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Developmental changes of L-arginine transport at the blood-brain barrier in rats

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ABSTRACT

L-Arginine is required for regulating synapse formation/patterning and angiogenesis in the developing brain. We hypothesized that this requirement would be met by increased transporter-mediated supply across the blood-brain barrier (BBB). Thus, the purpose of this work was to test the idea that elevation of blood-to-brain L-arginine transport across the BBB in the postnatal period coincides with up-regulation of cationic acid transporter 1 (CAT1) expression in developing brain capillaries. We found that the apparent brain-to-plasma concentration ratio (Kp, app) of L-arginine after intravenous administration during the first and second postnatal weeks was 2-fold greater than that at the adult stage. Kp, app of L-serine was also increased at the first postnatal week. In contrast, Kp, app of D-mannitol, a passively BBB-permeable molecule, did not change, indicating that increased transport of L-arginine and L-serine is not due to BBB immaturity. Double immunohistochemical staining of CAT1 and a marker protein, glucose transporter 1, revealed that CAT1 was localized on both luminal and abluminal membranes of brain capillary endothelial cells during the developmental and adult stages. A dramatic increase in CAT1 expression in the brain was seen at postnatal day 7 (P7) and day 14 (P14) and the expression subsequently decreased as the brain matured. In accordance with this, intense immunostaining of CAT1 was observed in brain capillaries at P7 and P14. These findings strongly support our hypothesis and suggest that the supply of blood-born L-arginine to the brain via CAT1 at the BBB plays a key role in meeting the elevated demand for L-arginine in postnatal brain.

1. Introduction

L-Arginine is an indispensable precursor of nitric oxide (NO), which plays an essential role in regulating synapse formation and patterning (Gibbs, 2003), as well as angiogenesis (Ziche and Morbidelli, 2000). In addition, L-arginine is a building block for proteins and a biosynthetic precursor of creatine, which is involved in ATP homeostasis in the brain (Wyss and Kaddurah-Daouk, 2000). Therefore, there should be a high demand for L-arginine in the developing brain, where neural cells and blood vessels proliferate and differentiate by extending neural and vascular processes.

Since the enzymatic activities involved in de novo synthesis of L-arginine are insufficient in the brain of newborn infant (Glick et al., 1976), blood-to-brain transport across the blood-brain barrier (BBB), which is formed by complex tight junctions of brain capillary endothelial cells (Ohtsuki and Terasaki, 2007), would be required meet the increased demand for L-arginine in the postnatal brain. Indeed, it has been reported that the brain uptake of L-arginine from blood is 6 times greater in newborn rabbits and rats (6–30 h after birth) than in adults (Braun et al., 1980; Sershen and Lajtha, 1976), although there is no difference in the brain uptake of D-glucose between newborns and adults (Braun et al., 1980). Considering that the cerebral blood vessels in immature brain are impermeable to even small molecules (Ek et al., 2006), it seems likely that L-arginine transport function at the BBB is selectively upregulated in the developing brain. In this connection, transient elevation of L-serine uptake by the brain has already been observed in 5-day-old rats (Lefauconnier and Trouve, 1983), and Sakai et al. (2003) reported prominent expression of alanine-serine-cysteine transporter 1 (ASCT1/SLC1A4) in brain capillary endothelial cells of embryonic and first postnatal mouse brains (Sakai et al., 2003). Thus,

Abbreviations: ASCT1, alanine-serine-cysteine transporter 1; BBB, blood-brain barrier; CAT1, cationic amino acid transporter; eNOS, endothelial nitric oxide synthase; GLUT1, glucose transporter 1; HPLC, high performance liquid chromatography; NO, nitric oxide; P, postnatal; SLC, solute carrier

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increased expression of ASCT1 in brain capillaries appears to be responsible for the enhanced L-serine uptake by the developing brain.

On the other hand, blood-to-brain transport of L-arginine across the adult BBB is mediated by cationic amino acid transporter 1 (CAT1/*SLC7A1*) (Stoll et al., 1993). Since CAT1 forms a caveolar complex with endothelial nitric oxide synthase (eNOS) (McDonald et al., 1997), it is conceivable that CAT1 is functionally related to NO production. Considering that the Km value of mouse CAT1-mediated L-arginine transport is 70 μM (Stoll et al., 1993), it seems likely that the CAT1-mediated supply of L-arginine to adult brain would be mostly saturated at blood L-arginine concentrations of 100–170 μM (Aoyagi, 2003; Kamada et al., 2001; Tornquist and Alm, 1986). These lines of evidence prompted us to hypothesize that the elevated requirement for L-arginine in the developing brain would be met by increased supply across the blood-brain barrier (BBB), mediated by an increase of CAT1 protein expression in brain capillaries.

Therefore, the purpose of this work was to test this hypothesis by investigating the developmental changes of blood-to-brain L-arginine transport across the BBB and of CAT1 expression in brain capillaries by means of in vivo permeation analysis, immunohistochemistry and immunoblotting studies.

2. Materials and methods

2.1. Animals

Male Wistar rats at developmental and adult stages were purchased from Japan SLC (Hamamatsu, Japan) and maintained in a controlled environment. All experiments were approved by the Animal Care Committee, University of Toyama (A2011PHA-13 and 14) and Tohoku University (2012PhA-011).

2.2. Reagents

L-[2,3-^{3}H]Arginine ([^{3}H]L-arginine, 50.6 Ci/mmol) and D-[1-^{14}C] mannitol ([^{14}C]D-mannitol, 55 mCi/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO, USA). L-[^{3}H(G)] Serine ([^{3}H]L-serine, 29.5 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA, USA). All other chemicals were commercial products of analytical grade.

2.3. Blood-to-brain [^{3}H]L-arginine transport analysis

The rats were anesthetized with an intraperitoneal injection of pentobarbital (35 mg/kg) and then [^{3}H]L-arginine, [^{3}H]L-serine, or [^{14}C]D-mannitol [1.0 μCi/head (postnatal day 7 (P7) and P14), 2.5 μCi/head (P21), 3.0 μCi/head (P42)] was injected via the jugular vein. After collection of blood samples, rats were decapitated, and the brains were removed. All samples were dissolved in 2 N NaOH and subsequently neutralized. Radioactivity was measured in a liquid scintillation counter (LS6500; Beckman-Coulter, Fullerton, CA). The apparent tissue-to-plasma concentration ratio (Kp, app) was used as an index of the tissue distribution characteristics of test compounds. This ratio (Kp, app, mL/g brain) was defined as the amount of ^{3}H or ^{14}C per gram of brain divided by that per milliliter of plasma, calculated over the time period of the experiment. To determine the designated time points, the in vivo initial uptake of [^{3}H]L-arginine across the BBB was estimated by integration plot analysis after intravenous administration of [^{3}H]L-arginine to adult male rats (P56). Integration plot analysis and calculations were performed as reported previously (Kasai et al., 2011). Supplemental Fig. 1 shows that the Kp, app for [^{3}H]L-arginine increased linearly up to 5 min and the apparent initial uptake clearance, which should reflect the activity of blood-to-brain [^{3}H]L-arginine transport across the BBB, is estimated to be 9.34 ± 0.89 μL/(min·g brain) (mean ± SD). Moreover, we previously showed that the Kp, app value for [^{3}H]L-serine after intravenous administration to adult male rats increased linearly up to 10 min (Kasai et al., 2011). Thus, the decapitating times were determined to be 2 min for [^{3}H]L-arginine and 10 min for [^{3}H]L-serine after intravenous administration of the respective compounds.

2.4. High performance liquid chromatography (HPLC) analysis

The metabolism of [^{3}H]L-arginine in plasma and brain 2 min after intravenous injection was determined by HPLC according to a previous report (Tomi et al., 2009). The samples were treated and subjected to HPLC using a system equipped with a 4.6 mm × 150 mm Inertsil ODS-3™ column (GL Sciences, Tokyo, Japan).

2.5. Immunohistochemistry

Under pentobarbital anesthesia (50 mg/kg body weight, i.p.), rat brains at P7, P14, P21 and P42 (adult) were perfused transcardially with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB, pH 7.4). Brain sections (50 μm in thickness) were prepared on a microslicer (VT1000S; Leica, Nussloch, Germany). Immunohistochemistry were performed as described previously (Tachikawa et al., 2004). In brief, sections were immunoreacted overnight with guinea-pig antibody to CAT1 (2 μg/mL, (Tomi et al., 2009)) singly or in combination with rabbit glucose transporter 1 (GLUT1) antibody (0.5 μg/mL, (Sakai et al., 2003)). Subsequently, they were incubated with species-specific Alexa Fluor 488- (Invitrogen) and Cy3-conjugated secondary antibodies for 2 h (Jackson ImmunoResearch, West Grove, PA, USA). Photographs were taken using a confocal laser-scanning microscope (TCS-SP5; Leica).

2.6. Immunoblotting

Rat brains at P7, P14, P21, and P42 were homogenized in buffer containing (in mM): 10 Tris-HCl (pH 7.4), 1 EGTA, 250 sucrose, and a protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA), pH 7.4, using the nitrogen cavitation technique (900 psi, 30 min, 4 °C). The homogenates were centrifuged at 10,000*g* for 10 min. The supernatant fluids were further centrifuged at 100,000*g* for 60 min to obtain a crude membrane fraction from the pellets. Protein concentration was determined using DC Protein Assay kit (Bio-rad, Hercules, CA, USA). Protein samples (50 μg per lane) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto a PVDF membrane (Hybond™-P, GE Healthcare, Chalfont St. Giles, UK). The blotted membrane was incubated with affinity-purified CAT1 antibody at 0.2 μg/mL in Tris-buffered saline (TBS; 25 mM Tris-HCl and 125 mM NaCl, pH 7.4) containing 0.1% Tween 20 and 5% skimmed milk for 16 h at 4 °C, and visualized with an enhanced chemiluminescence kit (GE Healthcare). Intensities of individual bands were quantified by Image J (U. S. National Institutes of Health, Bethesda, Maryland, USA).

2.7. Statistical analysis

Unless otherwise indicated, all data are presented as the mean ± SEM. One-way analysis of variance followed by the modified Fisher's least-squares difference method was used to assess the statistical significance of differences among means of more than two groups.

3. Results

3.1. Developmental changes of blood-to-brain L-serine and L-arginine transport across the BBB

The apparent brain-to-plasma concentration ratio (Kp, app value) of L-arginine was significantly increased to 0.25 and 0.26 mL/g brain at P7 and P14, respectively, which was 2-fold greater than that at the adult