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Effect of Various Pathological Conditions on Nitric Oxide Level and L-Citrulline Uptake in Motor Neuron-Like (NSC-34) Cell Lines

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal motor neuron disorder that causes progressive paralysis. L-Citrulline is a non-essential neutral amino acid produced by L-arginine via nitric oxide synthase (NOS). According to previous studies, the pathogenesis of ALS entails glutamate toxicity, oxidative stress, protein misfolding, and neurofilament disruption. In addition, L-citrulline prevents neuronal cell death in brain ischemia; therefore, we investigated the change in the transport of L-citrulline under various pathological conditions in a cell line model of ALS. We examined the uptake of [^{14}C]L-citrulline in wild-type (hSOD1wt/WT) and mutant NSC-34/ SOD1^{G93A} (MT) cell lines. The cell viability was determined via MTT assay. A transport study was performed to determine the uptake of [^{14}C]L-citrulline. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed to determine the expression levels of rat large neutral amino acid transported 1 (rLAT1) in ALS cell lines. Nitric oxide (NO) assay was performed using Griess reagent. L-Citrulline had a restorative effect on glutamate induced cell death, and increased [^{14}C]L-citrulline uptake and mRNA levels of the large neutral amino acid transporter (LAT1) in the glutamate-treated ALS disease model (MT). NO levels increased significantly when MT cells were pretreated with glutamate for 24 h and restored by co-treatment with L-citrulline. Co-treatment of MT cells with L-arginine, an NO donor, increased NO levels. NSC-34 cells exposed to high glucose conditions showed a significant increase in [^{14}C]L-citrulline uptake and LAT1 mRNA expression levels, which were restored to normal levels upon co-treatment with unlabeled L-citrulline. In contrast, exposure of the MT cell line to tumor necrosis factor alpha, lipopolysaccharides, and hypertonic condition decreased the uptake significantly which was restored to the normal level by co-treating with unlabeled L-citrulline. L-Citrulline can restore NO levels and cellular uptake in ALS-affected cells with glutamate cytotoxicity, pro-inflammatory cytokines, or other pathological states, suggesting that L-citrulline supplementation in ALS may play a key role in providing neuroprotection.

Key Words: Amyotrophic lateral sclerosis, Large neutral amino acid transporter 1, L-citrulline, Glutamate, Nitric oxide, Hypoxia-inducible factor

INTRODUCTION

L-Citrulline is a non-essential neutral amino acid produced by arginine via nitric oxide synthase (NOS) (Förstermann and Sessa, 2012). It boosts the immune response by increasing NO production and polyamine synthesis, which contribute to tissue repair (Allerton *et al*., 2018). L-Citrulline supplementation improves NO synthesis and regulates arginine levels in the bloodstream more effectively than arginine supplementation (Moinard *et al*., 2016). Amyotrophic lateral sclerosis (ALS) is an incurable chronic motor neuron disorder associated with the loss of motor neurons and activation of glial cells in the spinal cord (Gubert *et al*., 2019). The pathogenesis of ALS entails impairment of glutamate uptake, leading to glutamate excitotoxicity, followed by neurodegeneration via disproportionate generation of oxidative free radicals, neurofilament aggregation, dysfunction of the axonal transport systems, and mitochondrial dysfunction (Renton *et al*., 2014). Lipopolysaccharides (LPS), bacterial endotoxins, stimulate microglia. In addition, tumor necrosis factor alpha (TNF-α) and interferon-γ are pro-inflammatory stimuli for microglia. These cytokines are elevated in various pathological conditions including spinal cord injury, stroke, multiple sclerosis, and neurodegenerative diseases (Lively and Schlichter, 2018).

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Furthermore, an enumerated study on the transport activities of L-citrulline on ALS cell lines demonstrated a relatively optimized framework for its effectiveness in ALS (Gyawali *et al*., 2021a). Consistent with previous reports, hereditary ALS is caused by mutations in the free radical-scavenging enzyme "superoxide dismutase-1" (SOD1), although the exact mechanism is unknown. Free radicals such as NO are hypothesized to play a key role in the pathogenesis of ALS (Lee *et al*., 2009a). NO production is stimulated by L-citrulline through *de novo* arginine production (Luiking *et al*., 2010). Furthermore, L-citrulline shows protective effect against cerebrovascular injury and neuronal cell death, thereby preventing NOS dysfunction (Yabuki *et al*., 2013). The hypoxia inducible factor-1 alpha (HIF-1α) transcription factor is essential for maintaining oxygen homeostasis. Hypoxia is linked to the pathophysiological process of ALS, and HIF deficiency causes motor neuron degeneration in ALS (Nomura *et al*., 2019). Therefore, in this study, we investigated the effects of L-citrulline on NO production. In addition, we aimed to determine the potential benefits of L-citrulline in several pathological conditions such as glutamate cytotoxicity, pro-inflammatory states, and high glucose conditions by using the mouse motor neuron-like NSC-34 cell lines that were transiently transfected with human SOD1(G93A).

MATERIALS AND METHODS

Materials

[^{14}C]L-Citrulline (specific activity of 56.3 mCi/mmol) was purchased from PerkinElmer (Boston, MA, USA). All other commercial grade chemicals were purchased from Sigma (St. Louis, MO, USA).

NSC-34 cell culture

Motor neuron-like cell lines (NSC-34) was pre-established by transfecting it with pCl neo expression vector comprising wild-type (hSOD1WT) and mutant SOD1G93A(hSOD1G93A) (Latif and Kang, 2021). Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), penicillin, and streptomycin was used for cell culture. For the mRNA and uptake studies, initial seeding was performed on collagen type I-coated 6 and 24 well plates at 3×10^5 and 1×10^5 cells/well, respectively. Experiments were performed when cells became confluent after 2 days of incubation at 37°C.

Cellular uptake study of L-citrulline

[^{14}C]L-Citrulline uptake was examined as previously described (Lee *et al*., 2017). Briefly, extracellular fluid (ECF) buffer containing [^{14}C]L-citrulline (44.4 μM) with and without inflammatory states were added to the NSC-34. The cell lines were incubated at pH 7.4 and 37°C for the designated time (5 min). Uptake was terminated by the addition of ice-cold ECF buffer. The cells were solubilized by overnight incubation in 750 μL of 1 N NaOH at room temperature. Radioactivity was measured using a liquid scintillation counter, LSC6500 (Beckman, Fullerton, CA, USA).

Cell to medium ratio (μL/mg protein) was calculated as follows: the radioactivity (dpm/μL) in the sample per milligram cell protein (dpm/mg protein) (Latif and Kang, 2022).

PCR analysis

Total RNA was isolated from cultured cells using the Qiagen RNeasy Mini Kit (Valencia, CA, USA), according to the manufacturer's instructions (Lee and Kang, 2016). The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) was used to reverse transcribe total RNA (2 μg). Real-time PCR was performed in 48-well plates using the Applied Biosystems StepOne apparatus and TaqMan probe assay. Probes for LAT1 (SLC7A5), hypoxia inducible factor-1 alpha (HIF-1α), and control GAPDH were purchased from Applied Biosystems. The analysis results were calculated in relation to the GAPDH product, and the results were calculated using the equation 2-ΔΔCt, which yields the amount of target normalized to an endogenous reference and relative to a calibrator. Ct is the threshold cycle for target amplification (Gyawali and Kang, 2019).

Cell viability via MTT assay and microscopy

The 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay was used to determine cell viability (Ryu *et al*., 2006). The MT model of ALS cells treated with 1 mM glutamate with or without 20 mM L-citrulline and/or L-arginine. After 24 h of pretreatment, MTT solution (5 mg/mL in phosphate buffered saline (PBS)) was added to each well. The cell lines were incubated for 3 h at 37°C before the MTT solution was removed from each well and washed with 1 ml of PBS/well. In each well, formazan precipitate was dissolved in 500 μL dimethyl sulfoxide (DMSO). The absorbance of each well was measured at 550 nm using an Infinite F200 PRO microplate reader (Tecan Trading, Männedorf, Switzerland). Images were obtained using an EVOS XL Core Cell Imaging System (Life Technologies, Bothell, WA, USA) with a 10× objective.

NO production assay

The levels of NO released by MT cells were measured using the Griess reagent system (Promega, Madison, WI, USA) (Sasabe *et al*., 2007; Jung *et al*., 2013). To examine the effect of several compounds on NO levels, MT cells were treated with 1 mM glutamate and/or co-treated with 20 mM L-citrulline, and L-arginine for 24 h. Then 100 μL of culture supernatant was collected from each sample (n=3) and added to a 96-well micro-plate. After 24 h of pre-treatment, the samples were incubated with 100 μL of modified Griess reagent (1% sulfanilamide in 5% phosphoric acid) at room temperature for 7 min. Finally, 0.1 N-1-napthylethylenediamine dihydrochloride in water was added followed by incubation at room temperature for 7 min. The absorbance was measured at 550 nm using an Infinite F200 PRO microplate reader (Tecan Trading AG, Männedorf, Swiss). A calibration curve was prepared using 0.1 M sodium nitrite in water as a standard.

High glucose and hypertonic pretreatment effects

NSC-34 cell lines after obtaining confluence were pretreated with 25 mM glucose-containing medium 24 h prior to uptake, according to a method described in a previous study (Gyawali *et al*., 2021b). Furthermore, for hypertonic stress, hypertonic media (380 mOsm/kg) were established by adding 100 mM sucrose to normal culture medium with an osmolality of 290 mOsm/kg. The cell lines were then subjected to the uptake of [^{14}C]L-citrulline at 37°C and pH 7.4 (Kang *et al*., 2002).

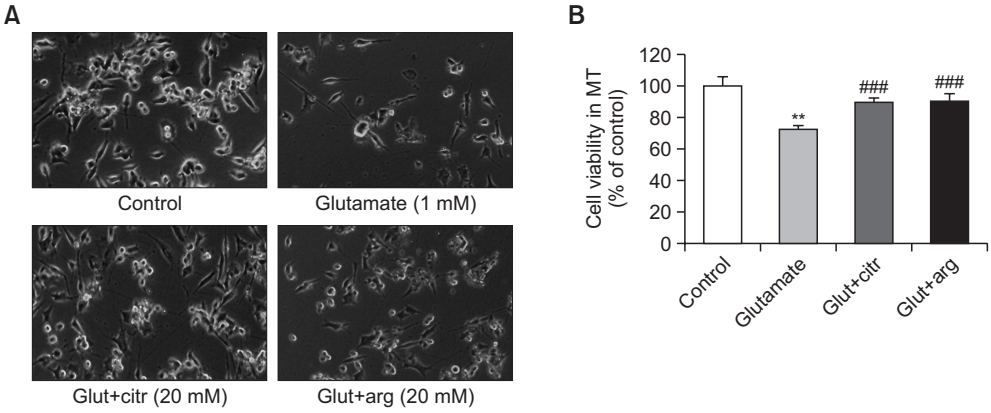

Fig. 1. Cell viability of MT cell line with glutamate cytotoxicity. (A) Images of the cells were obtained using EVOS XL Core Cell Imaging (100×). (B) MTT assay. MT cell lines were incubated with 1 mM glutamate (Glut) in the presence or absence of 20 mM L-citrulline (Citr) and L-arginine (Arg) for 24 h. Each column represents the mean ± SEM (n=3-4). $^{**}p<0.01$ vs. the control, $^{###}p<0.001$; vs. glutamate treatment.

Statistics

For statistical analysis, all data in figures were presented as mean ± standard error of mean (SEM). Data were analyzed using one-way analysis of variance (ANOVA) with Dunnett's post hoc test. A two-tailed unpaired t-test was used to compare the two groups. Differences were considered statistically significant if p was <0.05.

RESULTS

Effect of L-citrulline on cell viability against glutamate cytotoxicity in MT cell lines (MT)

To determine whether L-citrulline protects MT ALS cell lines from glutamate cytotoxicity, we examined cell viability using EXOS and MTT assays. When the MT cell lines were incubated for 24 h after treatment with 1 mM glutamate, there was a 72% increase in cell death compared to the control. Nonetheless, co-treatment with 20 mM L-citrulline and L-arginine had a restorative effect on cell death caused by glutamate cytotoxicity, with cell viabilities of 89.9% and 90.4%, respectively, compared to the control (Fig. 1). These findings demonstrated that L-citrulline treatment could prevent MT cell death caused by glutamate cytotoxicity.

Effect of glutamate induced cytotoxicity on [^{14}C] L-citrulline uptake and LAT1 mRNA expression by MT cell lines

A cellular uptake study in the ALS cell line (MT) was performed to investigate the changes in [^{14}C]L-citrulline transport caused by glutamate cytotoxicity. The uptake of [^{14}C] L-citrulline by MT cells treated with 1 mM glutamate 24 h before uptake was reduced to 78.3% compared to the control. In contrast, pre-treatment with 1 mM glutamate with 20 mM L-citrulline or L-arginine increased [^{14}C]L-citrulline uptake to near control levels by 107% and 104%, respectively (Fig. 2A). To determine whether increased [^{14}C]L-citrulline uptake was related to the expression level of the LAT1 transporter, we used quantitative real-time PCR to examine the expression level of LAT1 mRNA in MT cell lines. Our findings showed that the mRNA expression level of LAT1 significantly decreased after 1 mM of glutamate pre-treatment by 58.3% compared to the control. Conversely, LAT1 mRNA expression levels increased significantly after co-treatment with glutamate with 20 mM L-citrulline and L-arginine (Fig. 2B). These results suggest that increased [^{14}C]L-citrulline uptake by L-citrulline is associated with increased mRNA expression of LAT1 in glutamate exposed MT cell lines.

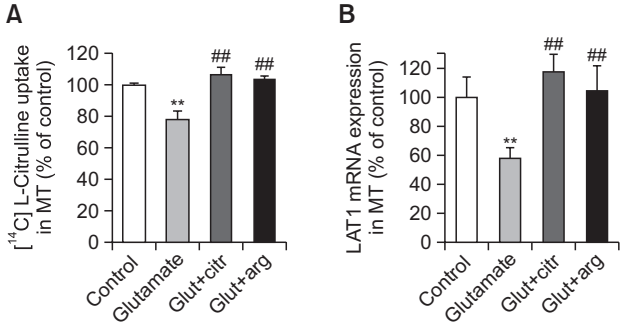

Fig. 2. [^{14}C]L-Citrulline uptake and LAT1 mRNA expression level in MT cell line with glutamate cytotoxicity. MT cells were incubated with 1 mM glutamate (Glut) with/without 20 mM L-citrulline (Citr) and L-arginine (Arg) for 24 h. (A) [^{14}C]L-Citrulline uptake was performed at pH 7.4 and 37°C for 5 min. (B) The transcription levels of LAT1 mRNA were determined by quantitative real-time PCR analysis and normalized to those of GAPDH. Each column represents the mean ± SEM (n=3-4). $^{**}p<0.01$ vs. the control, $^{##}p<0.01$ vs. glutamate treatment.

Effect of pro-inflammatory cytokines on [^{14}C]L-citrulline uptake and LAT1 mRNA expression level in MT cell lines

To rule out the effect of L-citrulline transport in the presence of the pro-inflammatory cytokines in MT cell lines, [^{14}C] L-citrulline uptake and LAT1 mRNA expression levels were measured 24 h after cells were exposed to LPS and TNF-α. Consequently, both the uptake and LAT1 mRNA expression levels were significantly reduced. However, the addition of 20 mM of L-citrulline to LPS and TNF-α treated MT cell lines significantly increased both the uptake and mRNA levels (Fig. 3). Based on the previous studies, we assume that NO production could have caused the decreased transport activity in the

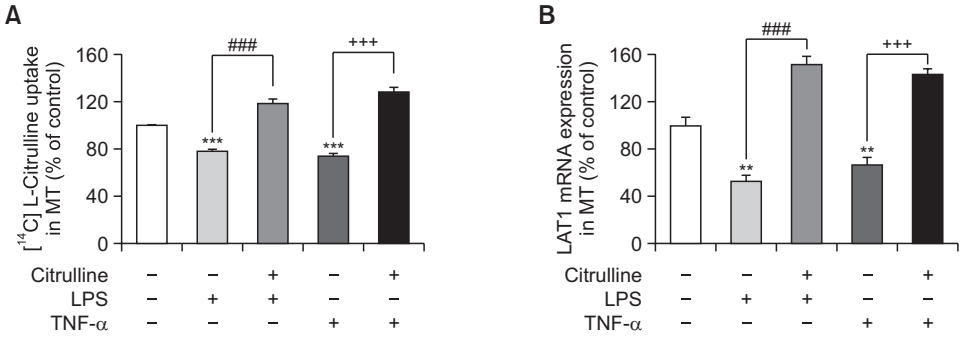

Fig. 3. [^{14}C]L-Citrulline uptake LAT1 mRNA expression level in SOD1 G93A mutant cell line exposed to various pro-inflammatory conditions. MT cells were incubated with 20 ng/mL of LPS and TNF-α with/without 20 mM L-Citrulline for 24 h. (A) [^{14}C]L-Citrulline uptake by MT cell lines was performed at pH 7.4 and 37°C for 5 min. (B) The transcription levels of LAT1 mRNA were determined by quantitative real-time PCR analysis and normalized to those of GAPDH. Each value represents the mean ± SEM (n=3-4). **p<0.01, ***p<0.001; significantly different from control. $^{###}p$<0.001; vs. LPS treatment and ^{+++}p<0.001; vs. TNF-α treatment.

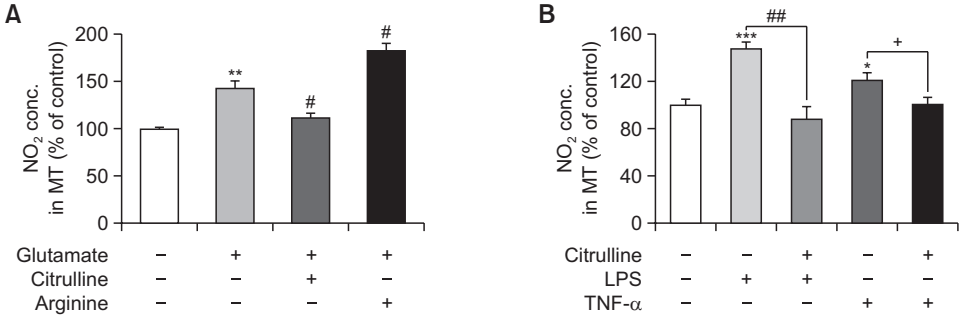

Fig. 4. NO levels in the MT cell line under inflammatory states. (A) NO concentrations were measured using Griess reagent after pre-treatment with glutamate (1 mM) and 20 mM of L-citrulline and L-arginine for 24 h. (B) NO concentrations were measured using Griess reagent after pre-treatment with LPS, TNF-α (20 ng/mL), and 20 mM of L-citrulline for 24 h. Each column represents the mean ± SEM (n=3-4). *p<0.05, **p<0.01, ***p<0.01 vs. the control, $^{#}p$<0.05 vs. glutamate treatment, $^{##}p$<0.01; vs. LPS treatment and ^{+}p<0.05 vs. TNF-α treatment.

inflammatory states which was later restored by the treatment with L-citrulline.

Effect of L-citrulline on NO production in MT cell lines with glutamate cytotoxicity and other inflammatory states

The Griess reagent was used to assess the effect of L-citrulline on NO production in MT cell lines with glutamate cytotoxicity and inflammatory cytokines. The results revealed that NO concentration was significantly increased (143%) after glutamate (1 mM) treatment compared to the control. Conversely, co-treatment of glutamate with L-citrulline (20 mM) restored NO levels near-control levels (112%). However, co-treatment with L-arginine (20 mM), an NO donor, increased NO production in the MT cell line by 183% compared to the control (Fig. 4A). These findings suggest that L-citrulline may reverse the increased NO production caused by glutamate cytotoxicity in ALS cells. Furthermore, NO production increased significantly in the presence of LPS and TNF-α, whereas the addition of citrulline restored NO levels (Fig. 4B).

Effect of hypertonic stress on L-citrulline uptake in ALS model cell lines

To investigate the L-citrulline transport system under hypertonic conditions, [^{14}C]L-citrulline uptake and LAT1 mRNA expression levels were measured in NSC-34 cell lines after 24 h of pre-treatment (Fig. 5). Compared with the isotonic state (280 mOsm/kg), hypertonic state (390 mOsm/kg) resulted in a significant decrease in the uptake rate and LAT1 mRNA expression level. However, compared to the hypertonic state, the addition of 20 mM unlabeled citrulline further reduced the uptake and mRNA expression levels.

Characteristics of L-citrulline uptake in ALS model cell lines under high glucose conditions

[^{14}C]L-Citrulline uptake and LAT1 mRNA expression levels were analyzed in ALS model cell lines after 24 h pretreatment to investigate the transport system of L-citrulline under the high glucose conditions. High glucose exposure facilitated a significant rise in the uptake rate and LAT1 mRNA expression levels (Fig. 6A, 6B). However, the addition of 2 mM unlabeled L-citrulline reduced the uptake and LAT1 mRNA expression levels while compared to the high-glucose condition. Previous studies suggested that the change in the uptake rate caused by high glucose levels was mediated by HIF-1 levels (Lee and Kang, 2015). The levels of HIF-1 mRNA expression in WT and MT cell lines under high glucose conditions were determined (Fig. 6C). In NSC-34 cell lines, high-glucose conditions down-regulated the HIF-1 mRNA expression significantly as compared to the control.

Differences in the [^{14}C]L-citrulline uptake level between WT

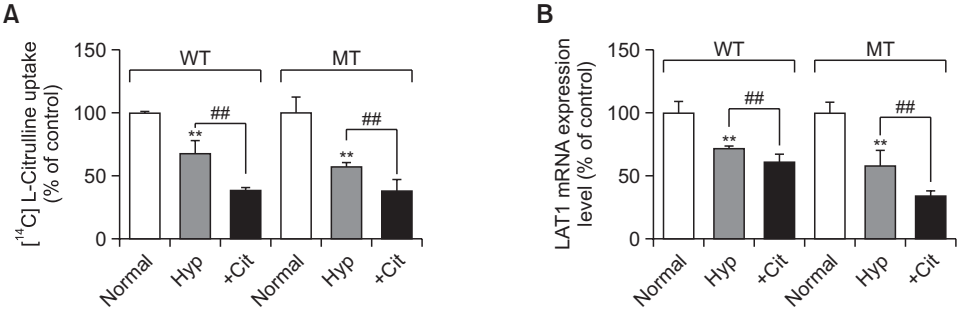

Fig. 5. Effect of L-citrulline under hypertonic condition. [^{14}C]L-Citrulline uptake by NSC-34 cell lines (WT; wild type, MT; mutant type) under hypertonic (Hyp) condition and addition of 20 mM L-citrulline (Cit) was performed at pH 7.4 and 37°C for 5 min. (A) LAT1 mRNA expression level on MT cells under hypertonic and high glucose condition. (B) The transcript levels of LAT1 were determined by quantitative real-time PCR analysis and normalized to those of GAPDH Each value represents the mean ± SEM (n=3-4). **p<0.01, significantly different from control. $^{\#\#}p$<0.01, vs hypertonic condition.

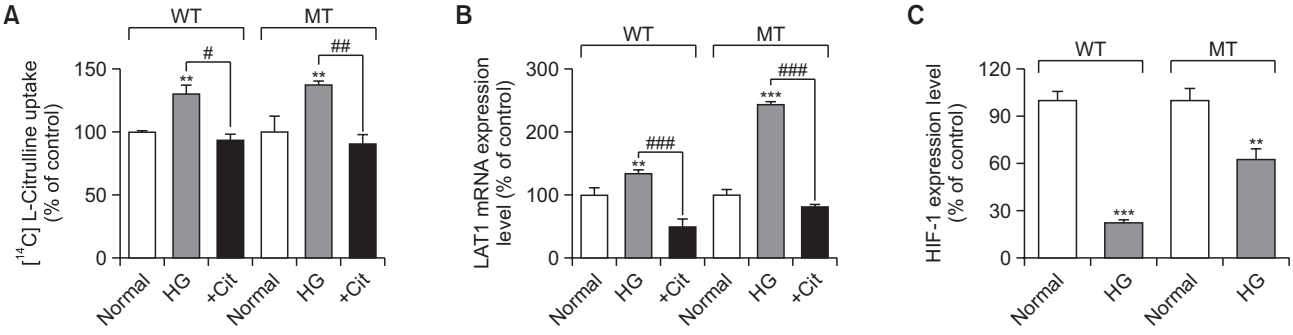

Fig. 6. Effect of L-citrulline under high glucose condition. (A) [^{14}C]L-Citrulline uptake by NSC-34 cell lines was performed at pH 7.4 and 37°C for 5 min under high glucose (HG) condition and addition of 20 mM of L-citrulline (Cit). (B) LAT1 mRNA expression level of NSC-34 cell lines. The transcript levels of LAT1 determined by quantitative real-time PCR analysis and normalized to those of GAPDH. (C) HIF-1α mRNA expression level on high glucose condition. The transcript levels of HIF-1 α determined by quantitative real-time PCR analysis. Each value represents the mean ± SEM (n=3-4). **p<0.01, ***p<0.001; significantly different from control. $^{\#}p$<0.05, $^{\#\#\#}p$<0.001 vs high glucose condition.

and MT cell lines under various pathological conditions

To obtain a clear picture of the difference in the uptake of [^{14}C]L-citrulline between the WT (control) and disease model cell lines (MT), we summarized the effects of various pathological conditions in both cell lines. Table 1 shows that pretreatment with 20 mM unlabeled L-citrulline resulted in increased uptake in both cell lines. Furthermore, in the presence of glutamate cytotoxicity, LPS and TNF-α resulted in a significant decrease in the uptake of [^{14}C]L-citrulline in MT cells compared to that in WT cells. However, co-treatment of these inflammatory conditions with unlabeled L-citrulline resulted in increased uptake, indicating the restorative effect of L-citrulline against pathological conditions. However, under hypertonic conditions, the uptake of [^{14}C]L-citrulline was significantly reduced, and the addition of L-citrulline resulted in a significant decrease in uptake. In contrast, in the presence of high glucose concentrations, although [^{14}C]L-citrulline uptake increased significantly, co-treatment with L-citrulline resulted in the decreased uptake.

DISCUSSION

Previous studies have demonstrated the inter-connection between motor neuron degeneration and the differential regulation of nitric oxide synthase NOS in ALS with L-arginine, yielding a neuroprotective effect (Lee *et al*., 2009a). L-Citrulline, a major precursor of L-arginine, has been found to protect against various conditions such as cardiac dysfunction, sickle cell disease, hypertension, and CNS dysfunction (Bahri *et al*., 2013; Lee and Kang, 2017).

To examine the effect of L-citrulline transport on various pathological conditions, MTT assay was performed to examine cell viability and determine whether L-citrulline could protect against glutamate cytotoxicity in MT cell line (Fig. 1). For this, 1 mM of glutamate was used because the previous study (Lee *et al*., 2016) reported that glutamate concentrations up to 1 mM undergo marked flooding into the extracellular space by ATP depletion. Several other studies have also reported that 1 mM glutamate is suitable for excitotoxicity (Sharp *et al*., 2003; Kuhlmann *et al*., 2008; Ahmed and Phillips, 2011). The MTT assay showed induction of cell death by 72% compared to the control when cells were incubated for 24 h after treatment with 1 mM glutamate. However, following co-treatment with 20 mM L-citrulline, L-arginine was observed to protect against glutamate cytotoxicity. Previous studies have shown that L-arginine has protective effects against motor neuron death associated with glutamate-induced excitotoxicity in ALS cell lines, which

Table 1. The effect of L-citrulline pretreatment and difference in the [^{14}C]L-citrulline uptake between WT and MT cell lines under various pathological conditions

	[^{14}C]L-Citrulline uptake (Cell/medium ratio)			
	WT	MT	Uptake	+ Citrulline Effect
Control	64.8 ± 4.6	33.3 ± 1.4***		
+ L-Citrulline (20 mM)	79.3 ± 3.1	47.8 ± 0.3$^{###}$	↑	↑
+ Glutamate (1 mM)	39.8 ± 0.9	26.1 ± 4.1$^{###}$	↓	↑
+ LPS (20 ng/mL)	51.9 ± 1.3	30.1 ± 0.6$^{###}$	↓	↑
+ TNF-α (20 ng/mL)	55.5 ± 0.9	28.8 ± 1.1$^{###}$	↓	↑
+ Hypertonic (390 mOsm)	45.2 ± 6.9	30.3 ± 0.9$^{#}$	↓	↓
+ High glucose (25 mM)	92.2 ± 2.8	76.5 ± 5.4$^{##}$	↑	↓

NSC-34 cell lines were exposed to various pathological states and incubated for 24 h and [^{14}C]L-citrulline uptake was performed. Each points represent the mean ± SEM (n=3). ***p<0.001, significant difference vs. WT control. $^{###}p$<0.001, $^{##}p$<0.01, $^{#}p$<0.05 significantly different from the values of their respective pathological states of WT.

may be related to arginase I activity (Lee *et al*., 2009b). In addition, we found that L-citrulline restored cells killed by glutamate cytotoxicity.

Similarly, the study of L-citrulline transport in the MT cell line with glutamate cytotoxicity showed that the uptake and mRNA expression levels of LAT1 were significantly increased in the MT cell line co-pretreated with L-citrulline and L-arginine compared to glutamate alone (Fig. 2). Our findings suggest that L-citrulline treatment may increase L-citrulline transport owing to increased levels of LAT1 mRNA in the MT cell line with glutamate cytotoxicity.

Previous studies have demonstrated that free radical nitric oxide (NO) has a cytotoxic effect in the ALS animal model (Almer *et al*., 1999; Hensley *et al*., 2006; Zhang *et al*., 2008). Similarly, with respect to the effect of L-citrulline transport in ALS cells exposed to inflammatory cytokines, the cells were pre-treated with 20 ng/mL TNF-α and LPS with or without 20 mM of L-citrulline for 24 h and the rates of [^{14}C]L-citrulline uptake and mRNA levels were analyzed. Consequently, the [^{14}C] L-citrulline uptake and LAT1 mRNA expression level of the ALS cells (MT) decreased significantly compared to the control; however, the addition of L-citrulline had a restorative effect (Fig. 3). Previous studies have demonstrated that TNF-α is associated with the stimulation of NO production, which is related to the induction of inducible nitric oxide synthase (iNOS) (Fonseca *et al*., 2003). Other studies have shown that LPS induces the production of NO, a member of the ROS superfamily (Hsu and Wen, 2002), which plays a major role in the pathogenesis of ALS (Pollari *et al*., 2014). We therefore presumed the decreased transport activity upon exposure to inflammatory cytokines to have resulted from NO production. NO assay results showed significant increase in NO levels on treatment with both LPS and TNF-α; however, treatment with 20 mM of L-citrulline had a restorative effect against increased NO levels. Therefore, NO production is the major cause of decreased transport activation in inflammatory states; however, further studies are required to elucidate the detailed mechanism.

In addition, NO levels (via Griess reagent) were measured in the MT cell line treated with glutamate, and NO concentrations in the glutamate-treated cell line were found to be significantly higher, whereas co-treatment with 20 mM L-citrulline and L-arginine reduced the concentration of NO in the glutamate-treated cell lines (Fig. 4). According to a previous study, iNOS is mainly responsible for NO generation via the release of chemotactic factors and glial activation (Dawson *et al*., 1993) and L-arginine supplementation decreases the level of iNOS and improves nNOS and arginase I levels in motor neurons (Lee *et al*., 2009a).

Assessment results of the pre-treatment effect of L-citrulline transport under hypertonic state showed that both the uptake and expression levels of mRNA were significantly decreased under hypertonic conditions compared to those under isotonic conditions (Fig. 5). However, when the cell line was co-pretreated with 2 mM L-citrulline, the uptake and LAT1 mRNA expression were downregulated. Conversely, previous studies on taurine have shown increased uptake of taurine in the hypertonic medium of rat brain capillary endothelial (TR-BBB13) cells compared to isotonic conditions (Kang *et al*., 2002). Another study on taurine uptake in epithelial intestinal cells (Caco-2) showed an increase in taurine transport in a hypertonic state (Satsu *et al*., 1999). However, the exact mechanism underlying the observed decrease in citrulline uptake is unknown. Previous studies have demonstrated that most cells respond to swelling or shrinkage by activating either metabolic or membrane transport processes (McManus *et al*., 1995). Furthermore, endogenous NO is synthesized during osmotic challenge (da Silva *et al*., 2014), and NOS activity is induced under hypertonic stress (Li *et al*., 2021). However, a decrease in LAT-1 transporter activity can be inferenced to have been the cause of the reduced uptake rate of [^{14}C]L-citrulline. In contrast, the uptake and expression levels of mRNA significantly increased when the MT cell line was treated with high glucose condition for 24 h. However, a significant reduction in the uptake and LAT1 mRNA expression levels was observed upon the addition of 2 mM L-citrulline to the MT cell line treated under high glucose conditions. High glucose levels have been reported to upregulate HIF-1 activity (Lee and Kang, 2015). However, its effects on ALS model cell lines are unknown.

To determine whether the increased [^{14}C] L-citrulline uptake was related to the expression level of HIF-1, we measured HIF-1 mRNA expression levels in MT cells. The expression level of HIF-1 was significantly decreased in cells exposed to high glucose conditions for 24 h (Fig. 6). Therefore, the increased uptake activity of [^{14}C]L-citrulline in ALS cells was associated with the upregulated expression level of HIF-1. Table 1 summarizes the effects of L-citrulline on ALS model cell lines under different conditions. Co-treatment of

unlabeled L-citrulline showed protective effects and resulted in increased [^{14}C]L-citrulline uptake under inflammatory states including glutamate, LPS, and TNF-α. In the case of hypertonic stress, L-citrulline uptake was downregulated even with unlabeled L-citrulline. In contrast, under high glucose conditions, L-citrulline uptake increased, whereas co-treatment with L-citrulline decreased [^{14}C]L-citrulline uptake.

The study results suggest that alterations in L-citrulline transport and transporter levels are associated with ALS pathogenesis. In addition, L-citrulline can restore the level of NO and its cellular uptake in the MT cell line with glutamate cytotoxicity. L-Citrulline can potentially increase the transport function of L-citrulline in LPS and TNF-α treated cells. Therefore, a delivery strategy for LAT1 mediated transport and supplementation of L-citrulline in ALS could potentially serve as a therapeutic approach thereby providing the neuroprotection.

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