The Journal of Applied Pharmacology, 14, 45-49(2006)

In vivo Brain-to-blood Efflux Transport of Choline at the Blood-brain Barrier

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(Received March 13, 2006; Accepted March 27, 2006)

Abstract – The purpose of this study was to clarify the efflux transport system of choline from brain to blood across the blood-brain barrier (BBB) in rats using the brain efflux index (BEI) method. [^{3}H]Choline was microinjected into parietal cortex area 2 (Par2) of the rat brain, and was eliminated from the brain with elimination half-life of 45 min. The BBB efflux clearance of [^{3}H]choline was about 124 mL/min/g brain, which was determined from combination of an elimination rate constant (1.54×10^{-2} min^{-1}) and the distribution volume in the brain (8.05 mL/g brain). The efflux of [^{3}H]choline was inhibited by unlabeled choline in a dose-dependent manner and was significantly inhibited by cationic substrates, such as hemicholinium-3 and tetraethylammonium (TEA). These results suggest that the BBB may act as an efflux pump for choline to reduce the excessive choline concentration in the brain interstitial fluid.

Keywords □ Blood-brain barrier, Choline, Efflux transport

INTRODUCTION

Choline serves both as a precursor to the neurotransmitter acetylcholine and as an essential component of membrane phospholipids (Klein *et al.*, 1993). In the brain, choline is acetylated in the neuronal cells by choline acetyltransferase to form acetylcholine, or phosphorylated by choline kinase to form phosphocholine. Because the brain has synthesized relatively small amounts of choline, most brain choline is derived from plasma across the blood-brain barrier (BBB), which is composed of tight junctions of the brain capillary endothelial cells (Wurtman, 1992). In the previous studies, it has been reported that choline uptake from blood into brain is mediated by carrier-mediated transport system with high affinity (Galea and Estrada, 1992; Sawada *et al.*, 1999; Friedrich *et al.*, 2001; Allen and Smith, 2001).

The BBB has various transporters and regulates the supply of nutrients and drugs into the brain (Pardridge, 2003; Spector, 1989). In addition, the BBB has several efflux transport systems and plays an important role in protecting the brain from potential toxins (Hosoya et al., 2002). Recently, the efflux transport systems of various neurotransmitters and neuromodulators such as gammaaminobutyric acid (GABA), acidic amino acid, and dehydroepiandrosterone sulfate (DHEAS) from brain to blood across the BBB have been characterized (Hosoya *et al.*, 1999; Asaba *et al.*, 2000; Kakee *et al.*, 2001). However, the elimination of choline from the brain to blood has not been studied. If the BBB acts as the efflux pump for choline, it would be very important information to understand the physiological functions of the BBB as well as the relation between choline and cholinergic neurodegenerative disorder such as Alzheimer's disease.

Therefore, the purpose of this study was to clarify the brain-to-blood efflux mechanism of choline at the BBB using the brain efflux index (BEI) method in rats.

MATERIALS AND METHODS

Materials

[Methyl-^{3}H]Choline ([^{3}H]choline, 86.0 Ci/mmol) and [carboxyl-^{14}C]inulin ([^{14}C]inulin, 1.92 mCi/g) were purchased from NEN Life Sciences (Boston, MA, USA). Choline, betaine, tetraethylammonium chloride (TEA) and hemicholinium-3 were purchased from Sigma Chemical Co. (St Louis, MO, USA). Ketalar (ketamine hydrochloride) was kindly obtained from Yuhan Co. (Seoul, Korea). Hionic-fluor and Soluene-350 were purchased from Packard Instruments (Meriden, CT, USA). All other chemicals were of reagent grade.

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Brain efflux index (BEI) study

In vivo brain efflux experiments were performed as described previously (Kakee *et al.*, 1996; Mori *et al.*, 2003). 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, which was located at 0.20 mm anterior and 5.5 mm lateral to the bregma using a dental drill (Eicom Co., Tokyo, Japan). Then, 0.50 μL of the applied solution containing [^{3}H]choline (80 nCi) and [^{14}C]inulin (4 nCi) in an extracellular fluid (ECF) buffer (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, 10 mM HEPES, pH 7.4) 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. 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 using a liquid scintillation counter (LSC 6500, Beckman, Fullerton, CA, USA) with the automatic external standard for quenching correction.

Determination of BEI from the brain

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; Mori *et al.*, 2003).

$$BEI(\%)=\frac{\text{Amount of test substrate effluxed at the BBB}}{\text{Amount of test substrate injected into the brain}}\times 100 \quad (1)$$

$$100\text{-}BEI(\%)=\frac{\text{(Amount of test substrate in brain/ Amount of reference in brain)}}{\text{(Concentration of test substrate injected/ Concentration of reference injected)}}\times 100 \quad (2)$$

As the percentage of taurine remaining in the brain is given by (100-BEI), K_{eff}, the apparent BBB efflux rate constant, was estimated by fitting the semilogarithmic plot of (100-BEI) versus time data to the nonlinear least-squares regression analysis program, MULTI. Moreover, the apparent efflux clearance across the BBB, $CL_{BBB,eff}$, was obtained from equation (3).

$$CL_{BBB,eff} = K_{eff} \times V_{brain} \quad (3)$$

where V_{brain} represents the distribution volume of choline in the brain, determined by the in vitro brain slice uptake study as described below.

Brain slice uptake study

The distribution volume of [^{3}H]choline in the brain was determined by the in vitro brain slice uptake study as reported previously (Kakee *et al.*, 1996). After preincubation of brain slices (300 μm) for 5 min at 37°C, the uptake was initiated by transferring the brain slices to 50 mL of oxygenated ECF buffer containing 0.05 mCi/mL of [^{3}H]choline and 0.01 μCi/mL [^{14}C] inulin at 37°C. At designated time, brain slices and an aliquot (500 μL) of incubation medium were collected. The associated radioactivity was measured by liquid scintillation counting.

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.

RESULTS

In vivo transport of [^{3}H]choline from brain to blood

The in vivo brain-to-blood efflux transport of choline was evaluated by means of the BEI method after intracerebral microinjection of [^{3}H]choline. The [^{3}H]choline was effluxed by 51.5% from the ipsilateral cerebrum for 40 min (Fig. 1). The K_{eff} value of [^{3}H]choline was found to be $1.54 \pm 0.16 \times 10^{-2}$/min (mean ± SD). In contrast, the remaining [^{14}C]inulin, a non-permeable marker, in the brain was not changed over the 40 min period after microinjection (Table I).

To obtain the information of the distribution volume in the brain, V_{brain} of choline, the uptake of [^{3}H]choline by brain slices was examined. Table II shows the slice-to-medium (S/M) concentration ratio of [^{3}H]choline. No significant difference in the S/M ratio was observed at 120 and 180 min after incubation, giving a steady-state S/M ratio of 8.05 ± 0.80 mL/g brain (mean ± SEM). Incorporating the values for K_{eff} ($1.54 \times 10^{-2} \pm 0.16 \times 10^{-2}$/min) and V_{brain} (8.05 ± 0.80 mL/g brain) into eqn. (3), an apparent $CL_{BBB,eff}$ of choline was found to be 0.124 ± 0.013 mL/min/g brain (mean ± SEM).

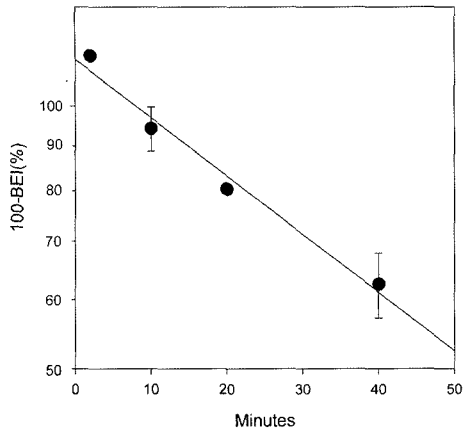

Fig. 1. Time-course of [^{3}H]choline in the ipsilateral cerebrum after intracerebral microinjection in the presence of [^{14}C]inulin as an internal reference. A mixture of [^{3}H]choline (80 nCi) and [^{14}C]inulin (4 nCi) dissolved in 0.5 μL ECF buffer was injected into Par2 region of rat brain. Rats were decapitated at 2, 10, 20 and 40 min after microinjection. The slope of the line represents the elimination rate constant of a tracer amount of [^{3}H]choline, i.e. 1.54×10^{-2} min^{-1}, obtained using non-linear least squares regression analysis.

Table I. Percentage remaining of [^{3}H]choline and [^{14}C]inulin in the ipsilateral cerebrum after intracerebral microinjection

Time after administration (min)	Percentage remaining in the brain relative to the injected amount	
	[^{3}H]choline	[^{14}C]inulin
2	41.9 ± 9.5	36.7 ± 8.3
10	33.9 ± 5.7	35.7 ± 5.8
20	30.9 ± 2.8	36.3 ± 4.6
40	23.7 ± 2.4	37.8 ± 3.3

[^{3}H]Choline (80 nCi) and [^{14}C]inulin (4 nCi) dissolved in 0.5 μL ECF buffer were injected intracerebrally into normal male rats for 1 s. Rate were decapitated at 2, 10, 20 and 40 min after administration. Each value represents the mean ± SEM (n = 3).

To examine the saturation of the efflux transport of choline across the BBB, various concentrations of unlabeled choline were coadministered with [^{3}H]choline (Table III). The efflux of [^{3}H]choline was inhibited in a dose-dependent manner. The BEI value fell significantly, by 59.2%, in the presence of 50 mM unlabeled choline.

Table II. Uptake of [^{3}H]choline by brain slices

Incubation time (min)	Slice-to-Medium (S/M) ratio (mL/g of brain)
60	5.66 ± 0.47
120	7.31 ± 0.24
180	8.05 ± 0.80

Rat brain slices were incubated with 0.05 μCi/mL of [^{3}H]choline and 0.01 μCi/mL of [^{14}C]inulin at 37°C. The radioactivity in the brain slices and incubation medium was measured at 60, 120 and 180 min. Each value represents the mean ± SEM (n = 3).

Inhibitory effects of various compounds on [^{3}H]choline efflux

The several compounds shown in Table IV were coadministered with [^{3}H]choline and their inhibitory effects on the choline efflux were investigated. [^{3}H]Choline efflux was inhibited more than 90% by hemicholinum-3 as a substrate for choline transporter and up to 30% by tetraethylamonium (TEA) as a substrate for organic cation transporter (OCT), whereas betaine had no effect on [^{3}H]choline efflux transport.

DISCUSSION

In this study, we obtained *in vivo* evidence to show that choline undergoes efflux from brain to the circulating blood across the BBB. A total of about 52% of the injected [^{3}H]choline was eliminated from the ipsilateral cerebrum within 40 min, and the apparent elimination half-life was about 45 min (Fig. 1). The apparent BBB efflux clearance of [^{3}H]choline, $CL_{BBB,eff}$ value of about 124 μL/(min·g brain) is similar to the apparent influx clearance of choline determined by the carotid artery perfusion

Table III. Concentration-dependent [^{3}H]choline efflux from the rat brain

Choline concentration in the injectate (mM)	Choline concentration in the brain (mM)	BEI (%)	% of Control
0	0	37.5 ± 5.3	(100)
5	0.17	25.3 ± 3.1	(67)
10	0.33	23.2 ± 1.3*	(62)*
50	1.7	15.3 ± 1.1**	(41)**

[^{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 various choline concentrations. 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).
*$p < 0.05$, **$p < 0.01$, significantly different from control.

Table IV. Effects of various compounds on [^{3}H]choline efflux from the rat brain across the BBB

Compounds	Concentration in the injectate (mM)	Concentration in the brain (mM)	BEI (%)
Control	0	0	37.5 ± 5.3
+ Choline	50	1.7	15.3 ± 1.1**
+ Hemicholinium-3	100	3.3	1.17 ± 1.49**
+ Betaine	100	3.3	33.2 ± 2.1
+ TEA	100	3.3	26.0 ± 0.7*

[^{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). *$p < 0.05$, **$p < 0.01$, significantly different from control. TEA; tetraethylammonium

method, 77 μL/(min·g brain) in parietal cortex area (Allen and Smith, 2001). Moreover, the efflux transport of [^{3}H]choline was inhibited by the unlabelled choline in a dose-dependent manner (Table III), suggesting that the efflux process of choline is saturable. Until now, the transport of choline across the BBB has been investigated *in vivo* (Cornford *et al.*, 1978; Allen and Smith, 2001) and *in vitro* brain capillary endothelial cells and isolated cerebral microvessels (Galea and Estrada, 1992; Sawada *et al.*, 1999; Friedrich *et al.*, 2001). These studies have demonstrated the presence of a carrier-mediated transporter with high affinity, and the characteristic of choline uptake across the BBB is similar to that of organic cation transporters (OCTs) system. Especially, Galea and Estrada suggested that choline transporters are localized at the abluminal membrane for choline transport out of the brain that may shows a part of brain function about choline efflux to blood.

The efflux transport of [^{3}H]choline was significantly inhibited in the presence of cationic substrates, whereas betaine had no effect (Table IV). GAT2/BGT-1, which is involved in GABA transport, is expressed at the BBB and betaine significantly inhibited [^{3}H]GABA uptake in the TM-BBB cells (Takanaga *et al.*, 2001). This result suggested that GAT2/BGT-1 is not responsible for the choline efflux. In addition, it has been reported that organic anion transporter 3 (OAT3), organic anion transporting polypeptide 2 (oatp2), system A transporter 2 (ATA2), and system ASC transporter 2 (ASCT2) are located at the abluminal membrane of the BBB (Asaba *et al.*, 2000; Ohtsuki *et al.*, 2002; Takanaga *et al.*, 2002; Tetsuka *et al.*, 2003). Since choline is a cationic compound, it could be possible that choline is transported through these transporters.

In conclusion, choline is eliminated from the brain to the circulating blood across the BBB via a carrier-mediated efflux transport system. This is the first direct *in vivo* evidence to prove the efflux mechanism of choline.

ACKNOWLEDGEMENTS

This work was supported by grants of the Research Institute of Pharmaceutical Sciences- Sookmyung Women's University and the SRC /ERC program (R11-2005-017) of the Korea Science & Engineering Foundation (KOSEF)/MOST.

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