Inositol Hexakisphosphate Kinase 2 is a Presymptomatic Biomarker for Amyotrophic Lateral Sclerosis

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder impairing both upper and lower motor neurons. Its main symptoms are limb weakness, bulbar palsy, and respiratory failure. The cause of ALS is unclear, but aging, environmental factors, and genetic factors are considered to be involved. More than 90% of ALS cases are sporadic, whereas the remaining 10% of cases are familial. The most common mutation in familial ALS is a mutation in the human superoxide dismutase (SOD1) gene, and G93A mSOD1 Tg mice have been used to elucidate the mechanisms of ALS neuropathogenesis [1, 2]. Riluzole is the only therapeutic agent for this condition; however, even with riluzole treatment, ALS is usually progressive and fatal [3, 4].

Inositol phosphates act as important signaling molecules in cells. Inositol 1, 4, 5-trisphosphate (InsP3) is a well-known inositol phosphate that acts as a second messenger for calcium release in cells. InsP3 serves as a precursor of highly phosphorylated inositol polyphosphates such as inositol tetrakisphosphate (InsP4), inositol pentakisphosphate (InsP5), and inositol hexakisphosphate (InsP6). There are also higher inositol phosphates, such as disphosphoinositol pentakisphosphate (InsP7) and bis-(disphospho)-inositol tetrakisphosphate (InsP8) [5–7]. InsP7 can compete for pleckstrin homology (PH) domain binding with PtdIns(3, 4, 5)P3 and serve as a phosphate donor to other proteins [8]. In mammals, the formation of InsP7 is mediated by a family of inositol hexakisphosphate kinases (InsP7Ks), which constitute three subtypes: InsP7K1, InsP7K2, and InsP7K3. Notably, InsP7K2 is usually localized exclusively in the nucleus, whereas the other two enzymes are found in both the cytoplasm as well as the nucleus [9–12]. When InsP7K2 is activated, it is translocated to the cytoplasm [13, 14].

We had previously reported that InsP7K2 induces cell death by converting InsP6 to InsP7 [14, 15]. InsP7K2 is abundant in the central nervous system, especially in the anterior horn cells of the spinal cord. To elucidate the role of InsP7K2 in ALS, we investigated the expression level of InsP7K2, employing G93A mSOD1 Tg mice.

MATERIALS AND METHODS


Mice

We used G93A mSOD1 Tg mice at postnatal week 6 (n = 6), week 12 (before the onset of motor neuron disorders, n = 6), week 17 (presenting motor neuron...
disorders, n = 7), and after week 17 (terminal stage of motor neuron disorders, n = 7) and compared them with wild-type littermates in the C57BL/6/J background (n = 31) of the same age. Specimens of the spinal cord were obtained from these mice.

**Immunostaining against InsP_{K2}**

Anesthesia was induced in the mice with 4% isoflurane/66% N2O/30% O2 and maintained with 1.5% isoflurane. The mice were perfused from the apex of the heart with phosphate-buffered saline (PBS) and perfusion-fixed with 4% paraformaldehyde (PFA) in PBS. Spinal cords were taken from the mice and immersion-fixed overnight at 4°C in 4% PFA with rocking and subsequently cryoprotected in 10% (2 h), 15% (2 h), 20% (2 h), and 25% (overnight) sucrose in PBS at 4°C. The slices were then embedded in OCT compound (Miles Scientific, DE, USA) and quickly frozen in isopentane. Coronal frozen sections (10 μm) were prepared on a cryostat and stored at −80°C until use. The frozen sections were thawed, washed thrice in PBS, permeabilized with 0.1% Triton X-100/PBS at room temperature for 5 min, and then blocked in 5% skim milk/3% bovine serum albumin (BSA)/PBS for 60 min. InsP_{K2} was detected with the use of anti-InsP_{K2} antibody that we prepared ourselves. The slides were incubated with primary antibodies (1:200) at 4°C overnight and with the secondary antibodies at room temperature for 2 h, and immunoreactivity was visualized by the ABC method. The immunohistochemical images were taken by OLYMPUS BX51 with the use of DP Controller software.

**Gene expression of InsP_{K}s**

Fresh spinal cord tissues were conserved in RNALater (Life Technologies, CA, USA) at −20°C and homogenized. Total RNA was extracted from these spinal cord tissues with Trizol RNA extraction reagent. Extracted total RNA was treated with DNase I and purified with RNeasy Mini Kit (Qiagen, Venlo, Netherlands). cDNA was synthesized using a SuperScriptIII cDNA synthesis kit (Invitrogen, CA, USA). The Step One Plus Real-Time PCR System (Applied Biosystems, CA, USA) was used with each custom-designed, gene-specific primer/probe set to amplify and quantify each mouse InsP_{K} mRNA. The reactions were performed by the comparative threshold (Ct) method ([16]). InsP_{K1}, InsP_{K2}, and InsP_{K3} mRNA expression in wild-type mice at postnatal week 6 was set as 1.0 for calibration.

**Protein expression**

The samples were homogenized and fractionated to the cytoplasm and the nucleus with a ProteoExtract Subcellular Proteome Extraction Kit (Merck KGaA, Darmstadt, Germany). These samples were separated by electrophoresis through 4%–12% gradient gels. After electrophoretic transfer to a polyvinylidene fluoride membrane (Immobilion-P; Millipore, MA, USA), the membranes were blocked with 4% BSA in PBS. The membranes were washed and incubated with the primary antibodies at 4°C overnight. After incubation with the primary antibodies, the membranes were washed with PBS-T (0.1% Tween 20) and incubated with secondary antibodies (Vector Laboratories, CA, USA) for 2 h at room temperature. The membranes were then examined with an enhanced chemiluminescence western blotting system (Amersham Pharmacia, NJ, USA). In this study, we compared the density ratio of InsP_{K2} protein expression in the cytoplasm and the nucleus among these mice.

**Statistical analysis**

Welch’s t-test was used for statistical analyses with SPSS Statistics (IBM, NY, USA). A P value < 0.05 was considered to indicate a statistically significant difference. Data are expressed as means ± SD.

**RESULTS**

**InsP_{K2} translocated from the nucleus to the cytoplasm in mSOD1 Tg mice**

The anterior horn cells of the spinal cord in a wild-type mouse were strongly stained with anti-InsP_{K2} antibody. InsP_{K2} was mainly stained in the nucleus in wild-type mice, whereas it was stained in the cytoplasm in the mSOD1 Tg mice in each period (Fig. 1). The ratios of the band density of the cytoplasmic/nuclear fractions in mSOD1 Tg mice were higher than those in wild-type mice at each developmental stage, especially in postnatal weeks 12 and 17, when they were significantly higher (Fig. 2).

**InsP_{K2} in mSOD1 Tg mice is expressed before the onset of clinical symptoms of ALS in the spinal cord compared with wild-type mice**

The gene expression levels of InsP_{K1} in mSOD1 Tg mice at each week were not significantly different from those in wild-type mice (Fig. 3). InsP_{K3} was very faintly expressed compared with InsP_{K1} and InsP_{K2} (data not shown). The gene expression levels of InsP_{K2} in mSOD1 Tg mice at postnatal week 12 were 2.6 times higher than those in wild-type mice at postnatal week 12. However, the gene expression levels of InsP_{K2} in mSOD1 Tg mice at postnatal week 17 were lower than those in wild-type mice at postnatal week 17 (Fig. 4).

**DISCUSSION**

ALS is a neurodegenerative disorder that is progressive, intractable, and fatal. ALS is often difficult to diagnose, especially in its early stages. The standard clinical criteria for the diagnosis of ALS are the revised El-Escorial criteria, also known as the Airlie House...
Fig. 1 Immunohistochemistry of inositol hexakisphosphate kinase 2 (InsP$_6$K2) in the anterior horn cells of mouse spinal cord at each developmental stage (Fig. 1A: reduced images of high-power field, Fig. 1B: high-power field). InsP$_6$K2 was stained mainly in the nucleus in wild-type mice, whereas it was stained in the cytoplasm in mSOD1 Tg mice at each developmental stage. The immunohistochemical images were taken at 400 × magnification.

Fig. 2 Immunoblotting against anti-inositol hexakisphosphate kinase 2 (InsP$_6$K2) antibody on cell fractions of mouse spinal cord at each developmental stage. The ratios of the band density of the cytoplasmic/nuclear fractions in transgenic mice expressing mutant superoxide dismutase-1 (SOD1) (mSOD1 Tg mice) mice were higher than those in wild-type mice. These findings show that InsP$_6$K2 is activated in the mSOD1 Tg mouse. The immunoblot figure was spliced from three sections. C, cytoplasm; N, nucleus.
Fig. 3  Quantitative PCR of inositol hexakisphosphate kinase 1 (InsP$_6$K1) mRNA expression in spinal cord of wild-type mice and transgenic mice expressing mutant superoxide dismutase-1 (SOD1) (mSOD1 Tg mice) at each development stage. The gene expression levels of InsP$_6$K1 in mSOD1 Tg mice at each week were not significantly different from those of wild-type mice.

Fig. 4  Quantitative PCR of inositol hexakisphosphate kinase 2 (InsP$_6$K2) mRNA expression in spinal cord of wild-type mice and transgenic mice expressing mutant superoxide dismutase-1 (SOD1) (mSOD1 Tg mice) at each developmental stage. The gene expression levels of InsP$_6$K2 in mSOD1 Tg mice at postnatal week 12 were 2.6 times higher than those of wild-type mice at postnatal week 12. However, the gene expression levels of InsP$_6$K2 in mSOD1 Tg mice at postnatal week 17 were lower than those of wild-type mice at postnatal week 17.
criteria. The revised El-Escorial criteria were designed to be of limited sensitivity for ALS, although their specificity was higher, particularly in the early stages of ALS [17–19]. Consequently, significant diagnostic delays are inevitable, leading to a delay in the establishment of neuroprotective therapies and recruitment into therapeutic trials, perhaps beyond the therapeutic window period. The neurophysiologically based Awaji criteria were developed for use in conjunction with the clinical criteria, in an attempt to minimize diagnostic delays [20]. With the use of the revised El-Escorial and Awaji criteria, ALS is diagnosed by clinical upper and lower motor neuron signs and electromyographic findings. A more useful diagnostic marker is required for ALS, such as a peripheral blood test, a cerebrospinal fluid (CSF) test, or an imaging study.

TAR DNA-binding protein 43 (TDP-43) has been identified as a major component of ubiquitin-positive inclusions in the brain and spinal cord of patients with frontotemporal lobar degeneration with ubiquitinated inclusions or sporadic ALS. Mutations in the corresponding gene have also been identified [21, 22]. TDP-43 levels in CSF are elevated in ALS patients compared with those in patients with other neurodegenerative or inflammatory diseases [23]. Thus, the sensitivity of CSF TDP-43 measurement for diagnosing ALS is relatively low.

Various biomarkers for ALS have been previously reported. Among the biochemical markers, phosphorylated NF-H [24] and the ratio of CD14 to S100 beta [25] in CSF are elevated in ALS patients, whereas cystatin C [26], CCL4, CXCL10 [27], angiotensin II [28] follistatin, interleukin-1 alpha, and kallikrein-5 [29] are decreased. On the other hand, among the biophysiological markers, a high rate of abnormalities of central motor conduction on transcranial magnetic stimulation was found in patients with ALS [30]. For bioimaging of ALS, voxel-based morphometry [31] and diffusion tensor imaging [32, 33] have been reported. However, to date, we have found no suitable specific biomarkers for ALS.

InsP₆Ks and inositol phosphates also play important roles in export of mRNA from the nucleus to the cytoplasm in Saccharomyces cerevisiae [34]. Moreover, InsP₆K₂ is mainly localized to the nucleus when the cell is in a normal state. Upon activation, InsP₆K₂ is translocated to the cytoplasm [14]. InsP₆K₂ activation generates InsP₆, from InsP₆. We previously demonstrated that InsP₆ competes with PtdIns(3, 4, 5)P₂ for binding to several mammalian PH-domain-containing proteins, including Akt, PIKE, and Tiam [8]. Because InsP₆ can compete with Akt for binding, a high concentration of InsP₆ would occupy more of the PH domain of Akt, thereby inhibiting Akt phosphorylation. Consequently, the activation of mTOR, which is located downstream in the Akt/PIP3 pathway, would be suppressed [35]. InsP₆K₂ is a proapoptotic protein regulated by HSP90 [36], which binds and inactivates it, as well as by casein kinase 2, which phosphorylates InsP₆K₂ to enhance its proteasomal degradation. Apoptotic stimuli stabilize, activate, and facilitate the nuclear translocation of InsP₆K₂, where it binds p53 and augments its apoptotic actions [37]. We reported that cell death was augmented in the presence of cytoplasmic TDP-43 aggregations and activated InsP₆K₂.

In the presence of both TDP-43 aggregation and activated InsP₆K₂ in the cytoplasm of cells, the expression levels of HSP90 and casein kinase 2 and the activity of Akt decreased [38].

As mentioned above, InsP₆K₂ is a part of the apoptotic pathway. In this study, InsP₆K₂ might have been activated in mSOD1 Tg mice. Notably, the expression of InsP₆K₂ and the translocation of InsP₆K₂ protein to the cytoplasm were increased at postnatal week 12 before the onset of motor neuron disorders in mSOD1 Tg mice. The gene expression levels of InsP₆K₂ in mSOD1 Tg mice at postnatal week 12 were significantly higher than those of wild-type mice at postnatal week 12. However, the gene expression levels of InsP₆K₂ in mSOD1 Tg mice at postnatal week 17 were lower than those in wild-type mice at postnatal week 17. It is considered that anterior horn cells in the spinal cord of mSOD1 mice were degenerated and reduced in its stage [39–41]. These results that InsP₆K₂ might be activated in mSOD1 Tg mice before the onset of motor neuron disorders. Moreover, InsP₆K₂ can serve as a diagnostic biomarker for an early stage of ALS.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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CONTRIBUTORS

YM, EN, and ST planned the experiments. SH provided the animals and information on the animals. YM and EN interpreted the data and wrote the manuscript. YM, EN, TS, and HO performed the experiments. YM provided the animals and information on the animals. The authors wish to acknowledge the Support Center for Medical Research and Education staffs, Tokai University and S. Kohara for excellent technical supports. The authors would thank Enago (www.enago.jp) for the English language review.

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