3 h, and then mixed with 10 mL hionic-fluor. The associated radioactivity was determined in a liquid scintillation counter (LSC 6500, Beckman, Fullerton, CA, USA) with the automatic external standard for quenching correction.

The BEI was defined by equation (1) and the percentage of substrate remaining in the ipsilateral cerebrum was determined using equation (2) (Kakee *et al*., 1996).

$$BEI(\%)=\frac{\text{Amount of test substrate effluxed at the BBB}}{\text{Amount of test substrate injected into the brain}}\times 100$$

$$100-BEI(\%)=\frac{\left(\dfrac{\text{Amount of test substrate in brain}}{\text{Amount of reference in brain}}\right)}{\left(\dfrac{\text{Concentration of test substrate injected}}{\text{Concentration of reference injected}}\right)}\times 100$$

Cell culture

TR-BBB cells were grown routinely in collagen type-I coated tissue culture dishes (Iwaki Co., Chiba, Japan) at 33°C under 5% CO_2 and 95% air as described previously (Hosoya et al., 2000). These cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY, USA) supplemented with penicillin-streptomycin (GIBCO, NY, USA), 10% fetal bovine serum (GIBCO, Grand Island, NY, USA) and 15 μg/L endothelial cell growth factor (Roche, Mannheim, Germany).

Measurements of [^{3}H]choline efflux in TR-BBB cells

After cell culture, which is the same as uptake experiment, ECF buffer containing 0.5 μCi [^{3}H]choline and 0.1 μCi [^{14}C]inulin was added to the TR-BBB cells, followed by the incubation for 60 min at 37°C. After 60 min incubation, the applied solution was removed from the cells, followed by the addition of ice-cold ECF buffer. Then, the ECF buffer at 37°C in the presence or absence of drugs was added to the cells, followed by the incubation for the desired times. After removal of incubation medium, the cells were solubilized in 1 N NaOH for overnight at RT. An aliquot was taken for the measurements of the amount of [^{3}H]choline remained in the cells.

Statistical analysis

Unless otherwise indicated, all data represent the mean ± SEM. An unpaired, two-tailed Student's t-test was used to determine the significance of differences between two group means. Statistical significance among means of more than two groups was determined by one-way analysis of variance (ANOVA) followed by modified Fisher's least squares difference method and $p<0.05$ was considered to be statistically significant.

RESULTS

To investigate the influence of various drugs on cho-

line efflux transport at the BBB, several drugs were coadministered with [^{3}H]choline into the rat brain and their inhibitory effects in the choline efflux transport were evaluated (Table I). [^{3}H]Choline efflux transport was inhibited more than 90% by hemicholinum-3, whereas betaine, an inhibitor of GABA transporter had no effect on [^{3}H]choline efflux transport. [^{3}H]Choline efflux was inhibited about 50% by verapamil, a substrate and an inhibitor of p-glycoprotein. Tacrine and PBN also inhibited [^{3}H]choline efflux transport more than 40% and 50%, respectively. But, [^{3}H]choline efflux transport was not significantly inhibited by acetyl L-carnitine. In addition, [^{3}H]choline was effluxed by TR-BBB cells, *in vitro* BBB model. As shown in Fig. 1, the amount of intracellular [^{3}H]choline was decreased in a time-dependent manner up to 30 min in TR-BBB cells. Donepezil hydrochloride and tacrine inhibited [^{3}H]choline efflux significantly at 15 and 30 min (Fig. 1).

DISCUSSION

It has been performed successfully to the delivery of several drugs to the brain by amino acid transporter at the BBB previously. It was reported that various drugs such as baclofen, melphan, sulfoximine, azaserine, L-DOPA and L-NAM (L-2-amino-7-bis[(2-chloroethyl) amino]-1,2,3,4-tetrahydro-2-naphthoic acid) were transported into brain through the BBB by large neutral amino acid transporter (Smith, 1993; Takada *et al*., 1992). In addition, choline transporter at the BBB was used for the delivery of cholinergic and nicotine-like drugs to the brain (Metting *et al*., 1998). It has been demonstrated that *in vivo* and *in vitro* transport of choline into the brain is carrier-mediated and saturable (Allen and Smith, 2001; Cornford et al., 1978). It was reported that the plasma choline concentration in healthy adults is approximately 10 μM (Cohen *et al*., 1995). This value is less than the calculated K_m for the BBB choline transporter (about 45 μM *in vivo*, and 20 μM *in vitro*), thus this transporter is not saturated under physiological conditions (Allen and Smith, 2001; Sawada et al., 1999). These characteristics imply that this carrier may deliver therapeutic molecules to the brain (Smith, 1993). In our previous study, we suggested the possibility of various amine drugs, such as tacrine, donepezil hydrochloride and PBN being transported into the brain through the choline transporter at the BBB (Kang *et al*., 2005). Therefore, in the present study, we evaluated the change of the efflux transport of choline from brain to circulating blood by several therapeutics of AD, such as donepezil hydrochloride and tacrine.

We proved that choline undergoes efflux from brain to blood across the BBB via a carrier-mediated efflux transport system for the first time. Choline efflux transport rate at the BBB is similar to blood-to-brain influx rate and this efflux process is saturable (Lee and Kang, 2006). Therefore, this efflux system is supposed to play an important role in the choline turnover in the brain. Our results showed that donepezil hydrochloride, tacrine and PBN inhibited significantly choline efflux at the BBB *in vivo* and *in vitro* (Fig. 1 and Table I). Especially, it is interesting that donepezil hydrochloride and tacrine inhibited efflux of choline. In the AD brain, choline uptake to the brain was decreased compared to normal brain and brain choline level in the AD patients was also decreased (Cohen et al., 1995; Nitsch et al., 1992). Furthermore, it has been reported that the decrease of choline level in AD brain may slow down acetylcholine synthesis because K_m of choline acetyltransferase is relatively high (30-100 μM) (Blusztajn and Wurtman, 1983). The decrease of acetylcholine synthesis caused by reduction of choline level in the brain led to the cholinergic deficit in AD (Nitsch et al., 1992). Therefore, the increase in the brain choline level can be one of the most effective strategies as an AD therapy leads to an increase of acetylcholine level in the brain. In the previous report, choline was accumulated in the brain slices during the preincubation with tacrine (Dolezal and Tucek, 1992). Dolezal and Tucek proposed that the potency of tacrine to increase the content and

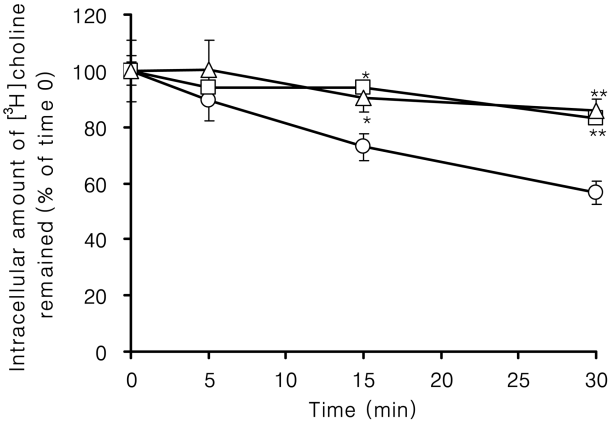

Fig. 1. Time-course of [^{3}H]choline efflux in TR-BBB cells. The cells were incubated with ECF buffer containing [^{3}H]choline (11.6 nM) for 60 min at 37°C. Then, the cells were incubated with ECF buffer in the absence (control, ○) or presence of tacrine (△) or donepezil hydrochloride (□) for the specified periods at 37°C, followed by the measurement of the amount of [^{3}H]choline remained in the cells. Each point represents the mean ± SEM (n = 3-4). $^*p<0.05$, $^{**}p<0.001$ significantly different from control.

Table I. Effects of various drugs on [^{3}H]choline efflux from *in vivo* rat brain across the BBB

Compounds	Concentration in the injectate (mM)	Concentration in the brain (mM)	BEI (%)	% of control
Control	0	0	37.5 ± 5.3	(100)
+ hemicholinium-3	100	3.3	1.17 ± 1.49	(3)[b]
+ betaine	100	3.3	33.2 ± 2.1	(89)
+ PBN	10	0.33	17.1 ± 1.1	(46)[a]
+ acetyl L-carnitine	100	3.3	25.7 ± 4.4	(69)
+ tacrine	100	3.3	22.0 ± 2.9	(59) [a]
+ verapamil	100	3.3	19.2 ± 3.8	(51) [a]

[^{3}H]Choline (80 nCi) and [^{14}C]inulin (4 nCi) dissolved in 0.5 μL ECF buffer were injected into Par2 region of the brain in the presence of several compounds.The brain concentration was estimated from the injectate concentration divided by the dilution factor, i.e., 30.3, which was reported previously. Data, determined at 40 min after intracerebral microinjection, are mean ± SEM (n = 3).
[a]$p < 0.05$, [b]$p < 0.01$, significantly different from control.
PBN, α-phenyl-n-tert-butyl nitrone

synthesis of acetylcholine in cerebrocortical prisms is due to its ability to diminish the efflux of endogenous choline from the nerve terminals. The decrease of choline efflux by tacrine at the BBB observed in the current study is consistent with this proposal. It means that the amount of choline near the abluminal membrane of capillary endothelium is increased by tacrine or donepezil hydrochloride. Therefore, we suggest that the inhibition of choline efflux from the brain at the BBB becomes one of the important strategies in raising brain acetylcholine level by enhancing the amount of choline in the brain. Betaine had no effect on the blood-to-brain choline efflux (Table I). GAT2/BGT-1, which is involved in GABA transport at the BBB and betaine significantly inhibited [^{3}H]GABA uptake by TM-BBB cells (Takanaga et al., 2001). This result suggested that GAT2/BGT-1 had no correlation with choline efflux. Verapamil, which is a substrate of p-glycoprotein, inhibited choline efflux transport in rat brain (Table I). The substrates of p-glycoprotein include some of organic cations (Young et al., 2003). It is not known whether choline is substrate of p-glycoprotein or not, but choline transport at the BBB was inhibited by p-glycoprotein substrate. Therefore, further studies are needed to obtain the information on the relationship between choline transport and p-glycoprotein.

In conclusion, AD therapeutics for example donepezil hydrochloride, tacrine and PBN inhibited brain-to-blood choline efflux transport at the BBB *in vivo* and *in vitro*. These findings suggest that other compounds structurally related to choline may also exhibit increased brain distribution via choline transport system and regulate choline level in the brain, leading to increase in acetylcholine synthesis.

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