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L-Citrulline restores nitric oxide level and cellular uptake at the brain capillary endothelial cell line (TR-BBB cells) with glutamate cytotoxicity

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ABSTRACT

Objective: Glutamate excitotoxicity provokes neuronal cell damage and death, leading to collapse of the blood-brain barrier (BBB). Recently, it has been reported that L-citrulline, a neutral amino acid and a major precursor of L-arginine in the nitric oxide (NO) cycle, can prevent both neuronal cell death and cerebrovascular cell loss in brain ischemia. Therefore, the objective of this study was to investigate the effect of L-citrulline on glutamate cytotoxicity in the BBB using the conditionally immortalized rat brain capillary endothelial cell line (TR-BBB cells) as an in vitro model of the BBB.

Methods: Cell viability was determined using MTT assay. Cellular uptake of [^{14}C] L-citrulline and expression levels of rat large neutral amino acid transporter 1 (rLAT1), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS) at mRNA level were performed using quantitative real-time polymerase chain reaction (PCR) analysis. NO production from TR-BBB cells was measured using Griess reagents. All experiments were performed after treatment of TR-BBB cells with glutamate alone or co-treatment with L-citrulline, L-arginine, and/or taurine for 24 h.

Results: L-Citrulline treatment increased cell viability, [^{14}C] L-citrulline uptake, and the mRNA levels of LAT1 and eNOS in TR-BBB cells treated with glutamate. However, iNOS mRNA expression was inhibited by L-citrulline. NO production and transcript level of iNOS were markedly increased by glutamate treatment alone. However, co-treatment with L-citrulline, taurine, or both L-citrulline and taurine decreased NO levels and mRNA levels of iNOS in TR-BBB cells treated with glutamate. In co-treatment of TR-BBB cells with L-arginine, a NO donor, and glutamate, NO levels were increased and expression levels of iNOS mRNA were similar compared to those in cells treated with glutamate alone.

Conclusion: L-Citrulline can restore NO level and its cellular uptake in TR-BBB cells with glutamate cytotoxicity. Supplying L-citrulline at the BBB may provide neuroprotective effect to improve cerebrovascular dysfunction such as a brain ischemia.

1. Introduction

Glutamate, a nonessential amino acid, is the most abundant excitatory amino acid in the brain (Hawkins and Viña, 2016). In the central nervous system, glutamate acts as a major excitatory neurotransmitter and a potent neurotoxin. Glutamate concentrations are 50–100 μM in the plasma, 12 μmol/g in the whole brain, and 0.5–2 μM in extracellular fluids (ECF) (Hawkins and Viña, 2016). Under pathological conditions of CNS such as ischemia and trauma, glutamate concentration levels in brain can be increased up to 50-fold (Coyle and Puttfarcken, 1993). Excitotoxic concentrations (mM) of glutamate provoke neuronal damage and death (Doré et al., 2000) by activating *N*-methyl-D-aspartate (NMDA) receptors (Sharp et al., 2003, 2005) that are glutamate-gated ion-channels located in endothelial cells, especially in brain endothelial cells (Chen et al., 2005; Krizbai et al., 1998; Sharp et al., 2003). Activation of NMDA receptors from glutamate results in calcium influx, calcium overload in mitochondria, energy failure, and the production of reactive oxygen species (ROS), eventually leading to cell death (Duchen, 2000). Therefore, glutamate-induced excitotoxicity plays a crucial role in endothelial damage and the BBB disruption (Janigro et al., 1994; Sharp et al., 2003). These BBB breakdown is involved in the pathogenesis of neurodegenerative disorders such as stroke, epilepsy, and multiple sclerosis (Ballabh et al., 2004; Dobble, 1999).

L-Citrulline is a neutral amino acid identified from watermelon (*Citrullus vulgaris Schrad*) (Mandel et al., 2005). It is a major precursor of arginine in nitric oxide (NO) cycle by nitric oxide synthase (NOS) (Hayashi et al., 2005; Wu, 1998). Cynober et al. (2010) have reported

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that L-citrulline is regenerated to L-arginine by argininosuccinate synthase and argininosuccinate lyase. This regenerated L-citrulline can be used in the cycling of L-arginine for NO production. NO production and NOSs activities in the CNS are closely associated with physiological and pathological cellular processes. NO derived by endothelial NOS (eNOS), found in endothelial cells (Bredt, 1999), is involved in physiological process. It also has an important role of the neuroprotective effect on the brain (Albrecht et al., 2003; Calabrese et al., 2007). However, NO produced by inducible nitric oxide synthase (iNOS) causes oxidative stress which is neurotoxic to the brain (Calabrese et al., 2007). Several studies have reported that L-citrulline administration can reduce endothelial cell damage in the thoracic aorta of rats fed high-fat and high-cholesterol diets (El-Kirsh et al., 2011). It can also improve intestinal microcirculation in endotoxin-treated mice (Wijnands et al., 2012). In the CNS, Yabuki et al. (2013) have reported that L-citrulline can prevent neuronal cell death and capillary loss in the hippocampal region caused by cerebral ischemia. Therefore, L-citrulline might have a neuroprotective effect to improve cerebrovascular dysfunction. We have previously reported that L-citrulline transport is mainly mediated by the large neutral amino acid transporter 1 (LAT1) in Na^+-independent transport system for neutral amino acids in TR-BBB cells (Lee and Kang, 2017a, b). Therefore, LAT1 in the BBB might be involved in the maintenance of L-citrulline concentration in the brain. However, the effect of L-citrulline on the BBB cell damage induced by glutamate has not been fully investigated yet. Therefore, the purpose of this study was to determine the effect of L-citrulline on glutamate cytotoxicity in brain capillary endothelial cells using the conditionally immortalized rat brain capillary endothelial cell line (TR-BBB cells) as an in vitro model of the BBB (Hosoya et al., 2000). We further examined whether LAT1 expression and activity were regulated by L-citrulline treatment in TR-BBB cells with glutamate cytotoxicity.

2. Materials and methods

2.1. Materials

[^{14}C]L-Citrulline (specific activity, 56.3 mCi/mmol) was purchased from PerkinElmer (Waltham, MA, USA). Bovine serum albumin and Bio-Rad DC protein assay kit were obtained from Bio-Rad Laboratories (Hercules, CA, USA). High Capacity RNA-to-cDNA kit, TaqMan Gene Expression Master Mix, gene-specific oligonucleotide primers, probes for LAT-1, eNOS, iNOS, and endogenous control GAPDH were purchased from Applied Biosystems (Foster City, CA, USA). Griess Reagent System was purchased from Promega (Madison, WI, USA). Glutamate, L-citrulline, L-arginine, taurine and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents were commercial products of reagent grade.

2.2. Cell culture

TR-BBB cells established from transgenic rats harboring a temperature-sensitive simian virus 40 large T-antigen as an in vitro BBB model were cultured at 33 °C as described previously (Hosoya et al., 2000). TR-BBB cells were kindly provided by Professor Tetsuya Terasaki (Tohoku University, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, San Diego, CA, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen, San Diego, CA, USA), and 15 μg/L endothelial cell growth factor (Roche, Mannheim, Germany) at 33 °C in a humidified atmosphere with 5% CO_2/95% air. For cellular uptake study, TR-BBB cells were initially seeded at density of 1×10^5 cells per well into rat tail collagen type I-coated 24-well cell culture plates (IWAKI, Tokyo, Japan) and the cells were cultured for two days at 33 °C until confluence.

2.3. Glutamate cytotoxicity, survival of TR-BBB cells, and microscopy

To determine cell viability, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltertazolium bromide (MTT) assay was performed (Ryu et al., 2005). Briefly, TR-BBB cells were treated with glutamate (1 mM) with or without L-citrulline (20 mM), L-arginine (20 mM), and/or taurine (20 mM). At 24 h after the treatment, MTT solution (5 mg/mL in phosphate buffered saline (PBS)) was added to each well. After incubating at 37 °C for 3 h, MTT solution was removed from each well followed by PBS wash (1 mL/well). Formazan precipitate in each well was then dissolved in 200 μL dimethyl sulfoxide (DMSO). Absorbance of each well was measured at wavelength of 550 nm using an Infinite F200 PRO microplate reader (Tecan Trading AG, Switzerland). For microscopy, images were obtained using an EVOS XL Core Cell Imaging System (Life Technologies, Bothell, WA, USA) with a 10× objective.

2.4. Uptake study of [^{14}C]L-citrulline

[^{14}C] L-citrulline uptake study was performed as described previously (Kang et al., 2002). Briefly, TR-BBB cells in 24-well culture plates were washed three times with 1 mL extracellular fluid (ECF) buffer (pH 7.4) per well at 37 °C. After adding ECF buffer (pH 7.4) containing [^{14}C]L-citrulline (44.4 μM) to each well, TR-BBB cells were then incubated at 37 °C for 5 min. Uptake of [^{14}C]L-citrulline was terminated by adding ice-cold ECF buffer. Cells were then solubilized by incubation with 750 μL of 1 N NaOH at room temperature overnight. Aliquots (0.5 mL) were collected from each well and mixed with 4.5 mL Ultima gold reagent. Radioactivity was measured with a liquid scintillation counter (LS6500; Beckman, Fullerton, CA, USA). Protein levels were evaluated using a DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as standard. Cell to medium ratio (μL/mg protein) was calculated as follows: radioactivity (dpm/μL) in the sample per milligram of cellular protein (dpm/mg protein).

To determine the effect of L-citrulline on [^{14}C]L-citrulline uptake in TR-BBB cells with glutamate cytotoxicity, TR-BBB cells were pre-treated with 1 mM glutamate alone or together with 20 mM L-citrulline, 20 mM L-arginine, and/or 20 mM taurine for 24 h. Then, [^{14}C]L-citrulline uptake was performed for 5 min.

2.5. Quantitative real-time PCR analysis of LAT-1, eNOS, and iNOS mRNA expression levels in TR-BBB cells

Total RNA was isolated from cultured TR-BBB cells using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed in 48-well plates using StepOnePlus Real-Time PCR system (Applied Biosystems) and MGB Taqman® probe assay with TaqMan® Gene Expression Master Mix (AB Applied Biosystems) according to the manufacturer's protocols. Probes for LAT1, eNOS, iNOS, and endogenous control GAPDH (Rn00569313_m1, Rn02132634_s1, Rn00561646_m1 and Rn99999916_s1, respectively) were purchased from Applied Biosystems. Each reaction contained 10 μL Taqman Universal PCR Master mix (Applied Biosystems), and 1 μL cDNA in a total volume of 20 μL. Real-time PCR reactions were performed at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. All PCR products were subjected to melting curve analysis to confirm the specificity of amplification. Expression levels of specific genes were normalized against levels of GAPDH housekeeping gene as internal control. A negative control lacking cDNA was assayed in parallel to monitor genomic contamination. Comparative C_t method was used for relative mRNA quantification based on the mRNA level of the GAPDH housekeeping gene following the manufacturer's protocols.