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Effect of baicalin and berberine on transport of nimodipine on primary-cultured, rat brain microvascular endothelial cells[1]

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Key words

nimodipine; baicalin; berberine; P-glycoprotein

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Abstract

Aim: To investigate whether baicalin and berberine affects the transport of nimodipine (NMD) across the blood-brain barrier (BBB). **Methods:** Primary-cultured, rat brain microvascular endothelial cells (rBMEC) were used as an *in vitro* model of the BBB. When cells became confluent, the steady-state uptake of NMD by rBMEC with or without baicalin and berberine was measured. The effects of baicalin and berberine on the efflux of NMD from rBMEC were also studied. **Results:** Baicalin (2–5 μg/mL) increased the uptake of NMD, and baicalin (10–20 μg/mL) decreased the uptake. The steady-state uptake of NMD was higher than that of control group in the presence of 0.01–1 μg/mL berberine, but was lower in the presence of 2–10 μg/mL berberine. **Conclusion:** The bidirectional effect of baicalin and berberine on the uptake of NMD by rBMEC was found. Higher concentration showed an inhibitory effect, and lower concentration demonstrated an increasing effect.

Introduction

Nimodipine (NMD) is a dihydropyridine calcium channel blocker and is currently used to prevent and treat the ischemic damage caused by cerebral arterial spasm in subarachnoid hemorrhage[1]. NMD has also been used in other cerebrovascular disorders, such as ischemic stroke[2] and multi-infarct dementia[3].

Huanglian (Rhizoma Coptidis) and Huangqin (Radix Scutellariae) are a familiar medicine couple in many traditional complex prescriptions. Baicalin and berberine are the phytochemical markers for the quality control of Radix Scutellariae and Rhizoma Coptidis in Chinese pharmacopoeia, respectively. Thus, we choose baicalin and berberine as the experimental samples.

Baicalin has multiple biological activities such as vasodilatory[4], antioxidant[5], and antitumor activities[6,7]. Recent studies have demonstrated that baicalin has a protective effect against brain edema and cerebral ischemic damage[8–10].

Berberine has varied pharmacological effects including anti-diarrheic[11], antimicrobial[12], and anti-inflammatory[13]; it also exhibits protective effects against ischemic damage after ischemia/reperfusion[14–16].

As both baicalin and berberine have beneficial effects on brain ischemic damage, the opportunity of a combination of them or herbs containing them with NMD is increasing. A major concern is that a herb-drug interaction may occur. Thus, we investigated whether baicalin and berberine affected the transport of NMD across the blood-brain barrier (BBB) using the primary cultured rat brain microvessel endothelial cells (rBMEC) in the present study.

Materials and methods

Materials NMD and felodipine were provided by Shandong Xinhua Pharmaceutical Factory (Shandong, China). Baicalin and berberine standards were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Rhodamine123 (Rho123) was purchased from Sigma-Aldrich (St Louis, MO, USA). Cosmic calf serum was purchased from Hyclone (Tauranga, New Zealand). Gelatin, trypsin, and collagenase type II were purchased from Sigma-Aldrich (USA). Dulbecco's modified Eagle's medium (DMEM, high glucose)

and Ham's F-12 nutrient mixture were purchased from Gibco BRL (Rockville, MD, USA). Bovine serum albumin (BSA) was purchased from SABC (Fraction V; Luoyang, He-nan, China). Sprague-Dawley rats (7–10 d old) were supplied by the Center of Experimental Animals, China Pharmaceutical University (Nanjing, China). All other chemicals were of analytical grade and commercially available.

Isolation and culture of rBMEC Primary rBMEC isolation and culture were operated according to previous reports[17]. Briefly, the isolated cerebral gray matter was digested by trypsin (0.05%) at 37 ℃ for 20 min, then filtrated through a 149 μm nylon mesh before the filter was collected. The filtrate was filtered through a 79 μm nylon mesh before the matter on the nylon mesh was collected. Then the matter was digested by collagenase type II (0.1%) at 37 °C for 25 min, centrifuged at room temperature for 5 min (200×*g*), and the cells were collected. Cells were cultured in DMEM and F-12 supplemented with 20% cosmic calf serum under the conditions of 37 °C with 95% air and 5% CO_2. Uptake and efflux experiments were performed in a 24-well plate when the cells reached confluence within 12–14 d.

Uptake experiment When the cells reached confluence within 12–14 d, uptake experiments were performed. Cultured rBMEC were pre-incubated at 37 °C in 1 mL Hanks' balanced salt solution (HBSS) (0.137 mol/L NaCl, 5.37 mmol/L KCl, 1.26 mmol/L $CaCl_2$, 0.81 mmol/L $MgSO_4 \cdot 7H_2O$, 0.37 mmol/L $Na_2HPO_4 \cdot H_2O$, 0.44 mmol/L KH_2PO_4, 4.17 mmol/L $NaHCO_3$, and 2.92 mmol/L *D*-glucose) for 30 min. After the pre-incubation, the solution was removed and the HBSS (1 mL) containing NMD (10 mg/L) or both NMD (10 mg/L) and agents for testing was added to each incubation well. The steady state of NMD in rBMEC was recorded at 90 min[17]. The uptake was terminated at 90 min by washing the cells 3 times with 1 mL of ice-cold HBSS. Then the blank HBSS (0.3 mL) was added to each incubated well, frozen, and melted repeatedly 4 times to break down the cells. The uptake of Rho123 was performed in a similar way. After pre-incubation for 30 min with HBSS (1 mL), the solution was removed, and the HBSS (1 mL) containing Rho123 (0.1 mg/L) or both Rho123 (0.1 mg/L) and agents for testing was added to each incubation well. The steady state of Rho123 in rBMEC was recorded at 120 min according to previous study in our lab. So the uptake was terminated at 120 min by washing the cells 5 times with 1 mL of ice-cold HBSS. Then the blank HBSS (0.5 mL) was added to each incubated well, frozen, and melted repeatedly 4 times to break down the cells.

Efflux experiment For the efflux study, rBMEC were incubated with NMD (10 mg/L) or Rho123 (0.1 mg/L) for 90 min, then the cells were washed 5 times with 1 mL ice-cold HBSS. HBSS (1 mL) with or without test agents was added to initiate the efflux at 37 °C. The efflux was terminated at the designed time points by using the same procedure as used in the uptake study mentioned above. Efflux was estimated from the amount of NMD or Rho123 remaining in the cells.

Analytical method The concentration of NMD in the cell suspension was analyzed by a HPLC system (LC-10AT, Shimadzu, Kyoto, Japan) which was equipped with a VP-ODS column (4.6×150 mm, 5 μm, Shimadzu, Japan) and a UV-detector (SPD-10Avp, Shimadzu, Japan). The mobile phase consisted of MeOH: H_2O (70:30, *v/v*, pH=7); the flow rate was set at 1.0 mL/min, and the analytical wavelength was 238 nm[18]. Internal standard (felodipine, 5 μg/mL, 10 μL) and 150 μL methanol were added to cell suspension (150 μL) and mixed vigorously for 10 min, then centrifuged (44912×*g* for 10 min); 200 μL of supernatant was transferred to another clean tube and centrifuged (44912×*g* for 10 min) again. After the second centrifugalization, 120 μL of supernatant was transferred to another clean tube and 20 μL was injected onto the HPLC system. For the standard sample, NMD was dissolved into a blank cell (cell without drug treatment) suspension at a concentration from 5 to 1200 ng/mL. The lowest limit of quantitation of NMD was 5 ng/mL in the cell suspension. A good linearity was obtained from 5–1200 ng/mL.

The concentrations of Rho123 in the cell suspension were determined by HPLC[19]. Twenty microliters of cell suspension was injected into a Shimadzu LC-10A_{VP} system (Shimadzu, Japan) consisting of an LC-10A liquid pump, a CTO-10AS_{VP} column oven, and a fluorescence detector (RF-10A_{XL}) set at an excitation wavelength of 485 nm and emission wavelength of 565 nm. Conditions were as follows: column, a Shim-pack ODS (4.6 μm, 150 mm×4.6 mm id, Shimadzu, Japan); mobile phase, 0.1% HAc (pH 4.0)–acetonitrile=3:2 (*v/v*); column temperature, 40 °C; flow rate, 1 mL/min. Methanol was also used to remove the protein as in the case of NMD. For the standard sample, Rho123 was dissolved into the blank cell (cell without drug treatment) suspension at a concentration from 0.5 to 50 ng/mL. The lowest limit of quantitation of Rho123 was 0.5 ng/mL in the cell suspension. The linear range of Rho123 was 0.5–50 ng/mL.

The protein content in the cultured cells was measured by the method of Brandford MM[20] using BSA as the standard. Net uptake, expressed as the concentration ratio (ng/μg protein), was obtained by dividing the apparent uptake amount of NMD or Rho123 (ng/mL) by protein content (μg protein/mL). All data were expressed as mean±SD. Statistical analysis was performed by using Student's *t*-test.

A difference of $P<0.05$ was considered statistically significant.

Results

Effects of tested agents on the rBMEC cytoactivity In order to investigate whether tested agents affected cell cytoactivity, cells were incubated at 37 °C for 90 min with 10 mg/L NMD, 120 min with HBSS, 0.1 mg/L Rho123, 20 μg/mL baicalin, and 10 μg/mL berberine, respectively. Cell activity was measured using the 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. The HBSS group was set as the control group. Data were shown in Table 1. No differences ($P>0.05$) were found relative to the control group. Thus, we believed that the tested agents did not damage the cells at the concentrations used.

Table 1. Effects of agents used on cell cytoactivity (mean±SD, n=4).

OD	Mean	SD
HBSS (120 min)	0.041	0.002
Nimodipine (10 mg/L, 90 min)	0.040	0.003
Rho123 (0.1 mg/L, 120 min)	0.041	0.002
Baicalin (20 μg/mL, 120 min)	0.041	0.004
Berberine (10 μg/mL, 120 min)	0.040	0.002

Effects of baicalin on the steady-state uptake of NMD The concentration-dependent, bidirectional effect of baicalin on the steady-state uptake of NMD was observed. Baicalin at 2, 5, and 10 μg/mL increased the steady-state uptake of NMD, whereas baicalin at 15 and 20 μg/mL decreased the uptake (Figure 1).

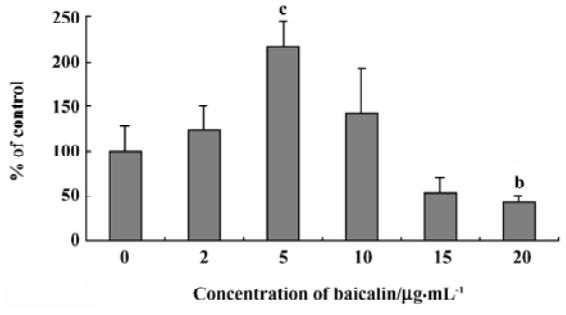

Figure 1. Effect of baicalin on the steady-state uptake of NMD by primary cultured rBMEC. 0: control; 2, 5, 10, 15, 20: NMD (10 mg/L) with baicalin 2, 5, 10, 15, 20 μg/mL, respectively. The uptake amount of NMD in the control group was 5.69±1.53 ng/μg protein. Data are mean±SD. n=4. $^bP<0.05$, $^cP<0.01$ *vs* control.

Effects of berberine on the steady-state uptake of NMD Similar to baicalin, berberine also affected the uptake of NMD in a dose-dependent, biphase manner. Figure 2 shows the profiles of the steady-state uptake of NMD by rBMEC in the presence or absence of berberine. Berberine increased the uptake of NMD by primary cultured rBMEC in a dose-dependent manner from 0.01 to 1 μg/mL, but decreased the uptake in a dose range from 2 to 10 μg/mL. The steady-state uptake of NMD was increased to 109%, 151%, and 202% of the control group in the presence of 0.01, 0.1, and 1 μg/mL berberine, respectively. The uptake of NMD decreased to 72%, 53%, and 46% of the control group by 2, 5, 10 μg/mL berberine, respectively.

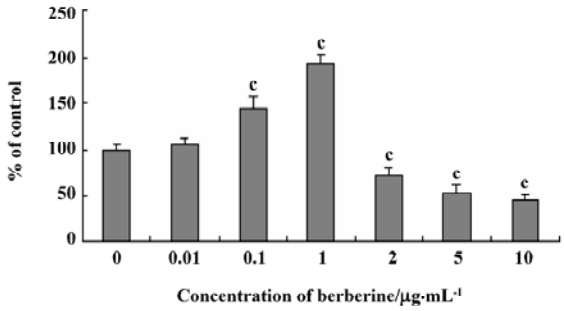

Figure 2. Effect of berberine on the steady state uptake of NMD by primary cultured rBMEC. 0: control; 0.01, 0.1, 1, 2, 5, 10: NMD (10 mg/L) with berberine 0.01, 0.1, 1, 2, 5, 10 μg/mL, respectively. The uptake amount of NMD in the control group was 5.71±0.64 ng/μg protein. Data are mean±SD. n=4. $^cP<0.01$ *vs* control.

Effects of baicalin and berberine on the steady-state uptake of Rho123 The transport of NMD across rBMEC was reported to be restricted by P-glycoprotein (P-gp)[17]. To clarify whether the observed effects of baicalin and berberine on the uptake of NMD were via P-gp modulation, we used Rho123, a typical P-gp substrate, as the positive control. The results were illustrated in Figure 3. The steady-state uptake of Rho123 was 106%, 123%, 108%, 86%, and 59% of the control group in the presence of 2, 5, 10, 15, and 20 μg/mL baicalin, respectively (Figure 3A). This result was similar to the observations in the uptake of NMD. Similar to the effect on the uptake of NMD, berberine exhibited a dose-dependent, bidirectional effect on the steady-state uptake of Rho123 by rBMEC. The steady-state uptake of Rho123 by primary cultured rBMEC was increased by berberine (0.01–1 μg/mL), but decreased by berberine (2–10 μg/mL; Figure 3B).

Effects of baicalin and berberine on the efflux of NMD To further understand the mechanism of the effect baicalin and berberine showed on the transport of NMD, we

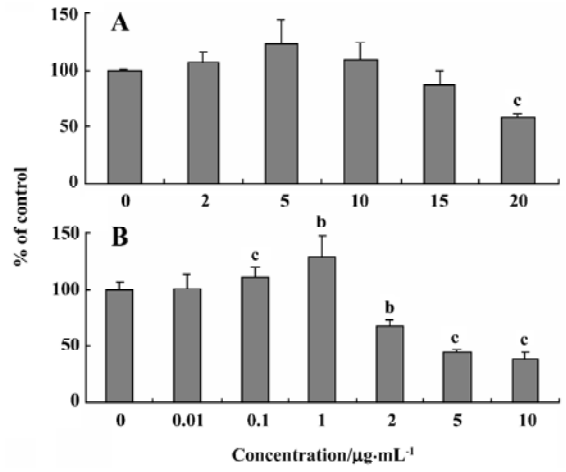

Figure 3. (A) effect of baicalin on the steady state uptake of Rho123 by primary cultured rBMEC. 0: control; 2, 5, 10, 15, 20: Rho123 (0.1 mg/L) with baicalin 2, 5, 10, 15, 20 μg/mL, respectively. The uptake amount of Rho123 in the control group was 0.21±0.01 ng/μg protein. Data are mean±SD. n=4. ^cP<0.01 *vs* control. (B) effect of berberine on the steady state uptake of Rho123 by primary cultured rBMEC. 0: control; 0.01, 0.1, 1, 2, 5, 10: Rho123 (0.1 mg/L) with berberine 0.01, 0.1, 1, 2, 5, 10 μg/mL, respectively. The uptake amount of Rho123 in control group was 0.20±0.01 ng/μg protein. Data are mean±SD. n=4. ^bP<0.05, ^cP<0.01 *vs* control.

examined the effects of baicalin and berberine on the efflux of NMD from rBMEC. In the presence of baicalin (5 μg/mL), the amount of NMD remaining in the cells was significantly higher than the control group. When the cells were incubated with 20 μg/mL baicalin, the amount of NMD remaining in the cells significantly decreased (Figure 4). These results suggested that 5 μg/mL baicalin inhibited the efflux of NMD, while 20 μg/mL baicalin stimulated the efflux of NMD from rBMEC. Similarly, the resident amount of NMD in the cells was significantly increased by berberine (1 μg/mL), but

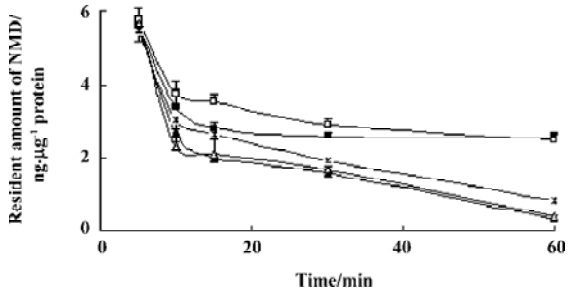

Figure 4. Effect of baicalin and berberine on the efflux of NMD from primary cultured rBMEC. Cells were pre-incubated in HBSS (1 mL) containing NMD (10 mg/L) for 90 min. Data are mean±SD. n=4. □, berberine (1 μg/mL); ■, baicalin (5 μg/mL); *, HBSS, control; △, berberine (10 μg/mL); ▲, baicalin (20 μg/mL).

decreased with berberine (10 μg/mL; Figure 4), which indicated that the efflux of NMD from rBMEC was inhibited by 1 μg/mL berberine, but promoted by 10 μg/mL berberine.

Effects of baicalin and berberine on the efflux of Rho123

As in the uptake experiment, the effects of baicalin and berberine on the efflux of Rho123 were also examined to provide a positive control. Similar with the effect on the efflux of NMD, 5 μg/mL baicalin and 1 μg/mL berberine inhibited the efflux of Rho123, while 20 μg/mL baicalin and 10 μg/mL berberine stimulated the efflux (data were shown in Figure 5).

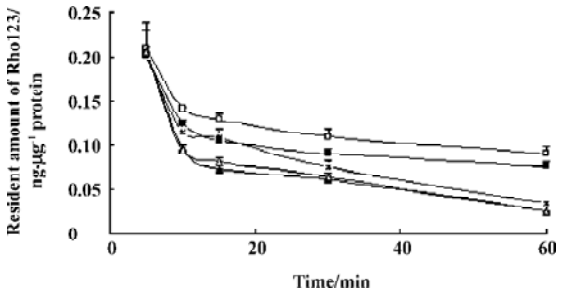

Figure 5. Effect of baicalin and berberine on the efflux of Rho123 from primary cultured rBMEC. Cells were pre-incubated in HBSS (1 mL) containing Rho123 (0.1 mg/L) for 90 min. Data are mean±SD. n=4. □, berberine (1 μg/mL); ■, baicalin (5 μg/mL); *, HBSS, control; △, berberine (10 μg/mL); ▲, baicalin (20 μg/mL).

Discussion

The present study demonstrated that both baicalin and berberine affected the transport of NMD across the BBB in a bidirectional manner.

The MTT assay demonstrated that tested agents did not damage cells at the concentrations used; we believed that the observed changes were not the result of nonspecific cytotoxicity.

We observed a concentration-dependent, bidirectional effect of baicalin on the steady-state uptake of NMD by primary cultured rBMEC; the uptake was significantly increased by 5 μg/mL baicalin, but decreased with 20 μg/mL baicalin. In the efflux experiment, baicalin (5 μg/mL) inhibited the efflux of NMD while baicalin (20 μg/mL) stimulated the efflux. Baicalin belongs to the class of flavonoids and in recent years, flavonoids had been described as P-gp modulators, however, contradictory effects have been reported. Critchfield *et al*[21] reported that quercetin increased adriamycin efflux from HCT-15 colon cells, whereas Scambia *et al*[22] found that quercetin inhibited Rho123 efflux in MCF-7 breast cells. Gwenaelle *et al*[23] demonstrated flavonoids as a new class of bifunctional modulators of P-gp and Yoshiharu *et al*[24] reported a concentration-dependent biphasic effect

of quercetin on the uptake of [^{3}H]vincristine, which was a similar phenomenon to our result. The effect of baicalin on the uptake and efflux of Rho123, the positive control, has the same tendency as the effect on NMD uptake; baicalin (5 μg/mL) inhibited the efflux and increased the uptake of Rho123, whereas 20 μg/mL baicalin stimulated the efflux and significantly decreased the uptake of Rho123. These results suggested that the effect of baicalin on the transport of NMD across the BBB might due to the modulation of P-gp function.

Berberine also showed a dose-dependent bidirectional effect on the transport of NMD across the BBB. Berberine increased the uptake of NMD from 0.01 to 1 μg/mL and decreased the uptake from 2 to 10 μg/mL. The efflux of NMD was inhibited by 1 μg/mL berberine and was promoted by 10 μg/mL berberine. We also adopted Rho123 as a positive control. Berberine demonstrated the similar effect on the transport of Rho123 as on the transport of NMD. Our results suggested that the effect of berberine on the transport of NMD across the BBB might result from the modulation of berberine on P-gp function. Sun *et al*[25] reported that berberine (30 μmol/L, corresponding to 10 μg/mL) decreased the uptake of carbamazepine by rBMEC, which was in agreement with our result. Verapamil had been reported to modulate P-gp ATPase activity in a bidirectional manner[26]. Whether berberine affected P-gp function via the same path still needs further investigation.

As the concentrations of baicalin (<10 μg/mL)[27] and berberine (<100 ng/mL level)[28] *in vivo* were so low, they might only be able to demonstrate inhibitory effects on P-gp function in *in vivo* studies. In addition, the detail mechanism of the bidirectional effect observed in the present study still needs further investigation.

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