Impact of Cationic Amino Acid Transporter 1 on Blood-Retinal Barrier Transport of L-Ornithine

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Purpose. To elucidate L-ornithine transport at the blood-retinal barrier (BRB).

Methods. Integration plot and retinal uptake index (RUI) were used to investigate the in vivo [^{3}H]L-ornithine transport across the BRB. In vitro transport studies of [^{3}H]L-ornithine were performed with TR-iBRB2 cells and RPE-J cells, the model cells of the inner and outer BRB, respectively. Immunohistochemistry was performed on cationic amino acid transporter 1 (CAT1/SLC7A1).

Results. The apparent influx permeability clearance of [^{3}H]L-ornithine was found to be 18. 7 μL/(min·g retina), and the RUI of [^{3}H]L-ornithine was reduced by L-ornithine and L-arginine, suggesting the blood-to-retina transport of L-ornithine at the BRB. [^{3}H]L-Ornithine uptake by TR-iBRB2 cells showed a time-, temperature- and concentration-dependence with a Michaelis-Menten constant (K_m) of 33.2 μM and a nonsaturable uptake rate (K_d) of 2.18 μL/(min·mg protein). The uptake was Na^+-independent, and was inhibited by L-ornithine, L-arginine, and L-lysine, suggesting the involvement of CAT1 in L-ornithine transport at the inner BRB. Immunohistochemistry revealed the luminal and abluminal localization of CAT1 at the inner BRB, and at the basal localization at the outer BRB. Retinal pigment epithelium–J cells showed that the basal-to-cell (B-to-C) uptake of [^{3}H]L-ornithine was greater than that of the apical-to-cell (A-to-C) uptake, and the B-to-C transport was inhibited by unlabeled L-ornithine, suggesting the involvement of CAT1 in the blood-to-cell transport of L-ornithine across the basal membrane at the outer BRB.

Conclusions. These suggest the involvement of CAT1 in L-ornithine transport at the luminal and abluminal sides of the inner BRB and the basal side of the outer BRB.

Keywords: L-ornithine, transport, blood-retinal barrier, blood-brain barrier, cationic amino acid transporter, gyrate atrophy of the choroid and retina, hyperornithinemia

L-Ornithine is a cationic amino acid produced in the urea cycle and ingested from the diet, and is physiologically important in the metabolism of ammonia to urea.[1] Clinical research has suggested the beneficial effects of L-ornithine in the body, and its ingestion is reported to contribute to health maintenance associated with body weight, body fat, hepatic dysfunction, burn injury, and fatigue, by means of unclear mechanisms,[2–5] supporting the use of L-ornithine as a dietary supplement. In mammals, L-ornithine is metabolized by ornithine aminotransferase (OAT) and ornithine decarboxylase (ODC), and they convert L-ornithine to pyrroline-5-carboxylate (P5C) and to putrescine, respectively.[6,7] The genetic defect of OAT leads to gyrate atrophy of the choroid and retina (GA), an autosomal recessive disease, and hyperornithinemia primarily shown by GA patients is known to cause the progressive retinal degeneration that leads to blindness.[8–11]

These pieces of evidence indicate the relevance of L-ornithine to health maintenance and pathogeneses, and suggest the physiological importance of regulation of the L-ornithine concentration in the retina where the blood-retinal barrier (BRB) is formed by retinal capillary endothelial cells (inner BRB) and RPE cells (outer BRB) to separate the neural retina and the circulating blood.[12–15] At the BRB, these responsible cells form tight junctions to restrict paracellular solute transport,[12,13] and previous studies have suggested that the retinal capillary endothelial cells express various transporters, such as equilibrative nucleoside transporter 2 (ENT2/SLC29A2), taurine transporter (TAUT/SLC6A6), L (leucine-referring)-type amino acid transporter (LAT1/SLC7A5) and glucose transporter (GLUT1/SLC2A1), involved in the blood-to-retina transport of nutrients across the inner BRB nourishing two-thirds of the retina,[14–19] suggesting the involvement of transporters at the inner BRB in regulating the L-ornithine concentration in the retina.

In the study of cationic amino acids transport, system y^+, system y^+L, and system $b^{0,+}$ transport cationic amino acids in an Na^+-independent manner, whereas system $B^{0,+}$ transport in an Na^+-dependent manner, and the transporters belonging to the SLC6A and SLC7A families have been reported to be responsible for these transport systems. System y^+L, $B^{0,+}$ and $b^{0,+}$ also recognize neutral amino acids as substrates, and the Na^+-dependent transport of neutral amino acids of system y^+L and $B^{0,+}$ have been reported during the Na^+-independent transport of system $b^{0,+}$.[20–24] Regarding the inner BRB, our studies have revealed the expression of cationic amino acid transporter 1 (CAT1/SLC7A1), CAT3 (SLC7A3), and y^+L amino acid transporter 2 (y^+LAT2/SLC7A6) at the inner BRB, and the involvement of CAT1 has been suggested in L-arginine transport at the inner BRB.[25] However, the detailed mechanisms for L-ornithine transport at the inner BRB and

its clarification would help in the better use of L-ornithine as a dietary supplement because a study in humans has revealed that the L-ornithine concentration in plasma dramatically increased from 60 μM to 300 μM after the ingestion of L-ornithine (100 mg/kg).[26]

At the outer BRB formed by RPE cells, the expression of CAT1, y^+LAT1 (SLC7A7), y^+LAT2, and $b^{0,+}$AT (SLC7A9) has been suggested, and an in vitro uptake study has suggested the involvement of CAT1 in L-ornithine transport across the outer BRB.[27] However, RPE cells possess clear polarity, and it is still unclear how CAT1 contributes to directional L-ornithine transport at the outer BRB. In particular, RPE cells are known as the primary lesion site in GA,[8,9] and previous studies with 5-fluoromethylornithine (5-FMO), an irreversible OAT inhibitor, have revealed the death of RPE cells induced by an increase in the ocular concentration of L-ornithine,[11] indicating that the clarification of the directional transport of L-ornithine would also improve our understanding of the association of L-ornithine with the pathogenesis of GA.

In this study, in vivo and in vitro investigations were carried out to examine L-ornithine transport at the inner and outer BRB. The transport at the inner and outer BRB was investigated using in vitro model cell lines, such as the conditionally immortalized cell lines of rat retinal capillary endothelial cells (TR-iBRB2 cells) and rat RPE cells (RPE-J cells).[28–30]

Methods

Animals and Reagents

Male Long-Evans (140–190 g) and Wistar rats (160–180 g) were obtained from Japan SLC (Hamamatsu, Japan), and used for immunohistochemistry and in vivo transport study, respectively. The animal experiments conformed to the provisions of the Animal Care Committee, University of Toyama, and the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. This study used chemicals that were commercially available and of reagent grade. L-[2,3-^{3}H]Ornithine ([^{3}H]L-ornithine, 60 Ci/mmol), [1-^{14}C]D-mannitol ([^{14}C]D-mannitol, 55 mCi/mmol) and [1-^{14}C]*n*-butanol ([^{14}C]*n*-butanol, 2 mCi/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO, USA).

In Vivo Transport Study

The apparent influx permeability clearance (K_{in}) of [^{3}H]L-ornithine in tissues was estimated by integration plot analysis as described elsewhere (Supplementary Material).[31] An amount of 400 μL Ringer-HEPES buffer containing [^{3}H]L-ornithine (4 μCi/rat) was administered via the femoral vein of Wistar rats anesthetized with pentobarbital (50 mg/kg body weight). Blood samples were collected, and the rats were decapitated. The collected tissues were lysed and neutralized with 2N NaOH and 2N HCl, respectively, and the radioactivity of [^{3}H]L-ornithine was measured using a liquid scintillation counter (LSC-7400; Hitachi Aloka Medical, Tokyo, Japan). Data were analyzed with Equation 1.

$$V_d(t) = K_{in,tissue} \times AUC(t) / C_p(t) + V_i \quad (1)$$

The distribution characteristics of [^{3}H]L-ornithine were assessed by the tissue uptake index method as described elsewhere.[31–33] An amount of 400 μL Ringer-HEPES buffer containing [^{3}H]L-ornithine (5 μCi/rat) and a diffusible internal reference [^{14}C]*n*-butanol (0.5 μCi/rat), was injected into the common carotid artery of anesthetized Wistar rats. The rats were decapitated 15 seconds after injection, and the collected tissues were treated with 2N NaOH and 2N HCl, and the radioactivity (disintegrations per minute; dpm) was measured using a liquid scintillation counter (LSC-7400). Data were analyzed using Equation 2 to calculate the retinal uptake index (RUI) and brain uptake index (BUI).

$$\begin{aligned} &\text{RUI}(\%) \text{ or } \text{BUI}(\%) \\ &= \left([^3\text{H}]/[^{14}\text{C}](\text{dpm in the retina or brain}) \right) / \\ &\quad \left([^3\text{H}]/[^{14}\text{C}](\text{dpm in the injectate solution}) \right) \times 100 \end{aligned} \quad (2)$$

In Vitro Transport Study

Rat-derived cell lines, TR-iBRB2 cells and RPE-J cells,[28,30] were adopted to investigate the transport of L-ornithine at the inner and outer BRB, respectively. Our previous studies support TR-iBRB2 cells as the model of the inner BRB because TR-iBRB2 cells exhibited the closely similar expression profiles of transporters to the primary cultured human endothelial cells.[17,18] Retinal pigment epithelium–J cells were also supported as the model of the outer BRB because RPE-J cells exhibit the unique bipolar localization of caveolae that was also observed for the primary culture of human RPE cells.[34] In addition, the amino acid sequences of transporters relevant to cationic amino acid transport are highly conserved in humans and rats, supporting the benefit of study with these model cells.

The TR-iBRB2 cells were cultured, and the [^{3}H]L-ornithine uptake was investigated as described previously.[28,29,35,36] The cells on BioCoat Collagen I Cellware 24-well dishes (BD Bioscience, Franklin Lakes, NJ, USA) were washed with extracellular fluid (ECF)-buffer three times, and then incubated in 200 μL ECF-buffer containing [^{3}H]L-ornithine (0.1 μCi, 8.33 nM) at 37°C. The RPE-J cells (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (Nissui Pharmaceuticals, Tokyo, Japan) with 4% fetal bovine serum, 20 mM $NaHCO_3$, 25 mM D-glucose, 0.1 mM nonessential amino acids, and antibiotics at 33°C under an atmosphere of 5% CO_2 in air.[30] The RPE-J cells were cultured on Not TC–treated culture dishes (Corning, Corning, NY, USA). Before the transport study, RPE-J cells were cultured on Transwell polyester membrane inserts (surface area 0.33 cm^2 and pore size 0.4 μm; Corning) for 6 days at 33°C, and maintained in culture medium containing 10 nM all-trans retinoic acid for another 2 days at 40°C. The integrity of the cell monolayer was evaluated by the transepithelial electrical resistance (TEER) using a voltohmmeter (Millicell ERS-2; Merck Millipore, Billerica, MA, USA). The directional transport in RPE-J cells was studied at 37°C and pH 7.4. After washing the cells with ECF buffer three times, the assay was initiated by adding ECF buffer with [^{3}H]L-ornithine (16.7 nM) and [^{14}C]D-mannitol (18.2 nM) to the donor side and ECF buffer to the receiver side. In the study of the apical-to-basal (A-to-B) and basal-to-apical (B-to-A) transport, a 20-μL aliquot of the receiver-side buffer was sampled at designated times, and then replaced with an equal volume of fresh buffer. In the study of the apical-to-cell (A-to-C) and basal-to-cell (B-to-C) uptake, the assay was terminated at the designated time, and the cells were lysed with 1N NaOH and neutralized with 1N HCl. The radioactivity was measured using a liquid scintillation counter (LSC-7400), and Equation 3 and Equation 4 were used to calculate the compound permeated and cell-to-medium (cell/medium) ratio, respectively.

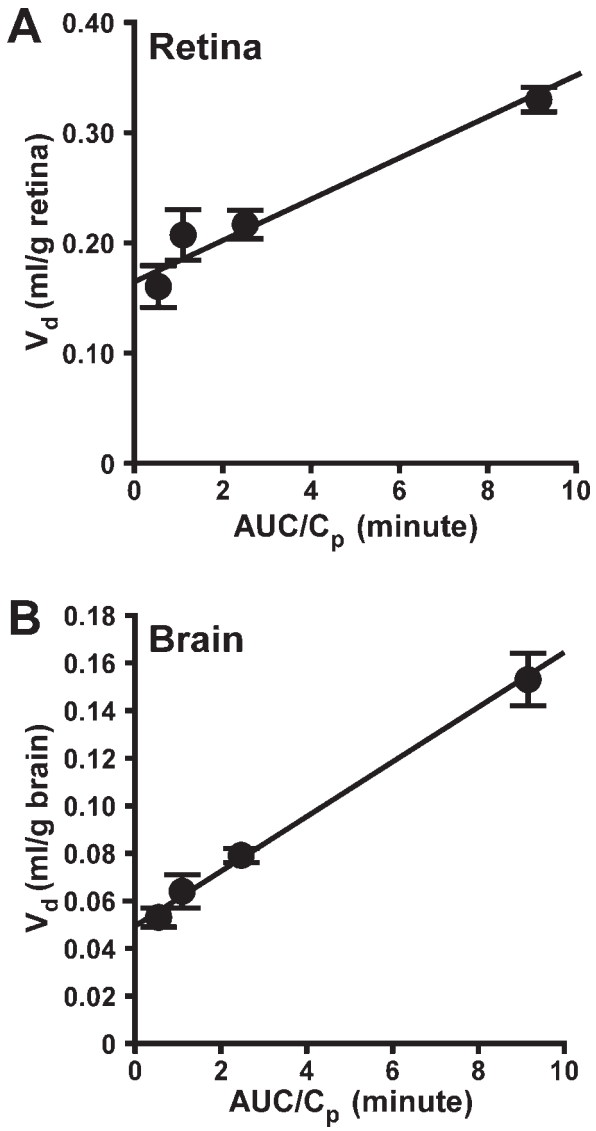

FIGURE 1. The initial uptake of [^{3}H]L-ornithine by the retina (**A**) and brain (**B**) in Wistar rats. In the integration plot analysis, [^{3}H]L-ornithine (4 μCi/rat) was injected into the femoral vein. Each point represents the mean ± SEM ($n = 3$–4).

$$\text{Compound permeated}(\mu\text{L/cm}^2) = \left\{\left([^3\text{H}]\text{ or }[^{14}\text{C}]\text{dpm on the receiver side}\right)/\left([^3\text{H}]\text{or}[^{14}\text{C}]\text{dpm per}\,\mu\text{L medium on the donor side}\right)\right\}/(\text{surface area}(0.33\text{cm}^2)) \quad (3)$$

$$\text{Cell/medium ratio} = \left([^3\text{H}]\text{dpm per cell protein(mg)}\right)/\left([^3\text{H}]\text{dpm per }\mu\text{L medium}\right) \quad (4)$$

The kinetic parameters were estimated using MULTI, a nonlinear least-square regression analysis program, and the data were fitted to a single saturable and single nonsaturable process model (Equation 5),[37] where V, [S], V_{max}, K_m, and K_d are the uptake rate, concentration of L-ornithine in ECF-buffer, maximum uptake rate, Michaelis-Menten constant, and nonsaturable uptake rate, respectively.

$$V = V_{max} \times [S] / (K_m + [S]) + K_d \times [S] \quad (5)$$

Confocal Microscopy

Immunohistochemistry was performed as reported previously (Supplementary Material).[25,38] The frozen sections (thickness 20 μm), prepared from Long-Evans rats, mounted on silanized glass slides were incubated with 10% goat serum (Nichirei, Tokyo, Japan) at room temperature. Guinea pig polyclonal anti-CAT1 antibodies (3.0 μg/mL) and rabbit polyclonal anti-GLUT1 antibodies (1.0 μg/mL) were used as the primary antibodies,[25] and Alexa Fluor 488-conjugated (Life Technologies, Carlsbad, CA, USA), Alexa Fluor 568-conjugated (Life Technologies), or Cy3-conjugated antibodies (Jackson Immuno Research, West Grove, PA, USA) were used as the secondary antibodies. The sections were treated with 4′,6-diamidino-2-phenylindole (DAPI) and VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA, USA), and were examined under a confocal microscope (LSM700; Carl Zeiss, Oberkochen, Germany). Immunocytochemistry was performed as described elsewhere (Supplementary Material), and RPE-J cells cultured on BioCoat Collagen I 8-well culture slides (Corning) were treated with 4% PFA at room temperature.

Western Blot Analysis

Western blot analysis was performed as described elsewhere (Supplementary Material).[25,38] The crude membrane fractions (10 μg protein) of TR-iBRB2 cells or RPE-J cells were analyzed, where the anti-CAT1 polyclonal antibodies (0.2 μg/mL) and the horseradish peroxidase–conjugated antibodies (Merck Millipore) were used as primary and secondary antibodies, respectively.

Protein Determination

In the present study, the protein content was determined using a DC protein assay kit (Bio-Rad, Hercules, CA, USA) and a microplate reader (Model680; Bio-Rad).

Statistical Analysis

The obtained data were statistically analyzed as described elsewhere (Supplementary Material).[31,35,36] The kinetic parameters are represented as the mean ± SD, and others are shown as the mean ± SEM.

RESULTS

In Vitro Transport Analysis

The blood-to-retina transport of L-ornithine across the BRB was investigated (Fig. 1A), and the integration plot analysis showed that the apparent influx permeability clearance ($K_{in,retina}$) of [^{3}H]L-ornithine was 18.7 ± 4.1 μL/(min·g retina), which is approximately 30-fold greater than that of [^{3}H]D-mannitol (0.626 μL/(min·g retina)).[39] Integration plot analysis was also used for the brain (Fig. 1B), and $K_{in,brain}$ of [^{3}H]L-ornithine was 11.5 ± 0.8 μL/(min·g brain), which was 1.64-fold lower than the $K_{in,retina}$ and 8.27-fold greater than that of [^{3}H]D-mannitol

Table 1. In Vivo Uptake of [^{3}H]L-Ornithine by the Retina and Brain in Wistar Rats

Inhibitors	RUI, %	% of Control	BUI, %	% of Control
Control	128.0 ± 7.0	100.0 ± 5.0	13.6 ± 1.5	100.0 ± 11.0
L-Ornithine	57.6 ± 7.3*	45.1 ± 5.7*	4.89 ± 0.91*	36.1 ± 6.7*
L-Arginine	63.5 ± 9.1*	49.7 ± 7.1*	5.36 ± 0.26*	39.5 ± 1.9*
L-Leucine	106.0 ± 9.0	83.0 ± 7.0	11.6 ± 2.1	85.5 ± 15.7

[^{3}H]L-Ornithine (5 μCi/rat) and [^{14}C]*n*-butanol (0.5 μCi/rat) were injected with or without (control) inhibitors (10 mM). Decapitation was performed 15 seconds after injection. Each value represents the mean ± SEM ($n = 3$–6).

*$P < 0.01$, significantly different from the control.

(1.39 μL/(min·g brain)).[16] The RUI of [^{3}H]L-ornithine was estimated as 128%, which was much higher than that of [^{3}H]D-mannitol (11.6%) (Table 1),[39] supporting the blood-to-retina transport of L-ornithine across the BRB. The retinal uptake of [^{3}H]L-ornithine was significantly inhibited by 10 mM L-ornithine and 10 mM L-arginine during no inhibition shown by 10 mM L-leucine (Table 1). The estimated BUI of [^{3}H]L-ornithine was 13.6%, which was higher than that of [^{3}H]D-mannitol (3.11%),[40] and was significantly inhibited by L-ornithine and L-arginine (Table 1). These results suggest the carrier-mediated influx transport of L-ornithine across the BRB.

In Vitro Uptake of [^{3}H]L-Ornithine by TR-iBRB2 Cells

The nature of L-ornithine transport across the BRB was investigated. In TR-iBRB2 cells, an in vitro model cell line of the inner BRB, a time-dependent increase was exhibited in [^{3}H]L-ornithine uptake for at least 5 minutes, and its initial uptake rate was 7.08 ± 0.45 μL/(min·mg protein) (Fig. 2A). In TR-iBRB2 cells, the temperature of 4°C significantly reduced [^{3}H]L-ornithine uptake by 84.7% during no effect shown in the assay with Na^+-free buffer (Fig. 2A). In Figures 2B and 2C, TR-iBRB2 cells exhibited [^{3}H]L-ornithine uptake in a concentration-dependent manner with a K_m of 33.2 ± 6.4 μM, a V_{max} of 0.47 ± 0.073 nmol/(min·mg protein) and a K_d of 2.18 ± 0.28 μL/(min·mg protein), and the contribution ratio of saturable process was estimated as 77% at the physiological concentration of L-ornithine in rat plasma (32 nM).[41] In addition, to clarify the substrate specificity of [^{3}H]L-ornithine uptake in TR-iBRB2 cells, the inhibitory effects of several compounds were examined (Table 2). L-Ornithine, L-arginine, and L-lysine markedly inhibited [^{3}H]L-ornithine uptake by more than 75%, and L-glutamine, L-leucine, and L-phenylalanine moderately inhibited it by more than 52% during no effect shown by L-histidine. The significant but slight effect on [^{3}H]L-ornithine uptake was also shown by L-glutamic acid.

Table 2. Inhibitory Effects on [^{3}H]L-Ornithine Uptake by TR-iBRB2 Cells

Compounds	% of Control TR-iBRB2 Cells
Control	100 ± 5
L-Ornithine	18 ± 1*
L-Arginine	21 ± 1*
L-Lysine	25 ± 1*
L-Histidine	104 ± 1
L-Glutamine	53 ± 3*
L-Leucine	48 ± 3*
L-Phenylalanine	61 ± 7*
L-Glutamic acid	106 ± 6*

[^{3}H]L-Ornithine (0.1 μCi, 8.33 nM) uptake was performed in the absence (control) or presence of 1-mM compounds at 37°C. The uptake by TR-iBRB2 cells was examined for 5 minutes. Each value represents the mean ± SEM ($n = 3$–6).

*$P < 0.01$, significantly different from the control.

Expression of CAT1 Protein at the BRB

To investigate the localization of CAT1 protein at the BRB, immunohistochemistry was performed using GLUT1 as a marker protein, because of its localization at both the luminal and abluminal membranes of retinal capillary endothelial cells and at both the apical and basal membranes of RPE cells,[19] and the fluorescence signal of CAT1 protein was detected in the retinal capillary endothelial cells and RPE cells (Fig. 3A). In the retinal capillary endothelial cells, the fluorescence signal of CAT1 was detected at the luminal and abluminal sides, and was colocalized with GLUT1 (Fig. 3B), suggesting the localization of CAT1 protein at the plasma membrane. In the RPE cells, the fluorescence signal of CAT1 protein was detected at the basal side, and was also colocalized with GLUT1 (Fig. 3C), suggesting the localization of CAT1 protein at the basal side. The expression of CAT1 in RPE-J cells was also confirmed in Western blotting, and the signal of CAT1 proteins was detected at a molecular mass of approximately 90 kDa, as detected in a positive control (TR-iBRB2 cells) (Fig. 4A).[25] Immunocytochemistry revealed the fluorescence of CAT1 at the lower side of RPE-J cells (Fig. 4B), supporting the localization of CAT1 protein at the basal side.

In Vitro Uptake of [^{3}H]L-Ornithine by RPE-J Cells

Directional L-ornithine transport across the outer BRB was investigated using RPE-J cells cultured on Transwell inserts. In Figure 5, there was no significant difference between the [^{3}H]L-ornithine and [^{14}C]D-mannitol in both A-to-B (Fig. 5A) and B-to-A (Fig. 5B) directions, and the permeability estimated for [^{3}H]L-ornithine and [^{14}C]D-mannitol was approximately 4.0 × 10^{-5} to approximately 4.7 × 10^{-5} cm/s, exhibiting 86.7 ± 9.8 Ω·cm^2 as the TEER measured for RPE-J cells. These results suggest the insufficient tight junctions and the deficient restriction of paracellular transport in RPE-J cells since our previous study with Caco-2 cells implied that a permeability of approximately 1.90 × 10^{-6} cm/s for D-mannitol and a TEER of approximately 290 Ω·cm^2 are required to study directional transport on Transwell inserts.[42] Therefore, the directional uptake of [^{3}H]L-ornithine was also investigated by examining the A-to-C and B-to-C directions, and asymmetric bidirectional uptake of [^{3}H]L-ornithine was observed (Fig. 5C). The B-to-C uptake of [^{3}H]L-ornithine was significantly greater than that of A-to-C, and the B-to-C uptake was significantly inhibited by unlabeled L-ornithine (1 mM).

Discussion

L-Ornithine is a cationic amino acid, and its beneficial effects have confirmed its usefulness as a dietary supplement.[1–5] L-

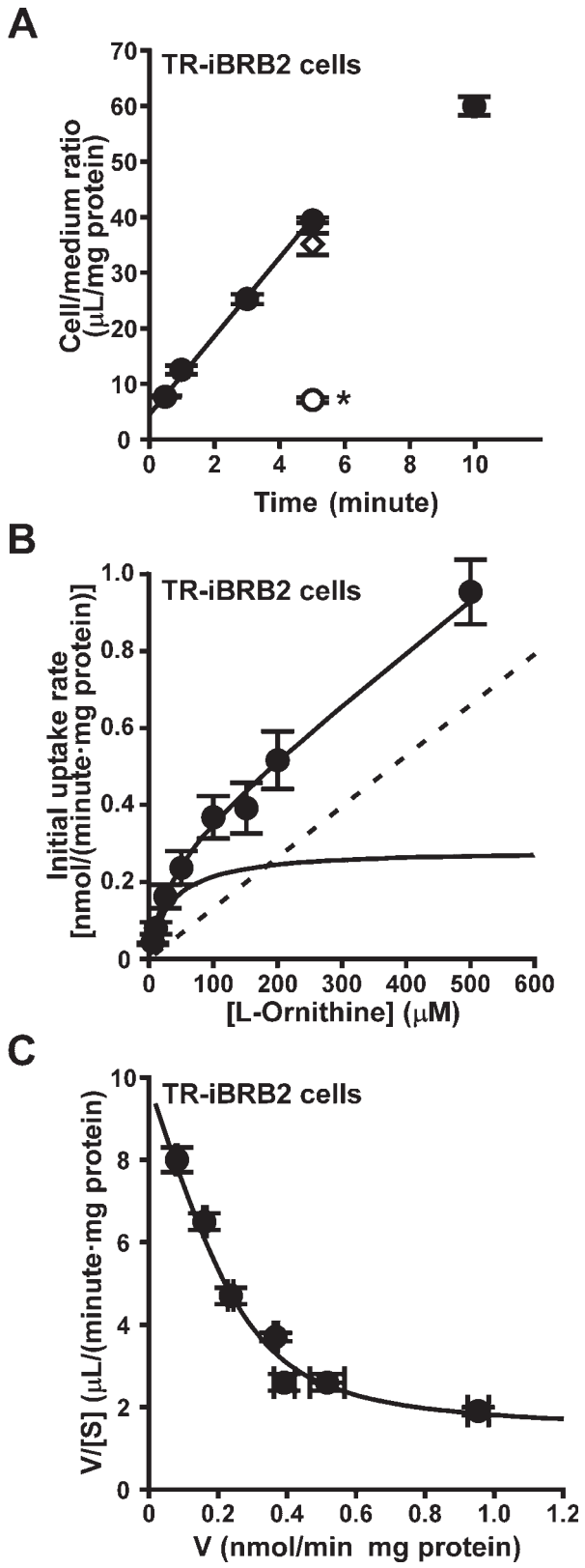

FIGURE 2. Uptake of [^{3}H]L-ornithine by TR-iBRB2 cells. (**A**) Time-course (*closed circles*) and Na^+-dependence (*open diamond*) of [^{3}H]L-ornithine (0.1 μCi, 8.33 nM) uptake was investigated at 37°C, and temperature-dependence was examined at 4°C (*open circle*). Concentration-dependent uptake of L-ornithine was investigated by means of Michaelis-Menten (**B**) and Eadie-Scatchard (**C**) plots of data obtained in the uptake of [^{3}H]L-ornithine (0.1 μCi, 8.33 nM) at 37°C for 5 minutes, over the concentration range 1 to 500 μM. Each *point* represents the mean ± SEM (n = 3–6). *$P < 0.01$, significantly different from the control.

Ornithine is also relevant to an autosomal recessive disease, GA, characterized by hyperornithinemia leading to progressive retinal degeneration,[8–11] suggesting that the regulation of L-ornithine concentration in the retina is essential for maintaining visual function. In this study, the importance of the BRB was proposed in regulating L-ornithine concentration in the retina, and the involvement of cationic amino acid transporters was investigated for L-ornithine transport at the BRB.

In the in vivo transport study, the integration plot showed that the $K_{in,retina}$ of [^{3}H]L-ornithine was much higher than that of the paracellular transport marker (Fig. 1A), suggesting the blood-to-retina transport of L-ornithine at the BRB. The inhibition study showed that the RUI of [^{3}H]L-ornithine was significantly inhibited by L-arginine, not by L-leucine (Table 1), suggesting the possible involvement of system y^+ in L-ornithine transport at the BRB, since system y^+ is a cationic amino acid transport system that is insensitive to neural amino acids.[20–25] Similarly, the in vivo study showed the possible involvement of system y^+ in the blood-to-brain L-ornithine transport at the blood-brain barrier because similar results were shown for [^{3}H]L-ornithine transport in the brain (Fig. 1B; Table 1).

The in vivo study suggested the involvement of a carrier-mediated process in the blood-to-retina transport of L-ornithine at the BRB. The inner BRB is known to nourish two-thirds of the retina,[14,15] and L-ornithine transport at the inner BRB is thought to be responsible for the retinal uptake of L-ornithine. The TR-iBRB2 cells, an in vitro model of the inner BRB, suggested the involvement of a carrier-mediated process in L-ornithine transport at the inner BRB because [^{3}H]L-ornithine was taken up in a time- and temperature-dependent manner (Fig. 2A). Kinetic parameters revealed that L-ornithine uptake by TR-iBRB2 cells is composed of a saturable and a non-saturable process (Figs. 2B, 2C), supporting the major contribution of a saturable process at the physiological condition in rat plasma.[41] In addition, no significant effect of Na^+-free medium on [^{3}H]L-ornithine uptake suggested the involvement of Na^+-independent transport, such as system y^+ and y^+L (Fig. 2A), and this is supported by the expression of CAT1, CAT3, and y^+LAT2 at the inner BRB.[25]

In the in vitro inhibition study, the involvement of system y^+ and y^+L in L-ornithine transport at the inner BRB was supported since [^{3}H]L-ornithine uptake by TR-iBRB2 cells was significantly inhibited by cationic and neutral amino acids, and this was also supported by negligible effect shown by L-glutamic acid, an anionic amino acid (Table 2). Among the responsible transporters for system y^+ and y^+L, the contribution of CAT1 to L-ornithine transport at the inner BRB was suggested because the K_m (33.2 μM) exhibited by TR-iBRB2 cells was comparable with the K_i (114 μM) of L-ornithine for CAT1-mediated L-arginine transport while it was much lower than K_m (910 μM) and K_i (8.3 mM) reported for CAT3 and system y^+L, respectively.[25,43,44] Although our previous reports support TR-iBRB2 cells as an excellent in vitro model of the inner BRB,[28] the insufficient formation of tight junction was supposed in TR-iBRB2 cells, showing the usefulness of protein localization to understand L-ornithine transport at the inner BRB. The immunohistochemistry investigation confirmed the expression of CAT1 at the inner BRB,[25] and the localization study suggested its contribution to the transcellular transport of L-ornithine, including influx and efflux,[45,46] at the luminal and abluminal, as CAT1 was localized at both membranes of the retinal capillary endothelial cells (Fig. 3B).

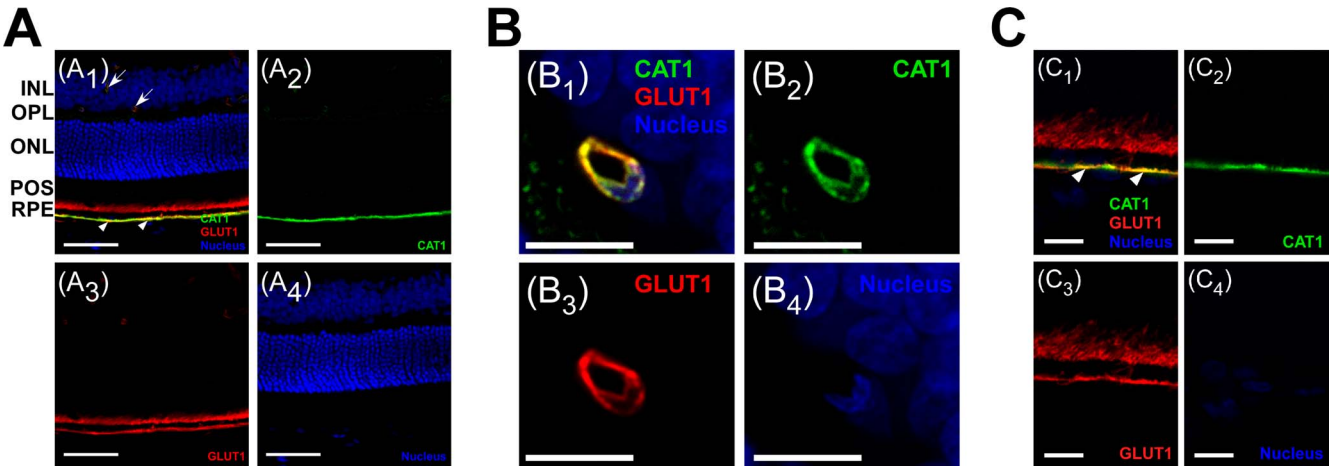

FIGURE 3. Immunohistochemical analysis of CAT1 protein in the retina. The retina of Long-Evans rat was stained with anti-CAT1 antibodies (*green* in A_1, A_2, B_1, B_2, C_1, and C_2) and anti-GLUT1 antibodies (*red* in A_1, A_3, B_1, B_3, C_1, and C_3), and the colocalization of both fluorescences appears in *yellow* (A_1, B_1, and C_1). Nuclei were stained with DAPI (*blue* in A_1, A_4, B_1, B_4, C_1, and C_4). Immunoreactivity to CAT1 protein was observed in the retinal capillary endothelial cells (A_1; *arrow*) and RPE (A_1; *arrowhead*). Under high magnification, colocalization of CAT1 and GLUT1 proteins was observed in the retinal capillary endothelial cells (B_1) and RPE cells (C_1; *arrowhead*). INL, inner nuclear layer; ONL, outer nuclear layer; OPL, outer plexiform layer; POS, photoreceptor outer segments. *Scale bars*: 50 μm (**A**), 10 μm (**B**), and 10 μm (**C**).

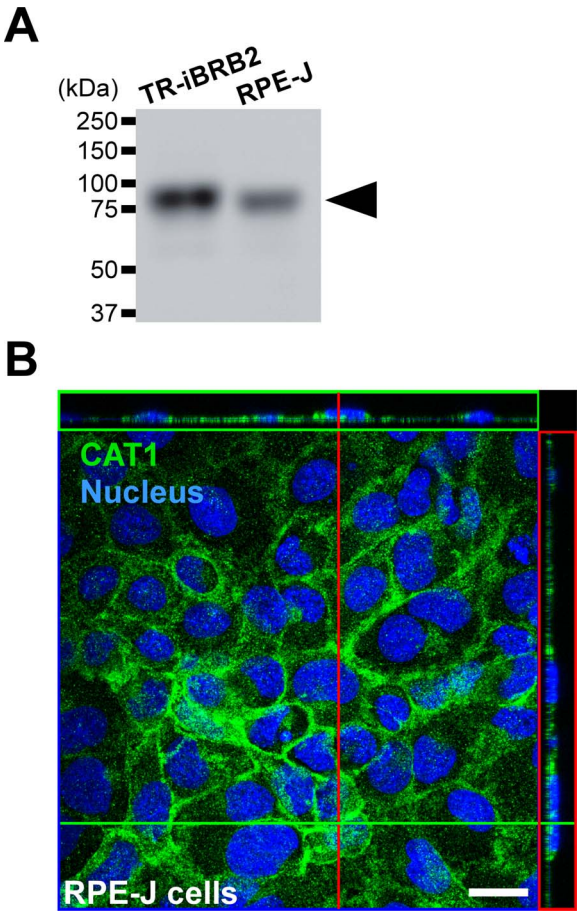

FIGURE 4. Expression of CAT1 protein in RPE-J cells. (**A**) Western blot analysis of CAT1 protein was performed with the crude membrane fraction (10 μg) prepared from RPE-J cells. TR-iBRB2 cells were used as a positive control, and an *arrowhead* indicates CAT1 protein (90 kDa). (**B**) Confocal microscopy of CAT1 protein was performed in RPE-J cells that were stained with anti-CAT1 antibodies (*green*) and DAPI (nuclei, *blue*). The vertical sections of the *xz*-plane and *yz*-plane are indicated by *green* and *red lines*, respectively, suggesting that CAT1 (*green*) is localized to the basal membrane of RPE-J cells. *Scale bars*: 20 μm.

In addition, the moderate sensitivity to neutral amino acids supports the non-negligible contribution of y^+LAT2 because they are not CAT1 substrates (Table 2),[20–25] and its involvement was suggested in nonsaturable L-ornithine transport processes at the inner BRB. It has been reported that the plasma concentration of L-ornithine is increased to approximately 300 μM by an excess intake of L-ornithine (100 mg/kg),[26] implying a change in L-ornithine transport across the BRB. Regarding CAT1 at the inner BRB, its involvement was suggested in L-arginine transport, and the physiological concentrations of L-arginine (140 μM) and L-ornithine in rat plasma (32 μM) suggests no effect of an excess on their CAT1-mediated transport at the inner BRB, as the K_m values shown by TR-iBRB2 cells for L-arginine (11.2 μM) and L-ornithine (33.2 μM) support the saturation of CAT1 under physiological conditions (Fig. 2B).[25,41] However, it is conceivable that excess L-ornithine intake would have a significant influence on the nonsaturable transport involving y^+LAT2 since its contribution ratio was estimated as 60.6% at an L-ornithine concentration of 300 μM.

In GA patients, RPE cells are the primary lesion site,[8,9] and L-ornithine accumulation is thought to be relevant to the degeneration of RPE cells, showing that the clarification of L-ornithine transport in RPE cells would improve our understanding of GA pathology. Although a previous study has suggested the involvement of CAT1 in L-ornithine transport in RPE cells,[27] its detailed mechanism remains unclear because RPE cells are polar. The study with RPE-J cells, an in vitro model cell line of the outer BRB,[30] suggested the involvement of CAT1 in the blood-to-cell transport of L-ornithine at the basal side of the RPE cells because of the greater B-to-C uptake of [^{3}H]L-ornithine than that of A-to-C (Fig. 5C). This difference in [^{3}H]L-ornithine uptake is supported by the results obtained in the immunohistochemistry that suggests the basal localization of CAT1 proteins in RPE cells (Figs. 3, 4), and this may be a potential source of lesion in RPE cells in GA. The different localization patterns of CAT1 proteins implies "no outlet," indicating the accumulation of L-ornithine in RPE cells and transcellular transport, indicating no accumulation of L-ornithine in the retinal capillary endothelial cells, although it remains unclear how other transporters expressed in RPE cells, such as y^+LAT1, y^+LAT2, and $b^{0,+}$AT, contribute to L-ornithine transport.[27] Furthermore, in clinical studies, L-ornithine

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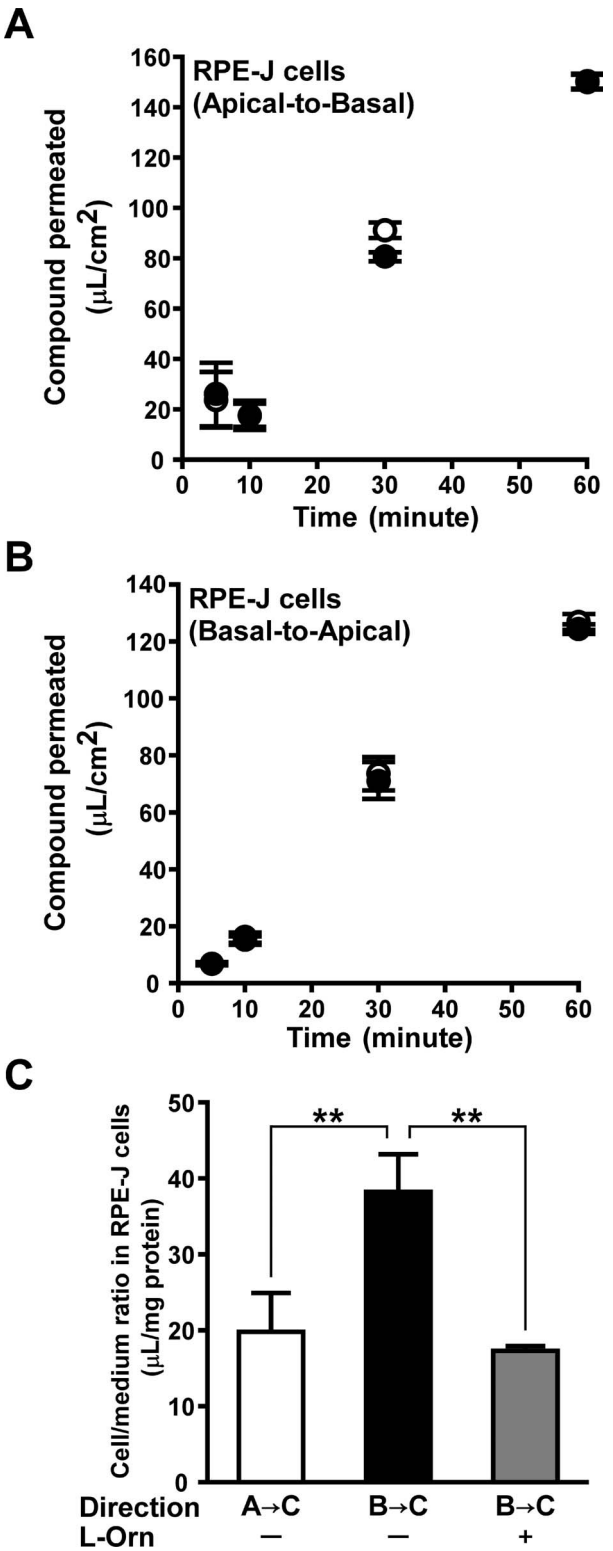

FIGURE 5. Uptake of [^{3}H]L-ornithine by RPE-J cells cultured on Transwell inserts. The transport in the apical-to-basal ([^{3}H]L-ornithine, 0.1 μCi, 16.7 nM, *open circles*; [^{3}H]D-mannitol, 0.1 μCi, 18.2 nM, *closed circles*) (**A**), and the basal-to-apical ([^{3}H]L-ornithine, 0.6 μCi, 16.7 nM, *open circles*; [^{3}H]D-mannitol, 0.6 μCi, 18.2 nM, *closed circles*) (**B**) directions were examined. (**C**) The [^{3}H]L-ornithine uptake in apical-to-cell (A-to-C, 0.1 μCi, 16.7 nM) and basal-to-cell (B-to-C, 0.6 μCi, 16.7 nM) directions was examined with or without 1 mM unlabeled L-ornithine at 37°C for 60 minutes. Each *point* and *column* represents the mean ± SEM ($n = 3$–4). $^{**}P < 0.05$, significantly different from the uptake in the direction B-to-C.

ingestion caused higher plasma concentrations of L-ornithine in OAT$^{+/-}$ than in OAT$^{+/+}$, and the in vitro study revealed the concurrent addition of 5-FMO and L-ornithine upregulates the expression of CAT1 mRNA.[26,47,48] These findings suggest that L-ornithine accumulation increases the expression of CAT1 at the basal side, showing a risk that L-ornithine ingestion could cause the degeneration of RPE cells in humans with impairment of L-ornithine elimination, such as OAT$^{+/-}$.

In this study, the involvement of a transport system was suggested in the blood-to-retina transport of L-ornithine, and CAT1 was suggested to contribute to L-ornithine transport at the luminal and abluminal sides at the inner BRB. At the outer BRB, CAT1, localized at the basal side, was suggested to contribute to the blood-to-cell transport of L-ornithine in RPE cells. This study also suggested non-negligible involvement of a transporter, such as y$^+$LAT2, at the BRB in the putative risk following the excess L-ornithine intake. These findings will help us better understand L-ornithine regulation in the retina.

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