Research Article

α-Synuclein Modification in an ALS Animal Model

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Amyotrophic lateral sclerosis (ALS) is a progressively paralytic neurodegenerative disease that can be caused by mutations in Cu/Zn-superoxide dismutase 1 (SOD1). Transgenic mice that overexpress mutant SOD1 develop paralysis and accumulate aggregates of mutant protein in the brainstem and spinal cord. Bee venom (BV), which is also known as apitoxin, is extracted from honeybees and is commonly used in oriental medicine for the treatment of chronic rheumatoid arthritis and osteoarthritis. The purpose of the present study was to determine whether BV affects misfolded protein aggregates such as alpha-synuclein, which is a known pathological marker in Parkinson disease, and ubiquitin-proteasomal activity in hSOD1G93A mutant mice. BV was bilaterally administered into a 98-day-old hSOD1G93A animal model. We found that BV-treated hSOD1G93A transgenic mice showed reduced detergent-insoluble polymerization and phosphorylation of α-synuclein. Furthermore, phosphorylated or nitrated α-synuclein was significantly reduced in the spinal cords and brainstems of BV-treated hSOD1G93A mice and reduced proteasomal activity was revealed in the brainstems of BV-treated symptomatic hSOD1G93A. From these findings, we suggest that BV treatment attenuates the dysfunction of the ubiquitin-proteasomal system in a symptomatic hSOD1G93A ALS model and may help to slow motor neuron loss caused by misfolded protein aggregates in ALS models.

1. Introduction

The neuropathological hallmarks of ALS are significant motor neuron loss, Bunina bodies, and the abnormal accumulation of insoluble ubiquitinated cytoplasmic inclusions in lower motor neurons. The neurodegenerative processes in motor neurons are complex. Although several genetic mutations are involved in motor neuron injury in familial ALS, less is known about the genetic and environmental factors that contribute to sporadic ALS. The etiology of ALS is a complex interplay between multiple pathogenic processes, which include misfolded protein aggregates, TDP-43 abnormalities, increased oxidative stress, mitochondrial dysfunction, ribosomal dysfunction, disturbance of intracellular trafficking, dysfunction of the ubiquitin-proteasomal and autophagic systems, increased glutamate excitotoxicity, and impaired axonal transport [1–6].

Parkinson's disease (PD), one of neurodegenerative movement disorders, is caused by selective degeneration of nigrostriatal dopamine neurons in the substantia nigra pars compacta (SNc). The physiological function of alpha-synuclein (α-synuclein), which is abundantly expressed in the central nervous system, is not fully understood. Its enrichment in presynaptic terminals and association with vesicles suggest a role for α-synuclein in synaptic dynamics. Synuclein has been extensively studied in the pathogenesis of Parkinson's disease (PD). Aggregation of α-synuclein in intracytoplasmic Lewy bodies is a key pathological feature of both sporadic and familial PD. In addition, duplication, triplication, or mutations in the α-synuclein gene cause some forms of familial PD [7]. Chung et al. have shown α-synuclein immunoreactivity in the brain and spinal cord of hSOD1G93A mice [8].

Bee venom (BV) is an oriental medicine that has been used for the treatment of inflammatory diseases such as rheumatoid arthritis and for the relief of pain [9]. BV consists of many biologically active enzymes, peptides, and biogenic amines, such as melittin (the major active ingredient of bee venom), apamin, adolapin, and mast cell-degranulating peptide [10]. BV has been reported to cause growth arrest or cytotoxic effects in hepatocellular carcinoma cells [11]. In addition, the proliferation of melanoma cells, activity of
apoptotic enzymes (bcl-2 and caspase-3) in leukemic cells, and activation of the ERK and Akt signaling pathways in renal cancer cells have all been shown to be regulated and/or suppressed by BV [12]. Furthermore, we demonstrated that BV treatment reduced antineuroinflammatory events and increased the survival of hSOD1G93A mice, an ALS animal model used in previous studies [13]. Moreover, we found an increase of modified α-synuclein (phosphorylation or nitration) in the spinal cord and brainstem of symptomatic hSOD1G93A transgenic mice [14].

On the basis of these findings, we investigated BV effects on α-synuclein modification in the brainstem and spinal cord of symptomatic hSOD1G93A mice. We found that BV treatment reduced the expression of α-synuclein modifications such as nitration and phosphorylation. In addition, BV treatment diminished ubiquitinated α-synuclein and recovered proteasomal activity in the spinal cord of hSOD1G93A mice. Taken together, we suggest that BV treatment could be a useful therapy to reduce cell loss or death caused by protein misfolding in a neurodegenerative disease model.

2. Materials and Methods

2.1. Animals. All of the mice were handled in accordance with the protocols approved by the Institutional Animal Care and Use Committees of the Korea Institute of Oriental Medicine. Hemizygous hSOD1G93A transgenic B6SJL mice were originally obtained from Jackson Laboratories (Bar Harbor, ME). Transgenic mice were identified by PCR as described previously [15]. All of the mice were maintained in standard housing with free access to water and standard rodent chow purchased from Orient Bio (Orient, Gyeonggi-do, Korea).

2.2. Bee Venom Treatment. Bee venom (BV) was purchased from Sigma (St. Louis, MO) and diluted in saline. At a dose of 0.1 μg/g, BV was bilaterally injected (subcutaneously) twice a week into 14-week-old male hSOD1G93A transgenic mice at the Zusanli (ST36) acupoint, as determined by the human acupoint landmark and a mouse anatomical reference [16], which is known to mediate anti-inflammatory effects in an ALS animal [13]. Control animals (hSOD1G93A) were bilaterally injected (subcutaneously) with an equal volume of saline at the ST36 acupoint.

2.3. Immunoprecipitation and Western Blotting Analysis. After the mice were sacrificed, the brainstem and spinal cord were immediately homogenized in ice-cold RIPA buffer containing 50 mM Tris-HCl pH 7.4, 1% NP-40, 0.1% SDS, and 150 mM NaCl and then centrifuged at 14,000 rpm for 30 min at 4°C. The protein concentration in the supernatant was determined using a BCA protein assay kit (Interchim, Paris, France). The proteasomal activity was measured according to the manufacturer’s instructions for the 20S Proteasome Activity Assay kit (Chemicon Inc., CA, USA). The proteasomal activity was determined as an increase in the fluorescence of the reaction products.

2.5. Proteasomal Activity Assay. Twenty days after saline or BV injection, the male hSOD1G93A transgenic mice were sacrificed. The brainstem and spinal cord from each mouse were removed and homogenized with RIPA lysis buffer and centrifuged at 14,000 rpm at 4°C for 20 min. Then, the supernatant fraction was collected into a new tube, and the protein concentration was determined using a BCA protein kit (Interchim, Paris, France). The proteasomal activity was measured according to the manufacturer’s instructions for the 20S Proteasome Activity Assay kit (Chemicon Inc., CA, USA). The fluorescence of the samples was evaluated with a spectrophotometer at excitation and emission wavelengths of 370 nm and 430 nm, respectively. Proteasomal activity was determined as an increase in the fluorescence of the reaction products.

2.6. Immunofluorescence. In this study, 16-week-old hSOD1G93A mice were sacrificed after anesthetizing with pentobarbital and perfusion with phosphate-buffered saline (PBS). The spinal cord tissue of the hSOD1G93A mice was fixed with 4% paraformaldehyde in PBS at 4°C overnight. The lumbar spinal cord was dissected, transferred into 30% sucrose, and then frozen. The lumbar spinal cord was
embedded in OCT compound, a freezing solution, and cut along the transverse plane on a Leica Microtome (Leica, Germany). Free-floating sections were washed three times with PBST and then incubated overnight at 4°C with primary antibodies (α-synuclein, 1:1000, and ubiquitin, 1:2000). The sections were washed three times in PBST and then incubated with Alexa Fluor 488 or 568 conjugated secondary antibodies (Molecular Probes, NY, USA). The ventral horns of the spinal cord sections were then imaged using an Olympus fluorescent microscope (Olympus, Tokyo, Japan) under identical exposure settings.

3. Results

3.1. BV Treatment Reduces Modified α-Synuclein Expression in the Brainstem and Spinal Cord of hSOD1<sup>G93A</sup> Mice. To assess whether BV treatment affects the expression and modification of α-synuclein, the total proteins were extracted in RIPA buffer and loaded onto a 15% SDS-PAGE. Consistent with previous studies, high molecular α-synuclein proteins were detected in the saline-treated brainstem and spinal cord (Figure 1(a)). However, phosphorylated or nitrated α-synuclein was reduced by BV treatment in the brainstem and lumbar spinal cord of hSOD1<sup>G93A</sup> mice (Figures 1(b) and 1(c)). Our data suggested that BV treatment attenuates α-synuclein modifications caused by motor neuron death in hSOD1<sup>G93A</sup> mice.

To provide further evidence of increases in phosphorylated α-synuclein in symptomatic hSOD1<sup>G93A</sup> mice, we examined the expression level of phospho-GSK3β with RIPA-extracted spinal cord and brainstem tissue in hSOD1<sup>G93A</sup> mice. As shown in Figure 1(d), the expression of phosphorylated GSK3β at Ser 9 was increased in the brainstem and spinal cord of hSOD1<sup>G93A</sup> mice. Interestingly, BV treatment reduced the phosphorylated levels of GSK3β in the brainstem and spinal cord of symptomatic hSOD1<sup>G93A</sup> transgenic mice compared to age-matched hSOD1<sup>G93A</sup> mice. This finding suggests that GSK3β activation may enhance α-synuclein phosphorylation at Ser 129 and contribute to motor neuron loss in symptomatic hSOD1<sup>G93A</sup> mice.

3.2. Modified α-Synuclein Is a Soluble Protein in hSOD1<sup>G93A</sup> Mice. To determine the solubility of modified α-synuclein in the brainstem and spinal cord of hSOD1<sup>G93A</sup> mice, we examined the solubility of α-synuclein with sequentially different buffers (high salt, RIPA buffer and 0.5% SDS buffer as an insoluble buffer). As shown in Figure 2(a), monomeric or modified α-synuclein was extracted in high salt buffer as a soluble protein, and BV treatment reduced the expression of modified α-synuclein in the brainstem of hSOD1<sup>G93A</sup> mice. Oligomeric (higher molecular weight) and modified (∼26 kDa) α-synuclein of the spinal cord in symptomatic hSOD1<sup>G93A</sup> mice were detected in high salt buffer as a soluble fraction, and modified α-synuclein was decreased by BV treatment (Figure 2(b)). However, insoluble α-synuclein was not detected in the brainstem or spinal cord of hSOD1<sup>G93A</sup> mice (Figures 2(a), and 2(b)).

3.3. BV Treatment Decreases Ubiquitinated α-Synuclein and Rescues the Reduced Proteasomal Activity in Symptomatic hSOD1<sup>G93A</sup> Mice. However, it has been controversial whether it induces neuronal toxicity. To investigate the effects of BV on ubiquitinated α-synuclein, we examined the expression level of ubiquitinated α-synuclein in the brainstem and spinal cord of hSOD1<sup>G93A</sup> mice.

As shown in Figure 3(a), ubiquitinated α-synuclein was reduced by BV treatment in the spinal cord of symptomatic
hSOD1<sub>G93A</sub> mice. In addition, we confirmed that ubiquitinated α-synuclein was localized in the motor neurons of hSOD1<sub>G93A</sub> mouse spinal cords (Figure 3(b)).

Next, to investigate whether modified α-synuclein expression affects the protein degradation system, we examined the proteasomal activity in the brainstem and spinal cord of BV-treated or age-matched control hSOD1<sub>G93A</sub> mice. BV treatment rescued 1.2-fold proteasomal activity in the spinal cord compared to that of age-matched symptomatic hSOD1<sub>G93A</sub> mice (P < 0.05, Figure 3(c)).

4. Discussion

In this study, we demonstrated that two subcutaneous injections of 0.1 μg/mL BV resulted in the reduced expression of α-synuclein and modified α-synuclein (phosphorylation, nitration, or ubiquitination) in the brainstem and spinal cord of symptomatic SOD1<sup>G93A</sup> transgenic mice. Furthermore, we observed that BV treatment diminished ubiquitinated α-synuclein and rescued proteasomal activity in the spinal cords of hSOD1<sub>G93A</sub> transgenic mice.

Neuroinflammatory responses are induced as a consequence of oxidative and excitotoxic neuronal damage, mitochondrial dysfunction, and protein aggregation [17, 18]. In addition, neuroinflammation may be a direct response to protein aggregation in neurodegenerative diseases [19]. Neuroinflammation and accumulated protein aggregates are related to oxidative stress and mitochondrial dysfunction; accumulated protein aggregates trigger microglial activation and neuroimmune responses [20]. In the case of ALS, abnormal protein aggregation, ubiquitination, and deposition in the CNS are salient features of ALS [21, 22].

The role of neuroinflammation in the disease progression of neurodegenerative diseases, including PD and AD [23] in animal models, has been highlighted in recent studies. For example, in the presence of a preexisting PD-relevant insult, such as 6-OHDA, the loss of parkin or α-synuclein overexpression and induction of neuroinflammation synergistically worsen the disease process [24]. Chung et al. have demonstrated that changes in proteins relevant to synaptic transmission and axonal transport, coupled with neuroinflammation, precede α-synuclein-mediated neuronal death [25].

Recently, several studies have shown that α-synuclein is expressed in symptomatic hSOD1<sub>G93A</sub> mice [8, 14]. In addition, our previous study demonstrated that α-synuclein modification increased in the brainstem and spinal cord of hSOD1<sup>G93A</sup> transgenic mice compared to age-matched controls [14]. Furthermore, we have shown that treatment with melittin, one of the components of BV, reduced α-synuclein modification and increased the activity of proteasomes in the spinal cord of mutant (G93A) hSOD1-expressing mice [14]. This study showed that BV treatment also decreased the expression level of soluble modified α-synuclein (phosphorylation or nitration) in the spinal cords of symptomatic hSOD1<sup>G93A</sup> transgenic mice (Figures 1 and 2). These findings suggest that anti-inflammation by BV treatment may be involved in the restoration of reduced proteasomal activity in symptomatic hSOD1<sup>G93A</sup> transgenic mice.

Inclusions formed mainly by monoubiquitinated α-synuclein are toxic to cells [26]. Baba et al. have shown that the oligomeric or protofibrillar forms are more toxic than the fibrillar forms of α-synuclein in disease [27]. In addition, soluble aggregated α-synuclein mediates dopaminergic neurotoxicity in Drosophila [28]. In an ALS animal model, we found that the level of modified soluble α-synuclein (∼26 kDa) was increased in the brainstem and spinal cord, and BV treatment reduced the modification of α-synuclein (Figure 2). Furthermore, we showed that the expression level of ubiquitinated α-synuclein was decreased in the spinal cord of symptomatic hSOD1<sup>G93A</sup> transgenic mice compared to age-matched control mice (Figures 3(a), and 3(b)). This result suggests that BV treatment may attenuate the impairment of α-synuclein expression in disease.
Figure 3: BV treatment attenuates ubiquitinated α-synuclein and recovered proteasomal activity in the spinal cord of symptomatic hSOD1<sup>G93A</sup> mice. The total homogenate was immunoprecipitated with an anti-α-synuclein antibody and then immunoblotted with an anti-ubiquitin antibody (a). Ubiquitinated α-synuclein of high molecular weight (∗) was detected in the spinal cords of saline-treated hSOD1<sup>G93A</sup> mice, but not in BV-treated mice. Left upper panel is a long-exposure blot and the lower panel is a short-exposure blot. α-synuclein and ubiquitin were colocalized in the lumbar spinal motor neurons of symptomatic hSOD1<sup>G93A</sup> mice (b). This image is representative of three independent experiments. Proteasomal activity was determined using a fluorescence enzymatic assay (c). BV treatment increased the proteasomal activity 1.5-fold in the spinal cords of symptomatic hSOD1<sup>G93A</sup> mice (n = 4) compared to age-matched controls (n = 4). Proteasomal activity was reported in arbitrary fluorescence units (mean ± SEM of three independent experiments). Values that were significantly different from relative controls are indicated with an asterisk when P < 0.01. A.F.U. = arbitrary fluorescence units (mean ± SEM).
of proteasomal activity by the expression or accumulation of modified proteins involved in neurodegenerative events. However, further study of whether fibrillar or oligomeric forms α-synuclein are more toxic than soluble α-synuclein in ALS animal or cell models is needed.

The ubiquitin-proteasome system is a major proteinase system that plays important roles in a vast array of cellular processes, including protein trafficking, antigen presentation, and protein degradation under physiological conditions [29]. Ubiquitinated proteins accumulate to produce protein aggregates in AD, PD, and ALS [30–32]. In the case of ALS, these aggregates are thought to be composed of ubiquitin, SOD-1, some subunits of the proteasome, and neurofilaments [31, 33]. Accumulation of nitrated proteins is also found in the brains of patients with AD, PD, and ALS or in mutant Cu/Zn-superoxide dismutase (SOD-1) or mutant α-synuclein transgenic animals [31]. However, there is still no consensus regarding the pathway responsible for α-synuclein degradation. It is unclear whether α-synuclein degradation depends on the proteasome, chaperone-mediated autophagy, or macroautophagy [34, 35].

Autophagy inhibition promotes the accumulation and subsequent aggregation of monoubiquitinated forms of the α-synuclein protein [26]. Recent evidence suggests that autophagy may play a compensatory role when the UPS function is damaged, and vice versa [36]. Inhibition of the UPS has been shown to activate autophagy-mediated protein degradation [37], and suppression of autophagy leads to the accumulation of ubiquitinated proteins in the cytosol [38]. In ALS models, several studies have shown that autophagy dysfunction contributes towards motor neuron loss [39–42]. Thus, further studies are needed to demonstrate whether an increase of α-synuclein modification in an ALS animal model is caused by autophagy misregulation.

5. Conclusion

This paper demonstrated that BV-treated hSOD1G93A transgenic mice showed reduced detergent-insoluble polymerization and phosphorylation of α-synuclein. Furthermore, phosphorylated or nitrated α-synuclein was significantly reduced in the spinal cords and brainstems of BV-treated hSOD1G93A mice and reduced proteasomal activity was revealed in the brainstems of BV-treated symptomatic hSOD1G93A. From these findings, we suggest that BV treatment attenuates the dysfunction of the ubiquitin-proteasomal system in a symptomatic hSOD1G93A ALS model and may help to slow motor neuron loss caused by misfolded protein aggregates in ALS models.

Conflict of Interests

The authors declare no conflict of interests.

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References


