**P-glycoprotein restricted transport of nimodipine across blood-brain barrier**

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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 cellular 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-infaract 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
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. Cosmic 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 % CO2. 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, CaCl2 1.26 mmol/L, MgSO4·7H2O 0.81 mmol/L, NaH2PO4·H2O 0.37 mmol/L, KH2PO4 0.44 mmol/L, NaHCO3 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-
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.

**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.

**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.
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. 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