P-glycoprotein restricted transport of nimodipine across blood-brain barrier[1]

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ABSTRACT

AIM: To examine whether the transport of nimodipine (NMD) from the circulating blood into the brain is restricted by P-glycoprotein (P-gp) in rat brain capillary endothelial cells (BCEC). **METHODS:** When cells reached confluence, a time course of NMD uptake was recorded by incubation with a medium containing NMD 10 mg/L at 37 ºC. Effects of various agents in the steady-state uptake of NMD were tested by co-administration with NMD and each compound to cells at 37 ºC for 90 min. The uptake of NMD was measured for 90 min. Effects of P-gp inhibitors on the efflux of NMD from primary cultured BCEC were studied by administration of erythromycin, clarithromycin, cyclosporin A (CsA), and Hanks' solution after the accumulation of NMD by BCEC at 37 ºC for 90 min. **RESULTS:** The uptake of NMD by primary cultured rat BCEC was time-dependent, and the steady-state uptake of NMD was increased in the presence of several substrates of P-gp in BCEC. The steady-state uptake was also significantly increased (P<0.01) when celluar ATP was depleted by treatment with sodium azide. Furthermore, efflux of NMD from BCEC was inhibited by erythromycin ,clarithromycin, and CsA. **CONCLUSION:** The permeability of NMD into the brain is restricted by P-gp and increased by co-administration with P-gp inhibitors.

INTRODUCTION

The blood-brain barrier (BBB) is known to restrict the nonspecific influx of hydrophilic substrates from the circulating blood to the brain interstitial fluid space. The brain capillary endothelial cells (BCEC) are known to be connected tightly, some substrates, including the cerebral transmitters and their metabolites, restricted transport across the BBB via paracellular passive diffusion. Recently, the 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[1,2]. Furthermore, immunohistochemical studies have revealed that P-gp is expressed at the luminal membrane of the brain capillary endothelial cells[1,2]. It plays an important role in restricting the permeation of many compounds, such as doxorubicin, cyclosporin A (CsA), vincristine[3], some peptides, and amino acid[4-8], by pumping them out of the brain into the circulating blood.

Nimodipine (NMD) was a calcium antagonist of dihydropyridine and was successfully used to treat central nervous system disorders such as multi-infarct dementia, stroke, and subarachnoid hemorrhage. Because NMD is also a substrate of P-gp, its transport across BBB may be modulated by P-gp. Our previous

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study showed that P-gp inhibitors CsA markedly enhanced the protection of NMD against brain damage induced by hypoxia-ischemia in mice and rats[9].

In this study, the uptake of NMD by primary cultured rat BCEC was investigated in order to study whether P-gp causes the decreased transport of NMD into the brain.

MATERIALS AND METHODS

Materials NMD was provided by Shangdong Xinhua Pharmaceutical Factory. CsA was provided by Sichuan Industrial Institute of Antibiotics. Cosimic calf serum was purchased from Hyclone. Gelatin, trypsin, and collagenase type II were purchased from Sigma Chemical Co. Dulbecco's Modified Eagle Medium (high glucose) (DMEM) and Nutrient mixture F-12 Ham's (F-12) were purchased from Gibco BRL. Bovine serum albumin (Fraction V, BSA) was purchased from SABC. The naked rats (7-10 days old) of Sprague-Dawley rats were supplied by Center of Experimental Animals, China Pharmaceutical University. All other chemicals were of analytical grade and commercially available.

Isolation and culture of brain capillary endothelial cells BCEC were isolated from cerebral gray matter of rat brains by the method reported previously[10] with some modification. Briefly, the isolated cerebral gray matter was digested by trypsin (0.05 %) at 37 ºC for 20 min, then filtered through 149 μm nylon mesh and the filtrate was collected. The filtrate was filtered through 79-μm nylon mesh and the matter was collected on the nylon mesh. Then the matter was digested by collagenase type II (0.1 %) at 37 ºC for 20 min, centrifuged at room temperature for 5 min (200×*g*), and collected the cells. The isolated BCEC were seeded in 24-well plate coated with gelatin (2 %) and cultured at 37 ºC with 95 % air and 5 % CO_2. Transport experiments were performed when cells reached confluence in 12-14 d. The primary cultured cells were identified to be capillary endothelial cells by the immunostaining method using Factor-VIII related antigen[11].

Transport experiment When cells reached confluence in 12-14 d, uptake experiments were performed. Briefly, the cultured cell monolayer was washed 3 times with 1 mL of pH 7.4 Hanks' solution (isotonic buffer) containing NaCl 0.137 mol/L, KCl 5.37 mmol/L ,$CaCl_2$ 1.26 mmol/L, $MgSO_4 \cdot 7H_2O$ 0.81 mmol/L, $Na_2HPO_4 \cdot H_2O$ 0.37 mmol/L, KH_2PO_4 0.44 mmol/L, $NaHCO_3$ 4.17 mmol/L, and glucose 2.92 mmol/L at 37 ºC. Cultured BCEC were preincubated at 37 ºC for 30 min in the Hanks' solution. After the preincubation, the solution was removed by suction, and the Hanks' solution (1 mL) containing both NMD (10 mg/L) and tested agents was added to each incubation well. To terminate the transport reaction, cells were washed 3 times with 1 mL of ice-cold Hanks' solution at the designated time. Then the blank Hanks' solution (0.6 mL) was added to each incubated well, frozen and melted repeatedly 4 times to break cells[3].

Efflux experiment For the efflux study, after the accumulation of NMD (10 mg/L) by BCEC at 37 ºC for 90 min, the cells were washed 5 times with 1 mL of ice-cold Hanks' solution. Then the Hanks' solution with or without erythromycin (100 μmol/L), clarithromycin (100 μmol/L), and CsA (50 μmol/L), respectively was added to initiate the efflux of NMD at 37 ºC. Termination of the efflux was performed by the same procedure as the uptake study mentioned above. Efflux was estimated from the amount of NMD remaining in the cells[3].

Analytical method The concentrations of NMD in the cells were determined by HPLC[12] with UV detection at 238 nm. The sensitivity of the assay was 10 μg/L Hanks' solution. Reproducibility was better than 15 % in tested ranges and a good linearity was obtained from 10 μg/L to 1000 μg/L Hanks' solution. Protein content in cultured cells was measured by the method of Bradford MM[13] using bovine serum albumin as the standard. Net uptake, expressed as the NMD concentration to protein concentration ratio (ng/μg protein), was obtained by dividing the apparent uptake amount of NMD by per μg protein. All the results were represented as mean±SD. Student's *t*-test was used for statistical analysis and statistical significance was defined as $P<0.05$ or $P<0.01$.

RESULTS

Time course of NMD uptake The time course of the uptake of NMD by BCEC at a concentration of 10 mg/L at 37 ºC was shown in Fig 1. The accumulation of NMD was time-dependent and the plateau of accumulation was observed at 60-90 min. Accordingly, the uptake at 90 min was studied in the following experiments to evaluate the transport characteristics of NMD mediated by P-gp.

Effect of MDR-reversing agents and metabolic inhibitors on the steady-state uptake of NMD CsA increased the uptake of NMD in a dose dependent man-

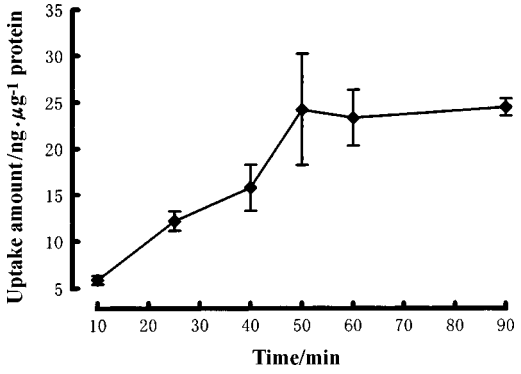

Fig 1. Time course for the uptake of nimodipine (NMD) by primary cultured BCEC. Cells were incubated with a medium containing nimodipine 10 mg/L at 37 °C. *n*=6.

ner from 0.1 to 50 μmol/L. In the presence of CsA 50 μmol/L, the steady-state uptake of NMD increased significantly ($P<0.01$) approximately by 21-fold. Furthermore, erythromycin, clarithromycin, azithromycin, and probenecid also increased the uptake of NMD by BCEC in the steady-state. Moreover, in the presence of sodium azide, a metabolic inhibitor, the uptake of NMD in the steady-state increased approximately by 2-fold (Tab 1). This result may be ascribed to the decreased activity of ATP-dependent P-gp function by metabolic inhibitors.

Cells were only administered with NMD (10 mg/L) and co-administered with CsA (50 μmol/L) and NMD (10 mg/L), respectively in the whole time course (Fig 2). From the figure, we can see the amount of NMD taken up was significantly increased ($P<0.01$) in the presence of CsA in the whole time course.

Tab 1. Effects of various agents on the uptake of nimodipine (NMD) by primary cultured BCEC. Cells were co-administered with NMD (10 mg/L) and each compound at 37 °C for 90 min. The uptake of NMD was measured for 90 min. *n*=6. Mean±SD. $^cP<0.01$ *vs* control.

Agents	Relative uptake (% of control)
Control	100
CsA (0.1 μmol·L^{-1})	213±23^c
CsA (1 μmol·L^{-1})	276±95^c
CsA (10 μmol·L^{-1})	554±77^c
CsA (50 μmol·L^{-1})	2148±218^c
Flunarizine (100 μmol·L^{-1})	119±16
Erythromycin (100 μmol·L^{-1})	551±78^c
Clarithromycin (100 μmol·L^{-1})	424±68^c
Azithromycin (100 μmol·L^{-1})	266±23^c
Probenecid (100 μmol·L^{-1})	899±33^c
Sodium azide (10 mg·L^{-1})	217±12^c

Effect of several MDR-reversing agents on the efflux of NMD from BCEC To investigate whether erythromycin, clarithromycin, and CsA inhibit the efflux of NMD from BCEC, the effects of the three substrates on the efflux of NMD were studied and were shown in Fig 3. In the presence of CsA (50 μmol/L), the amount of NMD remaining in the cells significantly increased ($P<0.01$), suggesting that CsA inhibits the efflux of NMD. By administration of erythromycin (100 μmol/L) and clarithromycin (100 μmol/L), the remaining amounts of NMD were also increased. These results suggest that the efflux of NMD was suppressed by these agents.

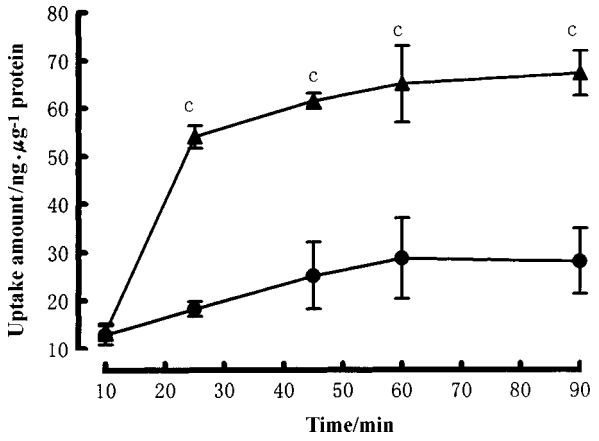

Fig 2. Effect of CsA on the uptake of nimodipine (NMD) by primary cultured BCEC. Cells were administered with NMD 10 mg/L alone (¡ñ) and co-administered with CsA 50 μmol/L and NMD 10 mg/L (¡ð) at 37 °C, respectively. *n*=4. Mean±SD. $^cP<0.01$ vs NMD alone group.

DISCUSSION

The movement of compounds across the BBB is not only dependent on the lipophilicity and molecular size of the compounds, but also is regulated by a specific carrier-mediated transport system which can eject them from endothelial cells into the blood stream. Until now, several mechanisms have been proposed to explain the exceptionally low distribution of some compounds into the brain, but P-gp-mediated efflux from BCEC is more likely mechanism than the others. More and more studies have shown that P-gp as a carrier-mediated transport system may function as a part of the BBB against the transfer of toxic compounds from circulating blood into brain interstitial fluid.

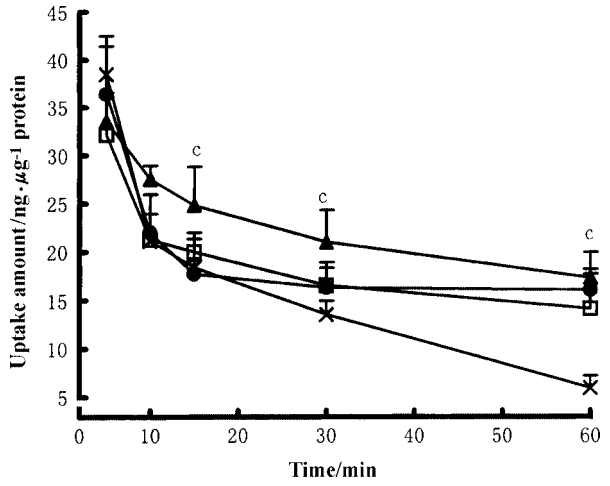

Fig 3. Effects of P-gp inhibitors on the efflux of nimodipine from primary cultured BCEC. Cells were administered with erythromycin 100 μmol/L (¡ñ), clarithromycin 100 μmol/L (¡ð), CsA 50 μmol/L (¡ø), and Hanks' solution (¡Á) at 37ºC for 60 min. *n*=4. Mean±SD. ᶜ*P*<0.01 vs Hanks' solution.

The present study demonstrated the transport of NMD out of the primary cultured BCEC by P-gp. It is well known that CsA, erythromycin, and clarithromycin are classical P-gp inhibitors[14,15]. These three drugs may increase the uptake of P-gp substrates by both BCEC and MDR-cells. Our study showed that CsA, erythromycin, and clarithromycin significantly enhanced the uptake amount of NMD by primary cultured BCEC. In the efflux experiment, the efflux of NMD by BCEC was increased in the presence of CsA, erythromycin, and clarithromycin. The results further give evidence that P-gp was involved in transport of NMD at BBB. Moreover, our studies demonstrated that among the three compounds, CsA showed a stronger effect on transport of NMD. These results indicated that there might exist some relationship between efflux of NMD from brain and P-gp at BBB.

In summary, the present study showed that MDR-reversing agents inhibited efflux of NMD from brain, increased uptake of NMD by BCEC. Taken together, these observations suggested that the transport of NMD at BBB was regulated by P-gp.

REFERENCES

1 Cordon-Cardo C, O'Brien JP, Casal D, Rittman-Grauer L, Biedler JL, Melamed MR, *et al*. Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. Proc Natl Acad Sci USA 1989; 86: 695-8.

2 Sugawara I, Hamada H, Tsuruo T, Mori S. Specialized localization of P-glycoprotein recognized by MRK16 monoclonal antibody in endothelial cells of brain and spinal cord. J Cancer Res 1990; 81: 727-30.

3 Tsuji A, Terasaki T, Takabatake Y, Tamai I, Yamashima T, Moritani S, *et al*. P-Glycoprotein as the drug efflux pump in primary cultured bovine brain capillary endothelial cells. Life Sci 1992; 151: 1427-37.

4 Tsuji A, Tamai I, Sakata A, Tenda Y, Terasaki T. Restricted transport of cyclosporin A across the blood-brain barrier by a multidrug transporter, P-glycoprotein. Biochem Pharmacol 1993; 46: 1096-9.

5 Tatsuta T, Naito M, Oh-Hara T, Sugawara I, Tsuruo T. Functional involvement of P-glycoprotein in blood-brain barrier. J Biol Chem 1992; 267: 20383-91.

6 Hughes CS, Vaden SL, Manaugh CA, Price GS, Hudson LC. Modulation of doxorubicin concentration by cyclosporin A in brain and testicular barrier tissues expressing P-glycoprotein in rats. J Neurooncol 1998; 37: 45-54.

7 Chikhale EG, Burton PS, Borchardt R. The effects of varapamil on the transport of peptides across the blood-brain barrier in rats: kinetic evidence for an apically polarized efflux mechanism. J Pharmacol Exp Ther 1995; 273: 298-303.

8 Liu XD, Liu GQ. P-glycoprotein regulated transport of glutamate at blood-brain barrier. Acta Pharmacol Sin 2001; 22: 111-6.

9 Liu XD, Pan GY, Xie L, Hou YY, Lan W, Su Q, *et al*. Cyclosporin A enhanced protection of nimodipine against brain damage induced by hypoxia-ischemia in mice and rat. Acta Pharmacol Sin 2002; 23: 225-9.

10 Qian ZY, Huang Q, Zhou LY, Sun ZF. Isolation and long-term cultivation of rat brain microvascular endothelial cells. Chin J Cell Biol 1999; 21: 42-5.

11 Audus KL, Borchardt RT. Characterization of an *in vitro* blood-brain barrier model system for studying drug transport and metabolism. Pharm Res 1986; 3: 81-7.

12 Liu XD, Xie L, Xu GQ, Wang J, Zhou YC, Liu GQ. Variability of nimodipine pharmacokinetics in healthy chinese males. Chin J Pharmacol Toxicol 1996; 10: 25-7.

13 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-54.

14 Schwarz UI, Gramatte T, Krappweis T, Oertel R, Kirch W. P-glycoprotein inhibitor erythromycin increase oral bioavailability of talinolol in humans. Int J Clin Pharmacol Ther 2000; 38: 161-7.

15 Wakasugi H, Yano I, Ito T, Hashida T, Futami T, Nohara R, *et al*. Effect of clarithromycin on renal excretion of digoxin: interaction with P-glycoprotein. Clin Pharmacol Ther 1998; 64: 123-8.