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Original Article

Ulinastatin up-regulates glutamate transporters in glial cells

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Abstract: General anesthesia impairs glutamate homeostasis at synapses by decreasing the production of glutamate transporters. Glutamate transporters in astrocytes are important to preserve cognitive function and memory in hippocampus. In this study we investigated whether ulinastatin increases the number of glutamate transporters in glial cells. We used human astrocytes to demonstrate glutamate uptake in glial cells. The cells were divided into control and ulinastatin-treated groups. After incubation for 3 hours in serum free media, ulinastatin was administered to ulinastatin-treated group, then media was changed to HBSS buffer with 0.1 mM of glutamate. And we determined glutamate absorption by astrocytes by measuring glutamate levels in the media and astrocyte lysates at a time. We also measured levels of the glutamate transporters. In the ulinastatin-treated group, the total amount of glutamate was decreased, and the amount in astrocytes was significantly increased, compared to the control group. Expression of glutamate transporter 1 and glutamate aspartate transporter was significantly increased in the ulinastatin group. In this study, administration of ulinastatin resulted in increased glutamate uptake by astrocytes. We also demonstrated glutamate transporters. Thus, we propose that ulinastatin may improve short memory impairment and cognitive disorder after anesthesia by increasing the number of glutamate transporters.

Keywords: Glutamate uptake, glutamate transporters, ulinastatin

Introduction

Glutamate is the one of the major neurotransmitters in brain, and the production and reuptake of glutamates is predominantly performed by astrocytes, which are the main glial cells in the hippocampus [1, 2]. Disruption of glutamate homeostasis in astrocytes is often observed in Alzheimer's disease [3], general anesthesia, and other cognitive disorders. These cognitive disorders are caused by neurovascular ischemic insult [4], chronic inflammation, or probably neurotoxicity of anesthetics [5, 6]; these conditions disturb glutamate homeostasis in astrocytes by reducing the production of glutamate transporters and the activity of glutamate transporters, and disrupt in glutamate release [7, 8]. Therefore, maintaining glutamate homeostasis in astrocytes is important to preserve glial function and cognitive function after anesthesia.

Ulinastatin is a serine protease inhibitor that has an anti-inflammatory effect by reducing reactive oxygen species (ROS) [9, 10]. It has been shown that ulinastatin reduces ROS via Toll-like receptor (TLR) 3 and 4, which are major receptors for ulinastatin [11]. Especially, TLR 3 affects the production of glutamate transporters such as glutamate aspartate transporter (GLAST) and glutamate transporter 1 (GLT-1) [12], which are major transporters for glutamate uptake in astrocytes [13]. Therefore, in this study we investigated whether ulinastatin increases glutamate uptake in astrocytes by increasing the expression of glutamate transporters.

Materials and methods

Experimental protocol

The cells used in this study were human brain progenitor-derived astrocytes (Gibco, St. Louis, MO, USA). We divided the cells into control and ulinastatin-treated groups. Astrocytes were prepared by plating 5×10^4 cells in each well of 24-well plates, and the cells were transferred to

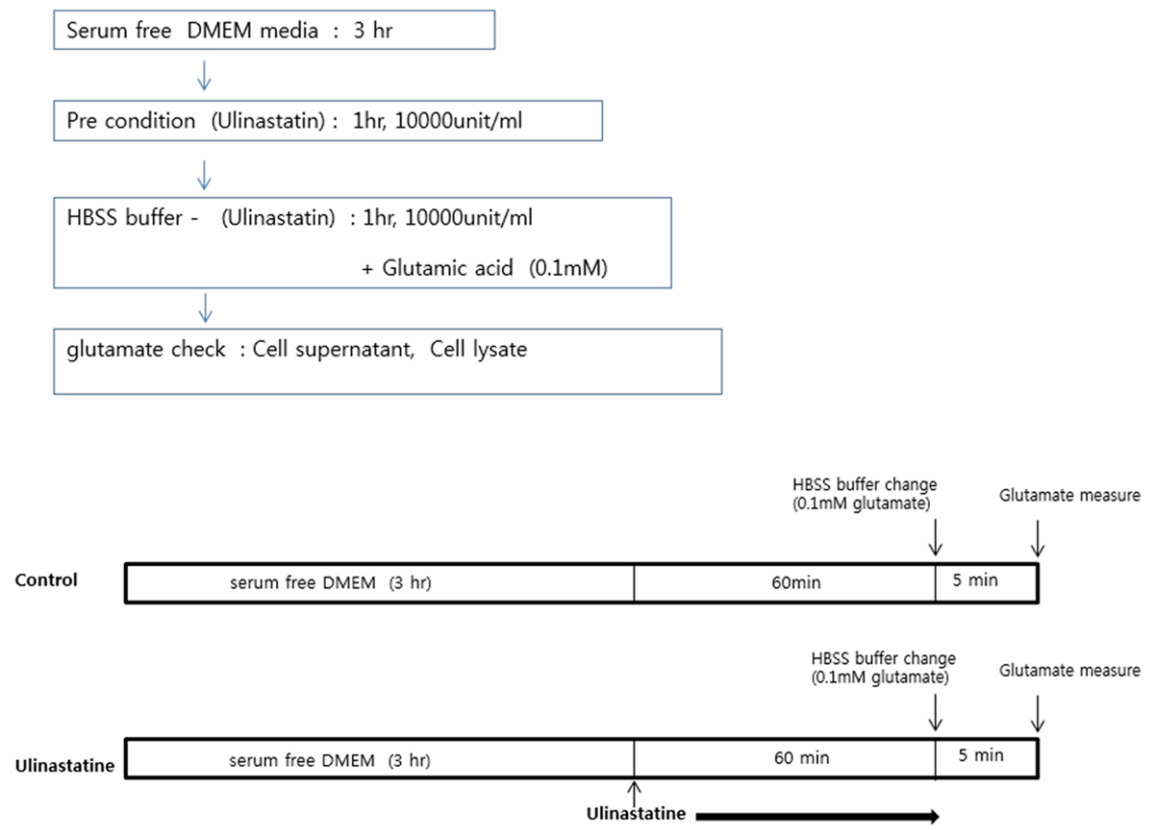

Figure 1. Experimental protocol. Effect of ulinastatin for glutamate reuptake in treated astrocytes. After glial cells were cultured with or without ulinastatin (10,000 unit/ml) for 1 hour, the medium were changed with glutamate uptake buffer and then the astrocytes were incubated with or without ulinastatin for 1 hour. The glutamate concentration of supernatant and cell lysate was detected by glutamate assay kit.

6-well plates to form a monolayer 1 day before the experiment. Three hours before starting the experiment, the medium was changed to medium containing 0.1% fetal bovine serum (FBS). Ulinastatin (Halim Medical Company, Korea) was added to the study group at a concentration of 10,000 units/ml [14]. After 1 hour, the medium was replaced with Hank's balanced salt solution (HBSS) containing 0.1 mM glutamate (Sigma Chemical Co., St. Louis, MO, USA), and cells were incubated for a further 5 minutes in the presence or absence of 10,000 units/ml ulinastatin. After incubation, the level of glutamate was simultaneously measured in the supernatant and cell lysate of both groups (**Figure 1**).

Measurement of glutamate uptake

We determined glutamate uptake by astrocytes by measuring the amount of glutamate in the media and in astrocyte lysates. We used this method rather than demonstrating glutamate transportation in real-time because we do not have the radioisotope system that is the preferred method for demonstrating the transmission of neurotransmitters in glial cells and neurons.

Measurement of glutamate absorption from media by astrocytes

Astrocyte cells were cultured in FBS-free Dulbecco's minimal essential medium (DMEM) at 37°C and 5% CO_2. At the end of the experiments, the glutamate level in the medium was measured using a glutamate assay kit (Abcam Co., Cambridge, MA, USA) in are action mixture containing 50 µl supernatant, 90 µl assay mix, 8 µl glutamate developer, and 2 µl glutamate enzyme mixture. After incubation at 37°C for 30 minutes the absorbance was measured at 450 nm using an ELISA reader uQuant (Bio-Tek Instruments.Inc, Winooski, VT, USA).

Table 1. Primer sequences for real-time PCR

Gene	Accession numbers	Sequence	Amplicon size	Annealing temperature
TLR3	NM198791.1	5'-GGAGGGTCCAACTGGAGAAC-3'	99nt	53°C
		5'-AACCAGCTCTCAACCTTGGTA-3'		
TLR4	CQI175880.1	5'-AACCAGCTCTCAACCTTGGTA-3'	100nt	53°C
		5'-CCACAGCCACCAGATTCTCT-3'		
GLAST	AF265360.1	5'-AAATGGGGAACTCCGTGATT-3'	106nt	53°C
		5'-ATCTTGGTTTCGCTGTCTGC-3'		
GLT1	AY044832.1	5'-ACATGTCCACGACCATCATT-3'	91nt	53°C
		5'CCCAGCTGCTTCTTGAGTTT-3'		
Beta actin	NM_031144	5'-GGTCCTAGCACCAATGAAGA-3'	105nt	53°C
		5'-ATCTGCTGGAAGGTGGACAG-3'		

Primer sequences of GLT-1, GLAST, TLR-3, and TLR-4 used for real-time PCR.

Measurement of glutamate in astrocyte cell lysates

Cell lysates were prepared as follows. After incubation, the astrocytes were harvested by scraping in HBSS buffer, homogenized by sonication for 3 seconds, and centrifuged at 12,000 rpm for 10 min. After centrifugation, the supernatant was collected and the protein amount of glutamate was determined by the Bradford method.

Real-time PCR

Preparation of total RNA: Total RNA was extracted from astrocyte cell culture. Cells were homogenized in 1 ml of Trizol reagent (Life Technologies, Carlsbad, CA, USA) and total RNA was isolated according to the manufacturer's protocol. The RNA concentration was determined by spectrometry using Nanodrop (ND-1000 spectrophotometer, Nanodrop Technologies, Wilmington DE, USA).

Real-time reverse transcription polymerase chain reaction (RT-PCR)

The expression levels of GLAST, GLT1, TLR-3, and TLR-4 mRNA were quantified by RT-PCR using LightCycler FastStart DNA Master SYBR Green I (Roche, Mannheim, Germany) according to the manufacturer's protocol. The reaction mixture (20 µl) contained LightCycler Fast Start DNA Master SYBR Green I, 4 mM $MgCl_2$, 0.5 µM of primer pairs, and 2 µl of the cDNA as template. The sequences of primer pairs are summarized in **Table 1**. The PCR reaction was performed at 95°C for 10 minutes, followed by 45 cycles at 95°C for 10 seconds, the appropriate annealing temperature for 10 seconds, and 72°C for size of amplicon (bp)/25 seconds. After amplification, melting analysis was performed at 65°C for 15 seconds and 40°C for 30 seconds. RT-PCR was performed in a LightCycler 1.5 (Roche Diagnostics). To control for variations, expression of all PCR products were normalized against that of beta actin.

Western blotting

To measure protein levels of the glutamate transporters GLAST and GLT-1, astrocytes were homogenized in lysis buffer (radio-immunoprecipitation buffer; Sigma, St. Louis, MO USA) containing a proteinase inhibitor Cocktail (Roche) to extract the proteins. Protein concentrations were determined by the Bradford method. Equal amounts of protein (60 µg) were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membranes. Membranes were blocked with 5% skim milk in 1× tris-buffered saline (TBS) at room temperature for 1 hr. For identification of GLAST and GLT-1, the membranes were incubated with a 1:1,000 dilution of rabbit monoclonal anti-GLAST antibody or rabbit polyclonal anti-GLT-1 primary antibody (Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C. The membrane was washed three times in 1× TBST buffer and incubated with a 1:2,000 dilution of horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody for detection of GLAST and GLT-1. For detection of actin as a normalization control, 1:3,000 diluted primary antibody (Sigma) and 1:5,000 diluted anti-rabbit IgG-HRP were used. An electro-chemiluminescence kit was used to develop the western blots (Amersham,

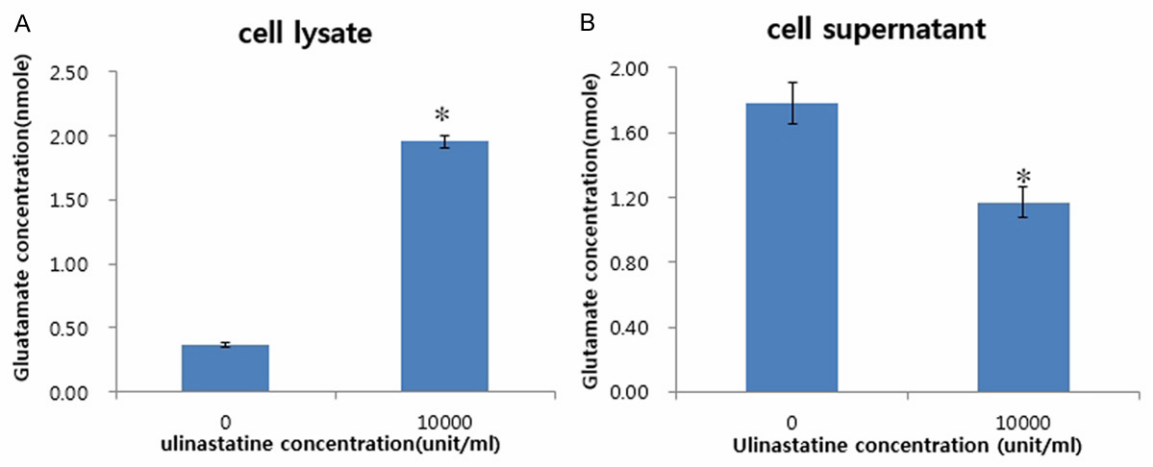

Figure 2. Glutamate uptake in astrocytes. This graph shows Glutamate in supernatant was significantly reduced in the ulinastatin treated group, as compared to the control group. And total amount of glutamate in ulinastatin treated cells lysate was five times higher than those of control group. *P<0.05 compared with the control group. A: Glutamate concentration of cell supernatant. B: Glutamate concentration of cell lysate.

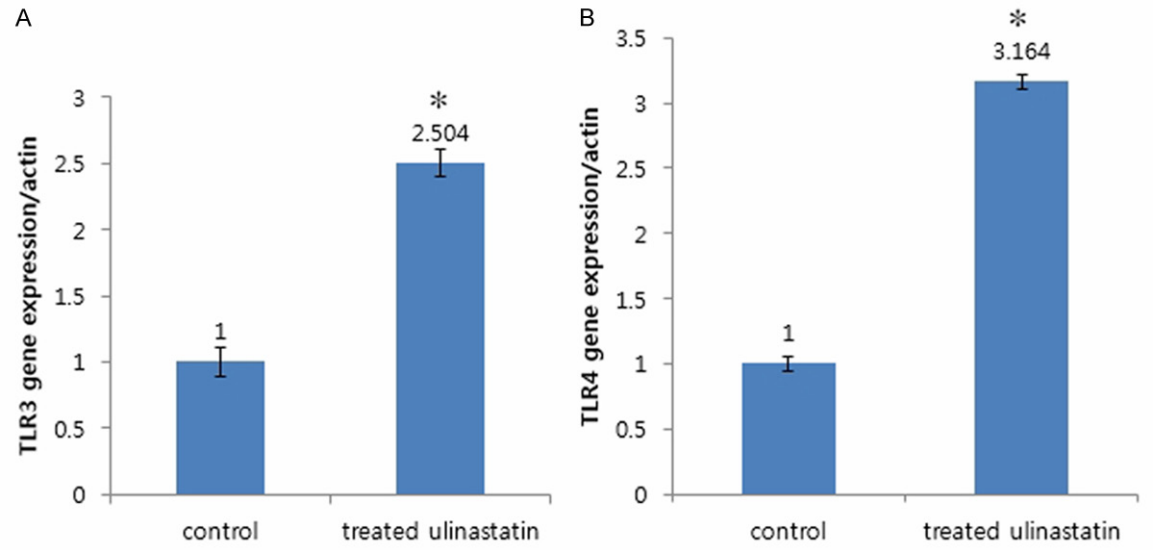

Figure 3. Up-regulated the expression of TLR-3, 4. Up-regulation of TLR-3, -4 mRNA expressions for ulinastatin (10,000 unit/ml) in astrocytes. Level of TLR-3 and TLR-4 mRNA were analyzed by real time PCR. Control; astrocytes were incubated in FBS free medium without ulinastatin, treated ulinastatin; astrocytes were incubated in FBS free medium with ulinastatin (10,000 unit/ml). A: TLR3 mRNA expression level, B: TLR4 mRNA expression level. *P<0.05 compared with the control group.

Boston, MA, USA). Image J software was used for densitometry analysis.

Results

Glutamate levels in supernatants and cell lysates

The glutamate level in media was significantly reduced in the ulinastatin-treated group, compared to the control group. Moreover the total amount of glutamate in lysates of ulinastatin-treated cells was increased almost 4-fold relative to the control group (**Figure 2**).

TLR-3 and TLR-4 expression by real-time PCR

We measured the expression of TLR-3 and 4 in astrocytes by real-time PCR at the end of incu-

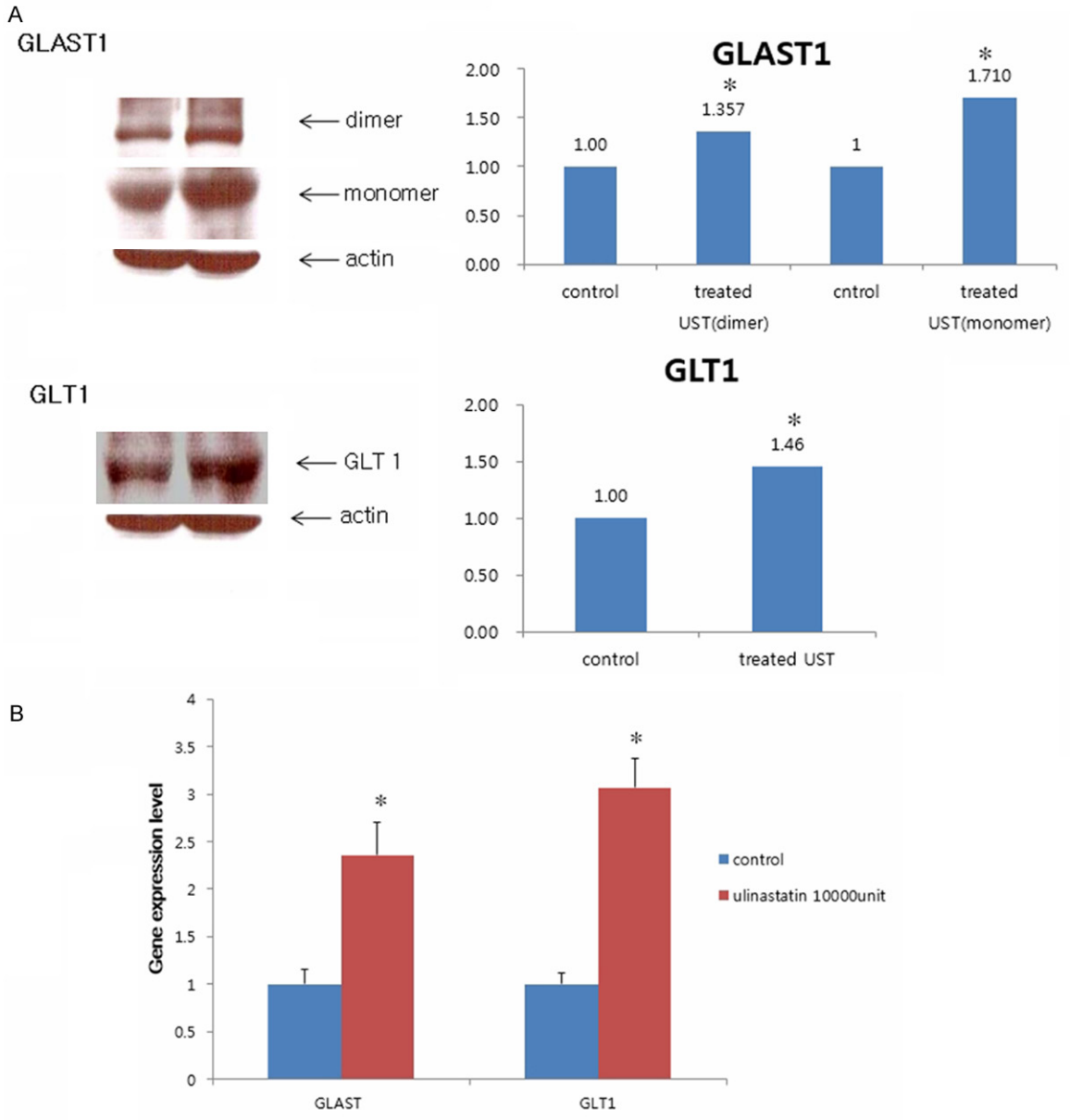

Figure 4. Up-regulation of GLAST and GLT-1. Up-regulation of glutamate transporter (GLAST and GLT1) in ulinastatin (10,000 unit/ml) treated mouse astrocytes. The astrocytes were analyzed by western blotting and real time PCR. The GLAST and GLT-1 protein band were quantified by densitometry and normalized for actin. Using the image J for densitometry analysis. ULI = ulinastatin. *P<0.05 compared with the control group. A: Western blotting of GLAST and GLT-1, B: Real time PCR of GLAST and GLT-1.

bation with or without ulinastatin. Both the expression of TLR-3 and TLR-4 were significantly up-regulated after treatment with ulinastatin (**Figure 3**).

GLT-1 and GLAST expression by western blot and PCR analysis

GLT-1 and GLAST transporters were significantly increased in the ulinastatin-treated group, as compared to the control group in both western blotting and PCR analysis (**Figure 4**).

Discussion

Nowadays, glial cells are thought to be functional units rather than barriers of neurons. It is known that dysfunction of astrocytes is one cause of cognitive disorder or short-term memory loss [15, 16]. Astrocytes, also known col-

lectively as astroglia, are characteristic star-shaped glial cells in the brain and spinal cord. The proportion of astrocytes in the brain is not well defined, but studies have found that the astrocyte proportion ranges from 20% to 40% of all glia in brain [17]. Astrocytes perform many functions, including biochemical support of endothelial cells that form the blood-brain barrier, provision of nutrients to the nervous tissue, maintenance of extracellular ion balance, and a role in repair [18]. They are dominantly located in hippocampus area, which has important roles in the consolidation of information from short-term memory to long-term memory [19], and involved in the release and absorption of neurotransmitters like glutamate, ATP, and GABA by regulating neurotransmitter transporters. Glutamate is the major excitatory transmitter in the brain and, once released to synapses, is rapidly cleared by glutamate transporters thus limiting glutamate excitotoxicity [20]. Indeed, inhibition of glutamate reuptake elicits short-term memory inhibition [7, 21]. Therefore, glutamate transporters play a crucial role in physiological glutamate homeostasis and neurotoxicity [22].

Neurotransmission is regulated by neurotransmitter concentrations at synapses in the neuronal circuits. Termination of neurotransmission is achieved by reuptake into presynaptic nerve terminals through high-affinity plasma membrane transporters. GABAergic, serotoninergic, glutamatergic, and dopaminergic transporters are involved in the neurotransmission systems of memory and cognitive processes. GLAST (EAAT1) is a primary glutamate transporter in the forebrain [23]. Duerson et al. [7] showed a lack of GLAST in the hippocampusin patients with Alzheimer's disease. Mookherjee P et al. [24] also showed that loss of GLT-1 accelerates cognitive function deficits in rat models of Alzheimer's disease. Ischemic insult or neurotoxic agents like halogenated anesthetics reduce the expression of glutamate transporters in astrocytes. Vinje et al. [25] reported that sevoflurane, a popular inhalation anesthetic, down-regulated glutamate transporters like GLAST and GLT-1 in glial cells in the brain. Lee S et al. [26] revealed that glutamate transporter type-3 (EAAT3) knockout mice had significant cognitive impairment after exposure to isoflurane compared to wild-type mice.

Postoperative cognitive dysfunction (POCD) is a recognized clinical phenomenon that presents with a decline of cognitive functions after anesthesia and surgery [27]. Approximately 30-40% of patients have POCD at hospital discharge after non-cardiac surgery. The incidence is 10% more for elderly patients at 3 months after surgery [6]. Although the cause of POCD is not clear, glutamatergic pathway is important to preserve cognitive function [28, 29], Rath et al. [8] reported that etomidate impaired glutamate homeostasis by reducing glutamate uptake in the cerebrocortical area, and Liachenko et al. [30] also revealed that isoflurane reduced glutamate release as well as glutamate reuptake. In this study, we showed that expression of glutamate transporters such as GLAST and GLT-1 was increased after ulinastatin administration and demonstrated that the uptake of glutamate into astrocytes was simultaneously increased. Therefore, we believe that administration of ulinastatin might reduce the incidence of POCD or other cognitive disorders because glutamate transporters decrease glutamate toxicity by increasing the uptake of glutamate and improve glutamate transporter production.

Ulinastatin (or urinary trypsin inhibitor, UTI) is a glycoprotein that acts as a trypsin inhibitor and potentiates local anti-proteolytic activity on the extracellular matrix (ECM), reduces the production of ROS, and also suppresses neutrophil accumulation and activity via suppression of cytokine-dependent signaling pathways such as ERK1/2, JNK, NF-kB, or Egr-1 pathways [9, 10]. Ulinastatin can be derived from urine or synthetically produced. In preclinical and clinical studies, ulinastatin protects against acute lung injury, graft ischemia–reperfusion injury, loss of renal function after cardiopulmonary bypass, severe burn injury, septic shock, and brain injury [11]. Ulinastatin also has an immune-suppresive function through its effect on Toll-like receptors. Toll-like receptors are a class of proteins that play a key role in the innate immune system. They are single membrane-spanning, non-catalytic receptors that are usually expressed in sentinel cells such as macrophages and dendritic cells and recognize structurally conserved molecules derived from microbes. Each type of toll-like receptor has specific ligands that bind different pathogens. Their signaling is followed by activation of the MyD88-dependent pathway or TRIF-dependent

pathway [31]. TLR-3 and -4 primarily signal through the TRIF-dependent pathway. Following activation by ligands of different origin, several reactions are possible via TLRs. Immune cells can produce signaling factors called cytokines, which trigger inflammation and induce adaptive immunityin dendritic cells [31]. Scumpia PO et al. [12] reported that up-regulated TLR-3 on astrocytes increases the production of glutamate transporters such as GLAST and GLT-1, though the effect of TLR-4 to glutamate transporters is still unclear. In this study we showed that ulinastatin upregulated the expression of toll-like receptors 3 and 4 on astrocytes, along with upregulated GLAST and GLT-1. Therefore, we propose that up-regulation of these glutamate transporters was mediated possibly by up-regulation of TLR-3 and 4.

In conclusion, this study provides evidence that ulinastatin improves glutamate uptake by up-regulating glutamate transporters such as GLAST and GLT-1. Furthermore, our results suggest that the correlation between the expression of TLR-3, 4 and glutamate transporters. In order to understand the role of TLR-3, 4 in glutamate transporter function, future studies using knock-out/in models are needed. Moreover, to confirm the effect of ulinastatin on cognitive function, we think that animal cognitive-behavior study is required.

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Disclosure of conflict of interest

None.

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