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The alteration of serine transporter activity in a cell line model of amyotrophic lateral sclerosis (ALS)

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ABSTRACT

The alteration of D-serine levels is associated with the pathogenesis of sporadic ALS and mutant SOD1 (G93A) animal model of ALS. However, the exact mechanism of D-serine transport is not known in ALS. To better understand the distribution of D-serine in ALS, we determined the activity and the expression of serine transporter in a motor neuronal cell line model of ALS (NSC-34/hSOD1^{G93A} cells). The uptake of [^{3}H]D-serine was significantly lower in NSC-34/hSOD1^{G93A} cells than in control NSC-34 and NSC-34/hSOD1^{wt} cells. In contrast, the uptake of [^{3}H]L-serine, precursor of D-serine, was markedly increased in NSC-34/hSOD1^{G93A} cells compared to control NSC-34 and NSC-34/hSOD1^{wt} cells. Both [^{3}H]D-serine and [^{3}H]L-serine uptake were saturable in these cells. The estimated Michaelis-Menten constant, K_m, for D-serine uptakes was higher in NSC-34/hSOD1^{G93A} cells than in NSC-34/hSOD1^{wt} cells while the K_m for L-serine uptake was 2 fold lower in NSC-34/hSOD1^{G93A} cells than in control cells. [^{3}H]D-serine and [^{3}H]L-serine uptakes took place in a Na^+-dependent manner, and both uptakes were significantly inhibited by system ASC (alanine-serine-cysteine) substrates. As a result of small interfering RNA experiments, we found that ASCT2 (SLC1A5) and ASCT1 (SLC1A4) are involved in [^{3}H]D-serine and [^{3}H]L-serine uptake in NSC-34/hSOD1^{G93A} cells, respectively. The level of SLC1A4 mRNA was significantly increased in NSC-34/hSOD1^{G93A} compared to NSC-34 and NSC-34/hSOD1^{wt} cells. In contrast, the level of SLC7A10 mRNA was relatively lower in NSC-34/hSOD1^{G93A} cells than the control cells. Together, these data suggest that the pathological alteration of D- and L-serine uptakes in ALS is driven by the affinity change of D-and L-serine uptake system.

1. Introduction

D-Serine is a co-agonist at the *N*-methyl-D-aspartate (NMDA) receptors mediating several physiological and pathological processes, including neurotransmission, memory formation, and neurotoxicity [1–3]. Abnormal levels of D-amino acid have been reported in aging and Alzheimer's disease. Recently, a direct link has emerged between D-serine and a chronic neurodegenerative disease called amyotrophic lateral sclerosis (ALS). ALS is a common adult-onset neuromuscular disease characterized by selective loss of motor neurons leading to progressive muscle wasting and fatal paralysis [4]. The link between D-serine and ALS was first indicated by findings that reported D-serine level elevation in sporadic ALS and also in the mutant SOD1 mouse models of ALS [5,6]. D-Serine is synthesized from L-serine by the enzyme serine racemase (SR) [7]. Previous studies have reported that D-serine levels are increased by mutant SOD1 (G93A), perhaps via generalized glial activation and resulting induction of SR expression [8]. Furthermore, previous studies have identified a unique mutation in the D-amino acid oxidase (DAO) gene, which is involved in the encoding of a D-serine degrading enzyme and reported to be associated with the classical familial ALS [9].

To understand the complete role of D-serine in ALS, the production, storage, release, transport, and degradation of D-serine must all be better understood. Especially, the transport mechanisms of D-serine remain uncertain in ALS. Specific transporters contribute to the rapid and efficient transport of D-serine and L-serine. The neuronal, Na^+ independent alanine-serine-cysteine 1 (Asc-1) transporter has a high affinity for serine, alanine, cysteine,

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and threonine, and is selective for the small neutral D- and L-amino acids [10,11]. Asc-1 is responsible for most of the D-serine uptake by synaptosomes [12]. Another candidate for D-serine uptake is the Na^+-dependent alanine-serine-cysteine-threonine 2 transporter (ASCT2), which has a high affinity for L-alanine, L-serine, L-threonine, L-glutamine, and L-asparagine, and has a comparatively lower affinity for D-serine [13,14]. In addition, alanine-serine-cysteine-threonine 1 transporter (ASCT1) has a high affinity for serine, alanine, cysteine, and threonine, and is selective for the small neutral L-amino acids [15,16].

In this study, we examined the changes of gene expression and activity of serine transporters, ASC1, ASCT1 and ASCT2, in the mutant SOD1 (G93A) transgenic cell line model of ALS.

2. Materials and methods

2.1. Materials

Serine, L-[^{3}H(G)] ([^{3}H]L-serine, 29.5 Ci/mmol) and serine, D-[^{3}H(G)] ([^{3}H]D-serine, 20 Ci/mmol) were purchased from PerkinElmer Inc. (Waltham, MA, USA). All other chemicals and reagents were commercial products of reagent grade and were purchased from Sigma Chemical (St. Louis, MO, USA).

2.2. Cell culture

Motor neuron-like cells (NSC-34) transfected with pCl-neo expression vector containing human wild-type (control NSC-34), hSOD1 WT (NSC-34/hSOD1^{wt} cells) and mutant hSOD1 G93A (NSC-34/hSOD1^{G93A} cells) were established previously [17,18]. The cells were cultured routinely in a humidified atmosphere of 5% CO_2/air. Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Salt Lake City, Utah, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Salt Lake City, Utah, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Hyclone, Salt Lake City, Utah, USA). On rat tail collagen type I-coated 24 well culture plates (IWAKI, Tokyo, Japan), initial seeding was done at 1×10^5 cells/well then the plates were incubated for 2 days at 37 °C.

2.3. In vitro uptake studies in NSC-34 cells

The [^{3}H]L-serine or [^{3}H]D-serine uptake by the cells was performed according to the previous study [19]. Briefly, extracellular fluid (ECF) buffer [^{3}H]L-serine (17 nM) or [^{3}H]D-serine (25 nM) in the presence or absence of unlabeled inhibitors was added to NSC-34, NSC-34/hSOD1^{wt}, and NSC-34/hSOD1^{G93A} cells, and then incubated at 37 °C for the designated time. The cells were then solubilized in 1 N NaOH, and radioactivity was measured. Na^+ free transport medium was prepared by choline chloride and *N*-methyl-D-glucamine (NMDG) instead of NaCl and $NaHCO_3$, respectively. Cell to medium ratio (μL/mg protein) was calculated as follows:

Cell to medium ratio (μL/mg protein) = ([^{3}H]dpm in the cell/amount (mg) of cell protein)/([^{3}H]dpm in the medium/amount (μL) of medium) × 100

2.4. Data analysis

For kinetic studies, the Michaelis-Menten constant (K_m), the maximum uptake rate (V_{max}), and the first-order constant for the non-saturable component (K_{ns}) of [^{3}H]L-serine or [^{3}H]D-serine were estimated from the following equation:

$$V = V_{max} \cdot C/(K_m + C) + K_{ns} \cdot C$$

where V and C are the initial uptake rate of [^{3}H]L-serine or [^{3}H]D-serine at 5 min and the concentration of L-serine or D-serine, respectively.

Statistical analyses were carried out by one-way ANOVA with Dunnett's post-hoc test.

2.5. SLC1A4, SLC1A5, and SLC7A10 small interfering RNA and small interfering RNA transfection

Transient knockdown of SLC1A4, SLC1A5, and SLC7A10 in NSC-34, NSC-34/hSOD1^{wt}, and NSC-34/hSOD1^{G93A} cells was achieved using small interfering RNA (siRNA) from Pharmacon, GE (Landsmeer, Netherlands). SLC1A4, SLC1A5, and SLC7A10 were targeted with a SMARTpool comprised of 4 different siRNAs and with each single siRNA individually. The end concentration of siRNA was 200 nM. The SLC1A4, SLC1A5, and SLC7A10 or control siRNA was delivered to cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Cells were used for Western blotting, and [^{3}H]L-serine or [^{3}H]D-serine uptake was analyzed at 48 h after the initiation of transfection.

2.6. Quantitative real time-PCR (qRT-PCR)

Total RNA was extracted from NSC-34, NSC-34/hSOD1^{wt}, and NSC-34/hSOD1^{G93A} cells by TRIzol reagent (MRC, TR118). RNA was measured in a spectrophotometer at 260 nm absorbance. RNA analysis was conducted as follows: Fifty nanograms of RNA were used as a template for quantitative RT-PCR amplification, using SYBR Green Real-time PCR Master Mix (Toyobo, QPK-201). Primers were standardized in the linear range of cycle before the onset of the plateau. Primer sequences are given in Table S1. GAPDH was used as an internal control. Two-step PCR thermal cycling for DNA amplification and real-time data acquisition were performed with an ABI StepOnePlusTM Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using the following cycle conditions: 95 °C for 1 min x 1 cycle, and 95 °C for 15 s, followed by 60 °C for 1 min x 40 cycles. Fluorescence data were analyzed by the ABI StepOnePlus software and expressed as Ct the number of cycles needed to generate a fluorescent signal above a predefined threshold. The ABI StepOnePlus software were used to set baseline and threshold values.

3. Results

3.1. Change of [^{3}H]D-serine transport in ALS cell line model

To investigate whether the activity of D-serine transport is altered in ALS cell line model or not, we performed an uptake study of [^{3}H]D-serine in NSC-34 cells. The uptake of [^{3}H]D-serine in NSC-34/hSOD1^{G93A} cells was significantly lower than in control NSC-34 and NSC-34/hSOD1^{wt} cells (Fig. 1A).

To clarify why the [^{3}H]D-serine uptake is decreased in NSC-34/hSOD1^{G93A} cells, initial uptake of [^{3}H]D-serine was analyzed according to the Michaelis-Menten equation. The initial uptake of [^{3}H]D-serine in NSC-34 cells showed a concentration-dependent manner (Fig. 1B). The obtained parameters are listed in Table 1. Both Eadie-Hofstee plot and Lineweaver Burk plot clearly showed that the K_m value was much larger in NSC-34/hSOD1^{G93A} cells, whereas the V_{max} value was similar in both cell lines (Fig. 1C and D). These results indicate that SOD1 mutation may affect the affinity of the transporter for D-serine. Indeed, the intrinsic uptake clearance of D-serine was 2 times lower in NSC-34/hSOD1^{G93A} cells (Table 1).

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