Involvement of Cationic Amino Acid Transporter 1 in L-Arginine Transport in Rat Retinal Pericytes

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Nitric oxide (NO), a known relaxant, is produced in cells from L-arginine (L-Arg). Because the relaxation of retinal pericytes alters the microcirculatory hemodynamics, it is important to understand the manner of NO production in retinal pericytes. The purpose of this study was to clarify the molecular mechanism(s) of uptake of L-Arg in retinal pericytes using a conditionally immortalized rat retinal pericyte cell line (TR-rPCT1 cells) which expresses the mRNAs of endothelial NO synthase and inducible NO synthase. L-Arg uptake by TR-rPCT1 cells exhibited Na⁺-independence and concentration-dependence with a K_m of 28.9 µM. This process was strongly inhibited by substrates of cationic amino acid transporters (CAT), such as L-ornithine and L-lysine. In contrast, L-valine, L-leucine, and L-glutamine, which are substrates of cation/neutral amino acid transport systems, such as system y⁺L, system B₀⁺, and system b₃⁺, did not strongly inhibit L-Arg uptake by TR-rPCT1 cells. In addition, the expression of mRNA and protein of CAT1 in TR-rPCT1 cells was observed by reverse transcription-polymerase chain reaction and immunoblot analyses. Taking these results into consideration, it appears that CAT1 is involved in L-Arg uptake by retinal pericytes and this is expected to play an important role in the relaxation of retinal pericytes, thereby modulating the microcirculatory hemodynamics in the retina.

Key words L-arginine; cationic amino acid transporter (CAT); CAT1; retinal pericyte; TR-rPCT1

Retinal pericytes communicate with retinal capillary endothelial cells, which form the inner blood–retinal barrier (BRB) and regulate the exchange of drugs and endogenous compounds between the circulating blood and retina, by direct contact.1,2 Because the ratio of capillary endothelial cells to retinal pericytes in the retinal capillaries is reported to be high (1 : 1),3,4 it is suggested that retinal pericytes play an important role in microcirculatory hemodynamics in the retina. It has been reported that pericytes contract and relax due to some physiological factors, thereby modulating blood flow in retinal capillaries.5–7 Since changes in the blood flow rate in the retinal capillaries affect the transport of various compounds across the inner BRB, it is important to investigate the mechanisms of contraction and relaxation of pericytes for controlling the transport of compounds across the inner BRB.

Nitric oxide (NO) is one of a number of endogenous dilators. Haefliger et al. have reported that the relaxation of cultured bovine retinal pericytes is induced by agents able to produce nitric oxide, such as sodium nitroprusside, through the production of cyclic guanosine monophosphate.8 In general, vasodilation of the capillaries leads to an increase in blood flow, thereby facilitating transport from the circulating blood to the retina of drugs/compounds with a movement that is rate-limited by blood flow.9 Because the relaxation of retinal pericytes causes the vasodilation of retinal capillaries, it is considered that NO is a key molecule involved in regulating the permeation of compounds across the inner BRB. L-Arginine (L-Arg) is known as a precursor for NO synthesis via endothelial NO synthase (NOS). This NO synthesis is dependent on the availability of extracellular L-Arg.10 Hence, membrane transport of L-Arg from extracellular fluid to pericytes represents a rate-limiting step in establishing the local NO concentration. However, little is known about the properties of L-Arg transport in retinal pericytes.

It has been reported that cationic amino acids, including L-Arg, are transported through the plasma membrane by distinct transport systems: system y⁺, system y⁻L, system B₀⁺, and system b₃⁺.11,12 System y⁺ mediates the extracellular Na⁺-independent transport of cationic amino acids and is encoded by CAT1/Slc7a1, CAT2A/Slc7a2a, CAT2B/Slc7a2b, and CAT3/Slc7a3.13,14 System y⁻L, which is encoded by y⁻LAT1/Slc7a7 and y⁻LAT2/Slc7a6, mediates the Na⁺-dependent transport of neutral amino acids as well as the Na⁺-independent transport of cationic amino acids.13 System B₀⁺ (ATB₀⁺/Slc6a14) and system b₃⁺ (b₃⁺AT/Slc7a9) transport both cationic and neutral amino acids, and represent an Na⁺-dependent and an Na⁺-independent transport system, respectively.12 Our previous studies have revealed that L-Arg is transported from the circulating blood to the retina15 and is taken up into Müller cells, which are glial cells in the retina,16 via cationic amino acid transporters (CAT). Therefore, it is hypothesized that these transport systems take part in the transport of L-Arg in retinal pericytes.

The purpose of this study was to clarify the molecular mechanism(s) which are responsible for the transport of L-Arg in pericytes. To examine the L-Arg transport and expression of L-Arg transporters in pericytes, we used TR-rPCT1 cells, which are a conditionally immortalized rat retinal pericyte cell line and an appropriate model having the characteristics of retinal pericytes in vivo.17,18 Using TR-rPCT1 cells, L-Arg uptake and the expression of NO synthase and L-Arg transporters were investigated. The aim of this study was to clarify the roles of cationic amino acid transporters (CAT) in the transport of L-Arg in retinal pericytes. To disclose the molecular mechanism(s) of uptake of L-Arg in retinal pericytes, the following processes were performed: (1) NO production was measured in TR-rPCT1 cells treated with L-Arg. (2) Uptake of L-Arg was measured in TR-rPCT1 cells expressing various CATs. (3) Expression of mRNA and protein of CATs was measured in TR-rPCT1 cells.
porters in the cells were assessed in this study.

MATERIALS AND METHODS

Animals Adult male Wistar rats (150–250 g) were purchased from Japan SLC (Hamamatsu, Japan). They were maintained in a controlled environment and all experiments were approved by the Animal Care Committee, University of Toyama.

Reagents Arginine monohydrochloride, L-[2,3-3H]-[^3H]Arg, 50.6 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade and available commercially.

Cell Culture TR-rPCT1 cells and TR-iBRB2 cells, a conditionally immortalized rat retinal capillary endothelial cell line,15,19 were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Nissui Pharmaceuticals, Tokyo, Japan) supplemented with 20 mM sodium bicarbonate, 100 µM benzylpenicillin potassium, 100 µg/mL streptomycin and 10% fetal bovine serum (Moregate, Bulimba, Australia). The cells were maintained at 33°C, which is the permissive temperature at which temperature-sensitive simian virus 40 large T-antigen is retained, and cells were main-

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Reverse Transcription-Polymerase Chain Reaction (RT-PCR) RT-PCR analysis was performed as described previously with minor modifications.17 Total RNA was prepared from TR-rPCT1 cells and rat tissues using a RNeasy Mini kit (Qiagen, Hilden, Germany) and TRizol reagent (Life Technologies, Carlsbad, CA, U.S.A.), respectively, according to the manufacturer’s protocol. Single-stranded complementary DNA (cDNA) was prepared from 1 µg total RNA using ReverTra Ace (Toyobo, Osaka, Japan) and oligo dT primer. The reactions were performed using ExTaq DNA polymerase (TaKaRa Shuzo, Kyoto, Japan) with the following thermal cycle program: 1 cycle of 94°C for 2 min, and 35 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min. The RT-PCR of each sample RNA in the absence of reverse transcriptase was used as a negative control. The RT-PCR products were separated by electrophoresis on an agarose gel in the presence of ethidium bromide (0.6 µg/mL) and visualized using ultraviolet light.

1-Arg Uptake Study Using TR-rPCT1 Cells The uptake studies were performed at 37°C, which is the normal physiological temperature. TR-rPCT1 cells were seeded at a density of 0.5×10⁴ cells/cm² on rat tail collagen type I-coated 24-well plates (BD Biosciences, Bedford, MA, U.S.A.) and were cultured at 33°C for 48 h. The cells were then washed three times with 1 mL extracellular fluid (ECF) buffer (pH 7.4) consisting of 122 mM NaCl, 25 mM NaHCO3, 3 mM KCl, 1.4 mM CaCl2, 1.2 mM MgSO4, 0.4 mM KH2PO4, 10 mM d-glucose and 10 mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) at 37°C. Uptake was initiated by applying 200 µL ECF buffer containing 0.1 µCi [3H]Arg (9.9 nM) at 37°C in the presence or absence of inhibitors. Na+-free ECF buffer was prepared by replacement with equimolar lithium. After a predetermined period, uptake was terminated by removing the solution, and cells were rinsed three times with ice-cold ECF buffer. Then, the cells were solubilized in 1 mL NaOH and subsequently neutralized with 1 M HCl. An aliquot was taken for measurement of the radioactivity and protein content using a liquid scintillation counter (LSC-5200; ALOKA, Tokyo, Japan) and a detergent-compatible protein assay kit (Bio-rad, Hercules, CA, U.S.A.) with bovine serum albumin as a standard, respectively. The uptake of [3H]Arg was expressed as the cell/medium ratio (µL/mg protein, Eq. 1).

Cell / medium ratio (µL/mg protein) = [3H]Arg concentration in the cells (dpm/mg protein) [3H]Arg concentration in the uptake buffer (dpm/µL)

(1)

In the analysis of the concentration-dependent uptake, the uptake data of L-Arg were fitted, using the non-linear least-square regression analysis program (MULTI),20 to a one saturable and one non-saturable process model as follows:

where Vmax is the maximum uptake rate of L-Arg, [S] is the concentration of L-Arg, Km is the corresponding Michaelis–Menten constant, and Kd is the non-saturable uptake rate constant.

Immunoblot Analysis The crude membrane fraction from TR-rPCT1 cells and TR-iBRB2 cells was prepared as described previously with minor modifications.1,15 TR-rPCT1 cells and TR-iBRB2 cells were homogenized by the

<table>
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<th>Table 1. Primer Sets for RT-PCR Analysis</th>
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<tr>
<td><strong>Target</strong></td>
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<tr>
<td>eNOS</td>
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<tr>
<td></td>
</tr>
<tr>
<td>nNOS</td>
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<td></td>
</tr>
<tr>
<td>CAT1</td>
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<td></td>
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<tr>
<td>CAT2A</td>
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<td>CAT2B</td>
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<td>CAT3</td>
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nitrogen cavitation technique (800 psi, 30 min, 4°C) in Tris-sucrose buffer (10 mM Tris–HCl, 250 mM sucrose, and 1 mM ethyleneglycol bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA); pH 7.4) containing a protease inhibitor cocktail (Sigma). The homogenized samples were centrifuged for 15 min at 10000 × g and 4°C and the supernatants were centrifuged for 60 min at 100000 × g and 4°C, and a crude membrane fraction was obtained from the pellets. The protein concentration was determined using a detergent-compatible protein assay kit (Bio-Rad) with bovine serum albumin as a standard. The protein (30 µg) was electrophoresed on a 10% Tris/glycine sodium dodecyl sulfate-polyacrylamide gel, and subsequently electrotransferred to a polyvinylidene difluoride membrane (Hybond-P; GE Healthcare, Buckinghamshire, U.K.). The membrane was then treated with blocking solution (20 mM Tris–HCl, 137 mM NaCl, 0.1% Tween-20, and 5% non-fat dry milk, pH 7.4) for 9 h at 4°C and incubated with guinea pig polyclonal anti-CAT1 antibody (0.2 µg/mL) for 12 h at 4°C. The membrane was rinsed with washing solution (20 mM Tris–HCl, 137 mM NaCl, 0.1% Tween-20, and 0.1% non-fat dry milk, pH 7.4), and incubated with horseradish peroxidase-conjugated anti-guinea pig immunoglobulin G (IgG). The bands were visualized using an enhanced chemiluminescence kit (ECL plus; GE Healthcare).

RESULTS

mRNA Expression of Nitric Oxide Synthase (NOS)
The expression of three kinds of NOS, i.e., endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS), in TR-rPCT1 cells was examined by RT-PCR analysis. As shown in Fig. 1, RT-PCR gave amplified products at the expected sizes of 243 bp and 179 bp for eNOS and iNOS mRNAs, respectively, in TR-rPCT1 cells as well as in respective positive controls (eNOS, rat brain; iNOS, rat liver). nNOS mRNA was detected in rat brain as a positive control, but not in TR-rPCT1 cells. These results indicate mRNA expression of eNOS and iNOS in TR-rPCT1 cells.

Characteristics of L-Arg Uptake by TR-rPCT1 Cells
To characterize the L-Arg transport in rat retinal pericytes, [3H]-L-Arg uptake by TR-rPCT1 cells was determined. The [3H]-L-Arg uptake exhibited a time-dependent increase for up to 20 s (Fig. 2A). In addition, there is no significant difference between the cell/medium ratio of [3H]-L-Arg at 20 s and 30 s (p = 0.212), suggesting that L-Arg uptake reached the steady-state after 20 s. The absence of Na+ did not significantly affect [3H]-L-Arg uptake by TR-rPCT1 cells for 10 s (Fig. 2B), indicating that [3H]-L-Arg uptake by TR-rPCT1 cells is predominantly medi-
ated by Na\(^+\)-independent process(es).

Figure 3 shows the kinetic analysis of L-Arg uptake by TR-rPCT1 cells. \(^{[3]H}\)-L-Arg uptake (0.5 \(\mu\)Ci/mL) was examined at 37°C for 10s, over the L-Arg concentration range of 10–3000 \(\mu\)M. Data were subjected to Eadie–Scatchard and Michaelis–Menten (inset) analyses. Each point represents the mean±S.E.M. (n=3).

Table 2. Effect of Various Inhibitors on \(^{[3]H}\)-L-Arg Uptake by TR-rPCT1 Cells

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Percentage of control</th>
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<tr>
<td>Control</td>
<td>100±1</td>
</tr>
<tr>
<td>L-Arg</td>
<td>1.69±0.06*</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>13.1±0.7*</td>
</tr>
<tr>
<td>L-NNA</td>
<td>28.7±1.1*</td>
</tr>
<tr>
<td>L-Ornithine</td>
<td>45.5±2.1*</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>48.0±0.8*</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>69.4±3.1*</td>
</tr>
<tr>
<td>L-Valine</td>
<td>80.2±9.4*</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>97.4±4.3</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>106±3</td>
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</tbody>
</table>

Uptake of \(^{[3]H}\)-L-Arg (0.5 \(\mu\)Ci/mL, 9.9 nM) by TR-rPCT1 cells was examined at 37°C for 10s in the absence (control) or presence of inhibitors at a concentration of 1 mM. Each value represents the mean±S.E.M. (n=3–6). *p<0.01, significantly different from the control. L-NMMA, N\(^\omega\)-monomethyl-L-arginine; L-NNA, N\(^\omega\)-nitro-L-arginine.

Expression of CATs in TR-rPCT1 Cells mRNA expression of CAT1, CAT2A, CAT2B, and CAT3 in TR-rPCT1 cells was examined by RT-PCR analysis (Fig. 4A). Bands of CAT1, CAT2B, and CAT3 were detected from TR-rPCT1 cells and rat brain, which was used as a positive control. CAT2A mRNA was detected in rat liver as a positive control, but not in TR-rPCT1 cells.

We also examined the protein expression of CAT1 in TR-rPCT1 cells (Fig. 4B). Using immunoblot analysis with the crude membrane fraction prepared from TR-rPCT1 cells and TR-iBRB2 cells, a positive control for the expression of CAT1,\(^{15}\) the CAT1 antibody recognized a single protein band at ca. 90 kDa.

DISCUSSION

In the present study, we evaluated the L-Arg uptake by TR-rPCT1 cells, conditionally immortalized rat retinal pericytes. TR-rPCT1 cells expressed mRNA of eNOS and iNOS, but not nNOS (Fig. 1), implying that NO is synthesized via eNOS and iNOS in TR-rPCT1 cells from L-Arg. The uptake of L-Arg by TR-rPCT1 increased up to 20 s and reached a steady-state of ca. 60 \(\mu\)L/mg protein after 20 s (Fig. 2A). In addition, this L-Arg uptake occurred in saturable and non-saturable manners (Fig. 3). The uptake clearance of the saturable process
(\(V_{\text{max}}/K_{\text{m}}\)) of \(l\)-Arg uptake by TR-rPCT1 cells was found to be 53.3 pmol/(min·mg protein). This value is 14-fold greater than that of the non-saturable process \(K_{\text{m}}\) of \(l\)-Arg uptake, suggesting that carrier-mediated transport system(s) of \(l\)-Arg play a major role in \(l\)-Arg uptake by TR-rPCT1 cells. In human, the concentration of \(l\)-Arg in the vitreous humor, which is corresponding to the concentration of retinal extracellular fluid, is reported to be 39.4 µM.\(^{20}\) Based on the concentration, the uptake rate of the saturable process \(V_{\text{max}}/([S]/(K_{\text{m}}+[S]))\) of \(l\)-Arg uptake was calculated to be 888 pmol/(min·mg protein), which is 6-fold greater than that of the non-saturable process \(K_{\text{m}}\). Therefore, it is suggested that carrier-mediated transport process(es) of \(l\)-Arg contribute to the uptake of retinal pericytes.

The uptake of \([3H]\)Arg by TR-rPCT1 cells for 10s was not altered in the absence of extracellular Na\(^{+}\) (Fig. 2B). This result suggests that Na\(^{+}\)-independent \(l\)-Arg transport systems, such as system \(y^*\), system \(y\)'L and system \(b^0,\text{L}\), are involved in the uptake of \(l\)-Arg in TR-rPCT1 cells. The \(K_{\text{m}}\) value for a saturable process of \(l\)-Arg uptake by TR-rPCT1 cells (28.9 µM) is similar to that obtained for \(l\)-Arg uptake via system \(y^*\) of rat and mouse primary-cultured glial cells and rat glioma cells (approximately 25 µM)\(^{21}\) and that obtained for \(l\)-Arg uptake by human system \(b^0,\text{L}\)-expressing \(Xenopus\) laevis oocytes (85 µM).\(^{22}\) Forray et al. have reported that \(l\)-Arg competitively inhibited \(l\)-lysine uptake by erythrocytes via system \(y^*\) with a \(K_{\text{m}}\) value of 3.2 µM.\(^{23}\) This \(K_{\text{m}}\) value is 9.0-fold lower than the \(K_{\text{m}}\) value of \(l\)-Arg uptake by TR-rPCT1 cell, ruling out the idea that system \(y^*\) is mainly involved in this \(l\)-Arg uptake process. In the inhibition study (Table 2), \([3H]\)Arg uptake by TR-rPCT1 cells was strongly inhibited by unlabeled \(l\)-Arg, \(l\)-ornithine, and \(l\)-lysine, which are substrates of system \(y^*\). \(l\)-Histidine is reported to be also a substrate/inhibitor of system \(y^*\) and system \(y\)'L\(^{12,25}\) and moderately inhibited \(l\)-Arg uptake by TR-rPCT1 cells (Table 2). On the other hand, the \(l\)-Arg uptake was not markedly altered in the presence of \(l\)-valine, \(l\)-leucine, and \(l\)-glutamine, which are substrate of neutral amino acid transport systems, such as system \(y^*\)L, system \(b^0,\text{L}\) and system \(b^0,\text{L}\)\(^{12,26}\).

Taking these points into consideration, it appears that system \(y^*\) is involved in the carrier-mediated uptake of \(l\)-Arg by TR-rPCT1 cells.

System \(y^*\) is reported to be encoded by CAT1, CAT2A, CAT2B, and CAT3.\(^{23}\) Of these transporters, mRNA expression of CAT1, CAT2B, and CAT3 in TR-rPCT1 cells was confirmed by RT-PCR analysis (Fig. 4A). It has been reported that the \(K_{\text{m}}\) values of \(l\)-Arg uptake via mouse CAT1, mouse CAT2B, and human CAT3 are 70 µM, 250–380 µM, and 360 µM, respectively.\(^{15,27,28}\) Among these values, the \(K_{\text{m}}\) value of \(l\)-Arg uptake by TR-rPCT1 cells (28.9 µM, Fig. 3) is similar to that of mouse CAT1. In addition, this \(K_{\text{m}}\) value of \(l\)-Arg uptake by TR-rPCT1 cells is also similar to the \(K_{\text{m}}\) values of \(l\)-Arg uptake by conditionally immortalized rat retinal capillary endothelial cells (\(K_{\text{m}}=11\) µM) and rat Müller cells (\(K_{\text{m}}=15\) µM), both of which involve CAT1 at least in part.\(^{1,15}\) Moreover, the expression of CAT1 protein in TR-rPCT1 cells was indicated from the immunoblot analysis (Fig. 4B). Consequently, it is suggested that CAT1 is a major contributor to \(l\)-Arg uptake by retinal pericytes.

Endogenous NO inhibitors, such as \(l\)-NMMA and \(l\)-NNA, strongly inhibited \(l\)-Arg uptake by TR-rPCT1 cells (Table 2). This is in good agreement with previous reports that the uptake of \(l\)-NMMA is mediated by system \(y^*\) in neuroblastoma and glioma hybrid cell lines.\(^{29}\) This result raises the interesting possibility that \(l\)-NMMA and \(l\)-NNA are able to inhibit the supply of an NO substrate, \(l\)-Arg, as well as NO synthesis via eNOS and iNOS in retinal pericytes.

In conclusion, our studies suggest that CAT1 is expressed in rat retinal pericytes and is involved in the transport of \(l\)-Arg, a precursor of NO, from the extracellular environment. Because the dilution of the retinal pericytes which is induced by the NO causes an increased blood flow in retinal capillaries, our findings could lead to the understanding of the physiological and pharmacological roles of retinal pericytes.

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**Conflict of Interest** The authors declare no conflict of interest.

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