Regular Article

Role of P-Glycoprotein in Regulating Cilnidipine Distribution to Intact and Ischemic Brain

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Summary: Cilnidipine is reported to show antihypertensive and neuroprotective actions in a rat brain ischemia model, but is barely distributed to normal brain, suggesting that its uptake into normal brain is inhibited by efflux transporter(s), such as P-glycoprotein (P-gp). Here, we investigated whether P-gp regulates the brain distribution of cilnidipine. Intracellular accumulation of cilnidipine was decreased in P-gp-overexpressing porcine kidney epithelial cells (LLC-GA5-COL150 cells) compared with control LLC-PK1 cells and the decrease was markedly inhibited by verapamil, a P-gp inhibitor. Further, cilnidipine concentration in the brain of P-gp knockout mice was significantly increased after cilnidipine administration, compared with that in wild-type mice. Moreover, when cilnidipine was administered to male spontaneously hypertensive rats (SHR) with tandem occlusion of the distal middle cerebral and ipsilateral common carotid artery, its concentration in the ischemic hemisphere was 1.6-fold higher than that in the contralateral hemisphere. This result was supported by visualization of cilnidipine distribution using matrix-assisted laser desorption/ionization-time of flight/mass spectrometry (MALDI-TOF/MS) imaging. Our results indicated that cilnidipine is normally excluded from the brain by P-gp-mediated efflux transport, but P-gp function is impaired in ischemic brain and consequently cilnidipine is distributed to the ischemic region.

Keywords: cilnidipine; P-gp; efflux transporters; ABC transporter; ischemia; MALDI-TOF/MS imaging; knockout mice; brain; distribution

Introduction

Influx of Ca2+ from blood into vascular smooth muscle cells induces vasoconstriction and blood pressure elevation, and is regulated by L-type Ca2+ channels. On the other hand, N-type Ca2+ channels in presynaptic cells regulate the extrusion of noradrenaline to the synaptic cleft. Cilnidipine is a dihydropyridine-type antihypertensive drug that inhibits not only L-type, but also N-type Ca2+ channels.1–3) It reduces blood pressure via inhibition of the L-type channels,4) and prevents development of tachycardia by suppressing the N-type channels on peripheral sympathetic-nerve terminals.5) Cilnidipine also has a cerebroprotective effect, because inhibition of N-type channels reduces the release of glutamate, which causes neuronal damage, from presynaptic sites in focal brain ischemia.6–9) However, cilnidipine is not well distributed to the brain, despite its high lipophilicity. The blood-brain barrier (BBB), which is formed from vascular endothelial cells linked via tight junctions,10) expresses several efflux transporters, such as P-glycoprotein (P-gp), multidrug resistance associated proteins (Mrps) and breast cancer resistance protein (Bcrp).11) Accordingly, even if cilnidipine is imported into vascular endothelial cells, it may be subject to ATP-dependent efflux transport to the blood, mediated by these transporters.12)

Interestingly, hypotensive doses of cilnidipine have been reported to reduce the size of the infarcted region in a rat model of cerebral ischemia.13) This result indicates that this drug can penetrate BBB in ischemic regions. Therefore, we speculated that cilnidipine is indeed a substrate of P-gp, but that the transport activity of P-gp is impaired in ischemic brain, permitting the entry of cilnidipine.
To investigate this hypothesis, we examined the contribution of P-glycoprotein to cilnidipine influx transport and brain distribution by means of in vitro and in vivo experiments, using P-gp-overexpressing cells, P-gp-knockout mice, and rats with unilateral brain ischemia.

**Materials and Methods**

**Chemicals and animals:** Cilnidipine was provided by Ajinomoto Co. Inc. (Kawasaki, Japan). P-gp knockout (mdra/b<sup>-/-</sup>) and FBV (wild-type of mdra/b<sup>-/-</sup>) mice were purchased from CLEA (Tokyo, Japan). Male spontaneously hypertensive rats (SHR) were purchased from Japan SLC Inc. (Shizuoka, Japan). P-gp-overexpressing porcine kidney epithelial cell line LLC-GA5-COL150 (MDR1 gene-transfected cells) and the parental LLC-PK1 cell line were obtained from RIKEN Cell Bank. Medium 199 (M199) was purchased from Life Technologies (Carlsbad, CA, USA). Mice and rats were maintained with free access to food and water until 8 weeks of age (body weights of 15–23 g and 190–220 g, respectively). All animal experiments were performed according to the Guidelines for the Care and Use of Animals at Takasaki University of Health and Welfare.

**Cell culture:** LLC-PK1 cells were grown in M199 containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in an atmosphere of 5% CO<sub>2</sub> and 95% relative humidity. LLC-GA5-COL150 cells were cultured under the same conditions in the presence of 150 ng/mL colchicine. For the uptake study, LLC-PK1 cells and LLC-GA5-COL150 cells were seeded on 12-well cell culture plates (Greiner bio-one, Tokyo, Japan) at cell densities of 0.5 × 10<sup>5</sup> and 1.5 × 10<sup>5</sup> cells/mL, respectively.

**General surgical preparation:** Focal brain ischemia was induced in male spontaneously hypertensive rats (SHR), 8 weeks of age, by tandem occlusion of the distal middle cerebral and ipsilateral common carotid artery, as previously described. 13,14) Twenty minutes after cilnidipine or Evans blue15,16) administration, the whole brain was excised and irrigated with saline, and blood was collected from the veins of both rodents with heparinized syringes 1 h after administration. Cilnidipine or Evans blue concentrations of blood and brain samples were measured as described below.

**Measurement of cilnidipine and Evans blue concentrations:** In vitro and in vivo samples were treated as follows. Blood samples were centrifuged at 1,800 x g and 4°C for 5 min. To measure cilnidipine concentration, 600 µL methyl tert-butyl ether (MTBE) was added to the supernatant. The mixture was vortexed and further centrifuged at 1,800 x g and 4°C for 5 min. To the organic layer was added 600 µL MTBE and the above procedure was repeated. The brain was divided into right and left hemispheres, and each was homogenized. The homogenates were then treated as described above for the blood supernatants. Cell lysates from the in vitro experiments were treated similarly. The collected organic layer from each sample was evaporated to dryness under a stream of nitrogen, and the residue was taken up in 200 µL methanol. Cilnidipine concentration was determined by means of LC-MS/MS (high-performance liquid chromatography–tandem mass spectrometry) for the brain, and HPLC for blood samples and cell homogenates. For LC-MS/MS measurement, a CN-3 column (5 µm, GL Science Inc., Tokyo, Japan) was used. The sample volume was 5 µL and the flow rate was 0.2 mL/min. The mobile phase was a mixture of 10 mM ammonium acetate (pH 5.0) and methanol. For HPLC measurement, a Mightyyl RP-18 Aqua 250–4.6 column (5 µm, Kanto Chemical Co. Inc.) was used. The sample volume was 50 µL and the flow rate was 1.0 mL/min. The mobile phase was a mixture of 5 mM ammonium acetate (pH 5.0) and acetonitrile.

To measure Evans blue concentration, blood samples were centrifuged at 1,800 x g for 10 min at 4°C, and the supernatants were used as plasma samples. Blood samples were homogenized in two volumes of saline and centrifuged at 13,000 x g, 4°C for 30 min. The supernatants in both plasma and brain samples were measured at 615 nm for absorbance using a spectrophotometer (Sunrise rainbow RC; Tecan, Kanazawa, Japan).

Brain-to-plasma concentration ratios (K<sub>pbrain</sub> values) were calculated using the formula: K<sub>pbrain</sub> (mL/g brain) = brain concentration (ng/g brain)/plasma concentration (ng/mL).

**MALDI-TOF/MS imaging:** Brain samples embedded in Tissue Tek O.C.T. compound (Sakura Fine Technical, Tokyo, Japan) were snap-frozen, and sliced at 9 µm using a cryostat (Leica CM1850, Tokyo, Japan). Tissue sections were sprayed with matrix solution [2,5-dihydroxybenzoic acid (DHB) 50 mg/mL in 70% methanol and 0.1% trifluoroacetic acid] using an ImagePrep matrix coater (Bruker Daltonics, Bremen, Germany), and dried for 1 h under vacuum. Various concentrations (0, 20, 40 and 80 pmol) of cilnidipine were dropped on the tissue sections as positive controls. Mass spectra were obtained using a positive linear mode in the range of mass to charge ratio (m/z) 300 to 2,000. MS imaging analysis was performed using a MALDI-TOF/MS, Ultraflextreme (Bruker Daltonics). Cilnidipine (m/z 492.52) was confirmed by the use of negative control tissue.

**Statistical analysis:** All data are presented as mean ± standard error of the mean (S.E.M.), except in Figure 4. Statistical analysis was done using the two-tailed t-test. Differences between

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means at the level of $p < 0.05$ or 0.01 were considered to be significant.

**Results**

**Efflux transport of cilnidipine by P-gp in LLC cells:** When LLC-PK1 and LLC-GA5-COL150 cells were exposed to 100 µM cilnidipine, the cell/medium (C/M) ratio of cilnidipine concentration linearly increased in LLC-PK1 cells from 0 to 120 s. On the other hand, the ratio in P-gp-overexpressing LLC-GA5-COL150 cells showed little increase and reached a steady state within 60 s (Fig. 1A). In addition, the C/M ratio of cilnidipine concentration in LLC-PK1 cells was higher than that in P-gp-overexpressing LLC-GA5-COL150 cells at 2 min after the start of uptake. When LLC-GA5-COL150 cells were incubated with verapamil or dinitrophenol (DNP), the C/M ratio of cilnidipine was significantly increased compared with the control (1.40- and 1.37-fold, respectively) (Fig. 1B).

**Uptake of cilnidipine into the brain in wild-type and P-gp KO mice:** When cilnidipine (1.0 mg/10 mL/kg B.W.) was administered intraperitoneally to mice, cilnidipine concentration in the brain of P-gp KO mice (62.1 ng/g brain) was 6.3-fold higher than that of FVB (wild-type) mice (9.89 ng/g brain) (Fig. 2A). Plasma concentration of cilnidipine in P-gp KO mice (20.8 ng/mL) was not significantly different from that in FVB mice (18.3 ng/mL) at 1 h after intraperitoneal administration of 1 mg/10 mL/kg body weight (B.W.) cilnidipine (Fig. 2B).

**Concentration of cilnidipine and Evans blue in the brain of control and ischemic rats:** Rats underwent ligation of the left common artery and occlusion of the distal middle cerebral artery after intraperitoneal administration of cilnidipine (0.1 mg/mL/kg B.W.). The Left/Right (L/R) ratio of cilnidipine $K_{p_{brain}}$ value was 1.62 mL/g brain in ischemic rats at 1 h after administration of cilnidipine (Fig. 3), but was 1.08 in the sham-operated control rats. In addition, when Evans blue was intravenously administered to left-brain ischemic rats and corresponding control rats, there was no significant difference in the L/R ratio of $K_{p_{brain}}$ value between the two groups (Fig. 3).

**Imaging of cilnidipine distribution in rat brain:** Cilnidipine concentration was increased in the ischemic penumbra site of the left hemisphere, as visualized with MALDI-TOF/MS (Fig. 4). Cilnidipine was barely distributed in the intact right hemisphere.

**Discussion**

The C/M ratio of cilnidipine was decreased in LLC-GA5-COL150 cells, compared with LLC-PK1 cells, after 2 min incubation (Fig. 1A). In addition, verapamil (a P-gp inhibitor) and DNP (an ATP synthesis inhibitor) each significantly increased the C/M ratio of cilnidipine in LLC-GA5-COL150 cells (Fig. 1B). These results indicate that cilnidipine is a substrate of P-gp. Moreover, our *in vivo* study showed that the concentration of cilnidipine in the brain of P-gp knockout mice was significantly higher than that
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Fig. 4. MALDI-TOF/MS imaging of cilnidipine distribution in the intact right hemisphere and ischemic left hemisphere of rat brain

The white dotted circle indicates the ischemic area. Blue and pink colors represent the absence and presence of cilnidipine in the brain, respectively. In the right panel, the images in the dotted circles illustrate the appearance of the indicated cilnidipine concentration.

In FVB mice, whereas the plasma concentration of cilnidipine showed no significant difference between them (Figs. 2A and 2B). P-gp restricts the permeability of many drugs across BBB into the brain in vivo.13,18 Accordingly, our results suggest that the intracerebral concentration of cilnidipine is regulated by P-gp in mice in vivo.

Since cilnidipine was reported to show a neuroprotective effect in ischemic rat brain,13 we speculated that this drug is taken up into the brain in the state of ischemia. Accordingly, we next investigated whether cilnidipine was preferentially distributed in the ischemic region in a rat model of focal brain ischemia.

The surgical operation was conducted according to the reported method.13 It is considered that the white dotted circle in our MALDI-TOF/MS imaging (Fig. 4) corresponds to the ischemic core site. Moreover, each hemisphere of the brains of sham-operated rats and left-brain ischemic rats was homogenized separately. Thus, the homogenate of the left hemisphere included the ischemic core and penumbra site. Indeed, the L/R ratio of cilnidipine Kpbrain values in the ischemic rats was significantly increased compared with that in sham-operated control rats (Fig. 3). Although focal brain ischemia caused down-regulation of P-gp, we confirmed that BBB permeability through the paracellular route was not affected for at least 40 min after brain ischemia (Fig. 3). This result is also supported by previous findings in animals subjected to brief ischemia.19,20

The involvement of P-gp was also supported by MALDI-TOF/MS imaging of the cilnidipine distribution (Fig. 4). We consider P-gp activity to have been impaired in the ischemic penumbra site owing to hypoxia-induced decrease in ATP, which is required to drive the transport process.21–23 This may be the reason why cilnidipine concentration was increased in the ischemic penumbra site.

In conclusion, our results suggested that cilnidipine distribution to the brain is physiologically regulated by P-gp-mediated efflux transport, but P-gp is downregulated in ischemic regions due to hypoxia-induced decrease in ATP, thereby resulting in an increase of cilnidipine concentration in the ischemic regions. Therefore, cilnidipine might contribute to reduction of cerebral infarction size by inhibiting N-type Ca2+ channels in ischemic regions of the brain.

References

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