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P-glycoprotein regulated transport of glutamate at blood-brain barrier[1]

LIU Xiao-Dong[2], LIU Guo-Qing
(*Center of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing 210009, China*)

KEY WORDS glutamates; blood-brain barrier; glycoproteins; verapamil; vincristine; cyclosporins

ABSTRACT

AIM: To study whether efflux of glutamate (Glu) at blood brain barrier (BBB) was regulated by P-glycoprotein (P-gp). **METHODS**: 1) After intracerebral microinjection [^{3}H]Glu 5 min, recoveries were determined in injected cerebrums in presence of multidrug-resistant (MDR) reversing agents verapamil (Ver), vincristine (VCR), and cyclosporin A (CsA); 2) apparent transfer constants (K_{in}) of [^{3}H]Glu from plasma to brain were determined after the *in situ* rat brain perfusion 2 min using solution containing MDR-reversing agents; 3) uptake amount of [^{3}H]Glu by primary cultured bovine brain capillary endothelial cells (BCEC) was analyzed; and 4) In presence of MDR-reversing agents and antibody of P-gp, C219, uptake amount of [^{3}H]Glu by luminal membrane vesicles derived from BCEC was also determined. **RESULTS**: In control rats, remaining percentage of [^{3}H]Glu in injected cerebrums was 25 % ± 16 % at 5 min after intracerebral injection. After pre-treating with CsA 10, 100 $\mu mol \cdot L^{-1}$, VCR 20 $\mu mol \cdot L^{-1}$ and Ver 100 $\mu mol \cdot L^{-1}$, the remaining percentages of [^{3}H]Glu were increased to about 2.2, 2.5, 2.3, and 2.7 folds of control, respectively. In the *in situ* rat brain perfusion experiment, VCR and CsA in perfusion medium concentration-dependently increased [^{3}H]Glu BBB permeability to brain. Co-administration of CsA 40 $\mu mol \cdot L^{-1}$ made BBB permeability of [^{3}H]Glu in cerebral cortex, hippocampus and stratum increase to about 9, 3, 7, and 4.6 folds of control, respectively. Steady-state uptake of [^{3}H]Glu by BCEC was also increased up to 2.5 folds in presence of 100 $\mu mol \cdot L^{-1}$ CsA. MDR-reversing agents and antibody of P-gp, C_{219}, level-dependently inhibited the uptake of [^{3}H]Glu by luminal membrane vesicles of BCEC. And this process is ATP-dependent. **CONCLUSION**: Efflux of Glu at BBB may be regulated by P-gp.

INTRODUCTION

Glutamate (Glu) is one of the most abundant free amino acids in the CNS. It participates in multiple metabolic functions and it is also involved in neuropathological processes such as ischemic insult and Huntington's disease. The regulation of extracellular Glu level is achieved by the high affinity uptake system located on neuron and glia, and also by the blood-brain barrier (BBB). At the abluminal membrane of endothelial cells there is a high affinity concentration transport, while at the luminal membrane there is a low capacity carrier[1]. Under physiological conditions Glu transport at BBB may be important in the outward direction. The efflux mechanism is not clear. The Glu transport at BBB was mediated by neither sodium-independent X_c^{-1} system[1], nor probenecid-sensitive anion transporters[2].

Recently, P-glycoprotein (P-gp), which functions as an ATP-dependent pump that transports drug out of multidrug-resistant (MDR) tumor cells, has been detected in brain capillary endothelial cells[3,4]. It modulates transport of many drugs at the BBB, such as doxorubicin, cyclosporin A (CsA), vincristine (VCR) and some peptides[4-7], leading to a low drug concentration in brain. The aim of the paper was to examine whether efflux transport of Glu from circulating blood to brain at the BBB was also regulated by P-gp *in vivo* and *in vitro*.

METHODS

Animals and chemicals Male Sprague-Dawley rats, weight 250-350 g, were supplied by Center of Ex-

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[2] Correspondence to Prof LIU Xiao-Dong.
Phn 86-25-327-1347. Fax 86-25-330-1655.
E-mail xdliu@jlonline.com
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perimental Animals, China Pharmaceutical University, No 98004. [2,3-^{3}H]Glu (specific activity, 666 – 740 TBq $\cdot$mol^{-1}) was purchased from Nuclear Institute of Shanghai. CsA was a gift from Dr SUN Guo-Qing (China Pharmaceutical University). Verapamil (Ver) and VCR were purchased from Sigma Co. Anti-P-gp monoclonal antibody C_{219} was donated by Dr WANG Jian-Xin. All other chemicals were of analytical grade and commercially available.

Efflux transport of Glu from the brain Efflux transport of Glu from the rat brain was determined using intracerebral microinjection technique as described previously[8]. In brief, VCR, Ver and CsA were dissolved in physiological buffer (mmol $\cdot$ L^{-1}: NaCl 122, $NaHCO_3$ 25, *D*-glucose 10, KCl 3, $CaCl_2$ 1.4. $MgSO_4$ 1.2, K_2HPO_4 0.4, and HEPES 10, pH 7.40). The rats were anaesthetized with pentobarbital sodium (50 mg $\cdot$ kg^{-1}, ip). After removing part of the scalp, a middle incision was performed to expose the reference point in the skull called the bregma. A small hole was drilled at the targeted region of parietal cortex area 2 (named as Par 2), ie, 0.2 mm anterior and 5.5 mm lateral to the bregma, and 4.5 mm deep. [^{3}H]Glu 1 μL (about 0.5 kBq) solution was injected into Par 2 at 30 s after injecting 50 μL physiological buffer or containing tested agent buffer. At 5 min after injecting [^{3}H]Glu, the rats were decapitated and injected cerebrums were obtained. The cerebrums were dissolved in 5 mL of formic acid by incubating at 70 ℃ for 2 h. H_2O_2 5 mL were added to the sample at room temperature. The aqueous solution 1 mL was used to determine radioactivity.

In another five rat experiments, after injecting 1 μL [^{3}H]Glu into Par 2, rats were immediately decapitated and radioactivity was determined in injected cerebrum. Comparison of the dpm injected with that recovered from the rat brains provided an estimate of the accuracy of the intracerebral injection and recovery techniques.

Influx transport of [^{3}H]Glu across the BBB The influx transport of the [^{3}H]Glu across the BBB was measured in pentobarbital-anaesthetized rats (50 mg $\cdot$ kg^{-1}, ip) using the *in situ* rat brain perfusion technique developed by Takasato *et al*[9]. The pterygopalatine, occipital and superior thyroid arteries were ligated, and the right common carotid artery was prepared for ligation. After iv administration of 300 U heparin, a catheter (PE-50) was placed in the right external carotid artery for retrograde perfusion. Before perfusion, the perfusate was filtered, oxygenated with a mixture of 95 % O_2 and 5 % CO_2 and heated to 37 ℃ in a water bath. The perfusate consisted of pH 7.40 bicarbonate-buffered physiological buffer (mmol $\cdot$ L^{-1}: NaCl 128, $NaHCO_3$ 24, KCl 4.2, NaH_2PO_4 2.4, $CaCl_2$ 1.5, $MgSO_4$ 0.9, and *D*-glucose 9) containing both [^{3}H]Glu (170 nmol $\cdot$ L^{-1}) and tested agents. Immediately after performing a heart-cut on the anesthetized rat and ligating the right common carotid artery, the perfusate solution was perfused by an infusion pump at a flow rate of 4.5 mL $\cdot$ min^{-1} into the right hemisphere of the rat brain. After a period of 2 min, the perfusion was terminated by the decapitation of the animal. The rat brain was removed. The (40 – 50 mg) right hippocampus, cerebral cortex and striatum were weighed and digested in 1.0 mL of mixture of formic acid and H_2O_2 (1:1) at 70 ℃ for 2 h. The brain sample and 50 μL of perfusate were taken for determining radioactivity.

The apparent unidirectional influx constants (K_{in}) were calculated as follows: $K_{in} = \frac{C_b}{TC_{pf}}$ where C_b and C_{pf} were the measured radioactivity in brain regions and perfusate, respectively. T was the net perfusion time.

Uptake of [^{3}H] Glu by primary cultured bovine brain capillary endothelial cells Bovine brain capillary endothelial cells (BCEC) were isolated from cerebral gray matter of bovine brains as described previously[10]. The isolated BCEC were seeded in 24-well plate coated with rat tail collagen and cultured at 37 ℃ with 95 % air and 5 % CO_2. Uptake experiment was performed when the cells reached confluence in 12 – 14 d. The cultured cells were washed thrice with 1 mL of pH 7.4 incubation buffer (mmol $\cdot$ L^{-1}: NaCl 141, KCl 4, $CaCl_2$ 2.8, $MgSO_4$ 1, *D*-glucose 10, HEPES 10, and 0.1 % bovine serum albumin). Uptake reaction was initiated by adding 250 μL incubation solution containing both [^{3}H]Glu (170 nmol $\cdot$ L^{-1}) and tested agents to each incubation well. At the designated time after start of incubation, the transport reaction was terminated by washing cells 3 times with 1 mL ice-cold incubation solution. The washed cells were solubilized by incubating them in 100 mmol $\cdot$ L^{-1} NaOH solution (300 μL) at room temperature for 120 min. After neutralization with 100 mmol $\cdot$ L^{-1} HCl (300 μL), 500 μL of solution was used for determining radioactivity. Protein content in the cultured cells was determined by the method of Bradford[11] using bovine serum albumin as the standard. Uptake, expressed as the cell-to-medium ratio (mL $\cdot$ g^{-1} protein) was obtained by dividing the uptake [^{3}H]Glu

amount per g protein by the [^{3}H]Glu concentration in incubation medium.

Uptake of [^{3}H] Glu by luminal membrane vesicle of BCEC Luminal membrane of BCEC was prepared as previously described[12]. The obtained membrane vesicle aliquots were incubated in ice-cold storage buffer (mmol·L^{-1}: mannitol 250, HEPES 10, pH 7.40) overnight, after which they were centrifuged at 90 000 × g for 1 h at 4 ℃. The supernatant was removed, and the pellet was suspended in the storage buffer at a concentration 2 g·L^{-1}.

Uptake of [^{3}H]Glu by membrane vesicles was measured by a rapid filtration method[13]. Membrane vesicle 10 μL containing 20 μg of protein were incubated with different concentration of tested drugs and 1 μmol·L^{-1} of [^{3}H]Glu in incubated buffer (mmol·L^{-1}: Tris-HCl 10, surcose 250, $MgCl_2$ 5, ATP 3, pH 7.40, final volume 50 μL). After 30 min, the reaction was stopped by adding 1 mL of an ice-cold stop buffer(mmol·L^{-1}: sucrose 250, Tris-HCl 10, $MgCl_2$ 5, ATP 3, 0.1 % bovine serum protein) followed by filtration over Whatmam GF/C filter (pre-wetted with the stop buffer). Then the filter was washed twice with 3 mL ice-cold stop buffer. The filters were dried and retained radioactivity was determined. To correct for retention of radioactivity material by the filter, control samples containing amount of radioactivity but without membranes were filtered as indicated above.

Data analysis All the results were represented as $x \pm s$. Student's t-test was used for statistical analysis and statistical significance was defined as $P < 0.05$ or $P < 0.01$.

RESULTS

Effects of MDR-reversing agents on efflux transport of [^{3}H]Glu from brain Fig 1 shows the effects of MDR-reversing agents on efflux transport of [^{3}H]Glu from the brain. In control rats, remaining percentage of [^{3}H]Glu in injected cerebrums was 25 % ± 16 % at 5 min after intracerebral injection. That is to say 75 % ± 16 % of [^{3}H]Glu eliminated from the injected cerebrum within 5 min. After pre-treating with CsA 10 μmol·L^{-1}, 100 μmol·L^{-1}, VCR 2 μmol·L^{-1}, 20 μmol·L^{-1}, and Ver 100 μmol·L^{-1}, the remaining percentages of [^{3}H]Glu were significantly ($P < 0.05$) increased to about 2.2, 2.5, 2.5, 2.3, and 2.7 folds of control, respectively. The results demonstrated that the efflux of [^{3}H]Glu from brain may be inhibited by CsA, VCR, and Ver.

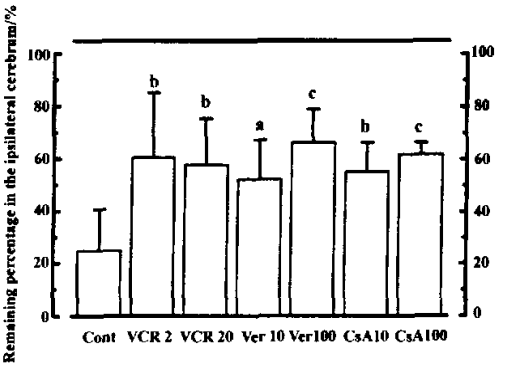

Fig 1. Effects of MDR-reversing agents (μmol·L^{-1}) on efflux of [^{3}H]Glu from cortex. Cont: control. n = 5. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Effects of MDR-reversing agents on influx transport of [^{3}H]Glu from the blood to brain Fig 2 shows the effects of VCR and CsA on influx transport of [^{3}H]Glu from circulating blood to cerebral cortex, hippocampus, and striatum. Increase in CsA concentration in the perfusate (0 – 40 μmol·L^{-1}) resulted in a concentration-dependent increase in permeability (K_{in}) of [^{3}H]Glu in the three brain areas. In cerebral cortex, in presence of CsA 40 μmol·L^{-1}, the value of K_{in} was increased to about 9-fold (3.2 ± 0.5) μL·s^{-1}·g^{-1} compared to control (0.35 ± 0.15) μL·s^{-1}·g^{-1}. In hippocampus and stratum, co-administration of CsA 40 μmol·L^{-1} made the value of K_{in} increase to 3.7-fold and 4.6-fold of control, respectively. VCR 20 μmol·L^{-1} also increased [^{3}H]Glu penetration into cerebral cortex ($P < 0.05$). The results indicated that penetration of [^{3}H]Glu into brain were enhanced by CsA and VCR. In contrast, Ver weakly inhibited the entrance of [^{3}H]Glu from plasma to brain ($P > 0.05$).

Effects of MDR-reversing agents on uptake of [^{3}H]Glu by BCEC The time course of [^{3}H]Glu uptake by BCEC at 37 ℃ and pH 7.40 was shown in Fig 3. The accumulation of [^{3}H]Glu is time-dependent and the steady-state was attained by 30 min. Accordingly, the uptake at 30 min was studied in the following experiments to evaluate the transport characteristics of [^{3}H]Glu.

Tab 1 gave the results of effects of MDR-reversing agents on the steady-state uptake of [^{3}H]Glu by BCEC. CsA, VCR and Ver increased the steady-state uptake of [^{3}H]Glu by BCEC. As clearly shown in Tab 1, CsA

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concentration-dependently increased the uptake of [^{3}H] Glu. In the presence of CsA 100 μmol·L^{-1}, the steady-state uptake of [^{3}H]Glu was increased ($P < 0.05$) to approximately 2.0-fold of the control.

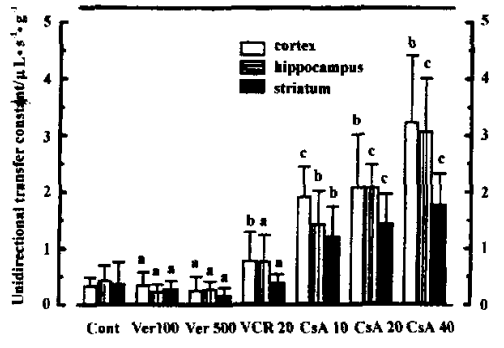

Fig 2. Effects of MDR-reversing agents (μmol·L^{-1}) on transport of [^{3}H]Glu from plasma to brain. Cont: control. $n = 5$. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.

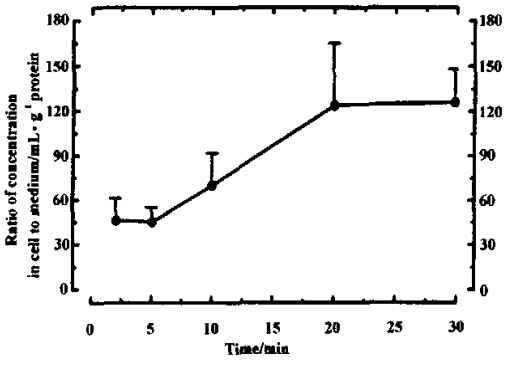

Fig 3. Time course for uptake of[^{3}H]Glu by primary cultured BCEC. $n = 4$. $\bar{x} \pm s$.

Tab 1. Effects of MDR-reversing agents on the Glu uptake by BCEC. $n = 6$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Agent/μmol·L^{-1}	% of Control
Control	100
Ver 100	132 ± 19
VCR 20	122 ± 18
CsA 1	117 ± 16
10	159 ± 23^b
100	204 ± 22^c

The uptake of [^{3}H]Glu was measured at 37 ℃ for 30 min. Control uptake was (122 ± 24) mL·g^{-1} of protein.

Effects of MDR-reversing agents on uptake of [^{3}H]Glu by luminal membrane of BCEC Uptake of [^{3}H]Glu by membrane vesicles was analyzed in the absence or presence of MDR-reversing agents. The results listed in Tab 2. When experiments were performed in the presence of ATP 3 mmol·L^{-1}, the uptake of [^{3}H]Glu by membrane vesicle was (0.91 ± 0.11) nmol·g^{-1} protein. However the uptake of [^{3}H]Glu reduced to (0.47 ± 0.16) nmol·g^{-1} protein in absence of ATP 3 mmol·L^{-1}. The results indicated that the uptake of [^{3}H]Glu by membrane vesicle was ATP-dependent. ATP-dependent stimulation of [^{3}H]Glu uptake from the incubation medium to membrane vesicle suggest that the membrane vesicles were mainly inside-out vesicles[13].

Tab 2. Effects of MDR-reversing agents on the uptake of [^{3}H]Glu by luminal membrane vesicle from bovine brain endothelial cells. $n = 6$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

	Agents	% of control
Control		100
CsA	0.1 μmol·L^{-1}	67 ± 9^b
	1.0 μmol·L^{-1}	64 ± 6^b
	10.0 μmol·L^{-1}	52 ± 9^c
C_{219}	0.48 mg·L^{-1}	99 ± 13
	4.8 mg·L^{-1}	57 ± 5^c
	48.0 mg·L^{-1}	48 ± 5^c
VCR	0.1 μmol·L^{-1}	65 ± 5^b
	1.0 μmol·L^{-1}	60 ± 4^c
	10.0 μmol·L^{-1}	60 ± 6^c
no ATP		53 ± 8^c

The uptake amount of control was (0.91 ± 0.11) nmol·g^{-1} of protein.

The effects of MDR-reversing agents on the uptake of [^{3}H]Glu by membrane vesicle were studied. As demonstrated in Tab 2, VCR and CsA inhibited [^{3}H]Glu uptake in a concentration-dependent manner. In order to confirm participation of P-gp in uptake of Glu by luminal membrane of BCEC, an anti-P-gp monoclonal antibody C_{219} on uptake of Glu was studied. After treatment with C_{219}, uptake of Glu decreased in a concentration-dependent manner as observed with various MDR-reversing agents (Tab 2). This result further demonstrated that P-gp is involved in transport of Glu at BBB.

DISCUSSION

More and more studies have shown that P-gp is a

major component of BBB, ensuring that toxins are pumped out of brain tissue across BBB and protecting the organism against brain damage. Schinkel *et al*, using mice with knock-out mutation in mdr1a (-/-), found that administration of neurotoxic pesticide ivermectin in mdr1a (-/-) and wild-type mice resulted in markedly (87-fold) higher levels in mdr1a (-/-) rat brain, and an increased sensitivity to the neurotoxin (100-fold), compared with wild-type mice. Glu is one of the internal neurotoxins, high level of Glu also results in neuron damage. So the efflux of [^{3}H]Glu from brain to plasma may be a part of the major components of BBB.

P-gp has numerous substrates including classic chemotherapeutical drugs such as vinca alkaloids and anthracyclines as well as modulators(eg, verpamil, *et al*). Some steroids[7] (eg, progesterone, testosterone), peptides and amino acids[16] (eg, *L*-Dopa) were substrates of P-gp. Interaction of substrates with numerous compounds results in increased levels of intercellular fluid and sensitivity by inhibiting function of P-gp. This phenomena is called "MDR-reversing" and the compounds are termed as MDR-reversing agents. Ver, CsA, and VCR are classic MDR-reversing agents. These three drugs may increase the uptake of P-gp substrates by both BCEC and MDR-cells. Our study showed that CsA, VCR and Ver significantly inhibited efflux of [^{3}H]Glu from brain, CsA, and VCR also enhanced entrance of [^{3}H]Glu from blood to brain. In prinary cultured BCEC, the uptake of Glu by BCEC was increased in the presence of VCR, Ver, and CsA. These results indicated that there may exist some relationship between efflux of [^{3}H]Glu from brain and P-gp at BBB.

The P-gp was expressed on the luminal membrane of BCEC[5-7]. In order to investigate the relationship, we also studied effects of MDR-reversing agents CsA and VCR on uptake of [^{3}H]Glu by BCEC and luminal membrane of BCEC. The uptake of [^{3}H]Glu by luminal membranes of BCECs were also inhibited by CsA and VCR as well as anti-P-gp monoclonal antibody C_{219} in a concentration-dependent manner. The results further gave evidence that P-gp involved in transport of [^{3}H]Glu at BBB. Our studies demonstrated that among the three compounds, CsA showed stronger effect on transport of [^{3}H]Glu, while Ver gave the worst effect. In *in situ* rat brain perfusion technique, Ver showed a weak inhibited entrance of [^{3}H]Glu from blood to brain. This phenomena suggested that transport of [^{3}H]Glu at BBB was also involved Ca^{2+}-channel.

The present study showed that MDR-reversing agents also inhibited efflux of [^{3}H]Glu from brain, increased influx of [^{3}H]Glu from circulating blood to brain and uptake by BCEC. [^{3}H]Glu uptake by luminal membrane of BCEC was also ATP-dependent, and was inhibited by MDR-reversing agents and anti-P-gp monoclonal antibody C_{219}. Taken together, these observations suggested that efflux of [^{3}H]Glu at BBB was also regulated by P-gp.

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P-糖蛋白调节血脑屏障上的谷氨酸转运[1]

刘晓东[2]，刘国卿（中国药科大学药代研究中心，南京 210009，中国）

关键词 谷氨酸盐类；血脑屏障；糖蛋白类；维拉帕米；长春新碱；环孢菌素类

目的：研究血脑屏障(BBB)上谷氨酸(Glu)外排是否受 P-糖蛋白(P-gp)调节. **方法**：使用在体脑灌流技术，皮层内注射 [^{3}H]Glu 5 min，测定回收率，Glu 单向转运常数(K_{in})和牛脑微血管内皮细胞(BCEC)及腔面膜摄取[^{3}H]Glu 作用. **结果**：对照鼠，皮层内注射 [^{3}H]Glu 5 min 回收率为 25 % ± 16 %，先用环孢菌素 A(CsA) 10，100 μmol·L^{-1}，长春新碱(VCR) 20 μmol·L^{-1}和维拉帕米(Ver) 100 μmol·L^{-1}处理，回收率分别增至对照鼠的 2.2，2.5，2.3 和 2.7 倍. VCR 和 CsA 增加 [^{3}H]Glu K_{in}. CsA 40 μmol·L^{-1}使 [^{3}H]Glu 在皮层、海马和纹状体中 K_{in}值分别增至对照组的 9，3，7 和 4.6 倍. CsA 100 μmol·L^{-1}使 BCEC 摄取[^{3}H]Glu 增至对照组的 2.5 倍. MDR-逆转剂和 P-gp 单克隆抗体 C_{219}抑制腔面膜摄取[^{3}H]Glu. **结论**：P-gp 调节血脑屏障上的 Glu 外排.

（责任编辑 朱倩蓉）