Rosmarinic Acid Alleviates Neurological Symptoms in the G93A-SOD1 Transgenic Mouse Model of Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that affects motor neurons in the brain and spinal cord, resulting in paralysis of voluntary skeletal muscles and eventually death, usually within 2–3 years of symptom onset. The pathophysiology mechanism underlying ALS is not yet clearly understood. Moreover, the available medication for treating ALS, riluzole, only modestly improves neurological symptoms and increases survival by a few months. Therefore, improved therapeutic strategies are urgently needed. In the present study, we investigated whether rosmarinic acid has a therapeutic potential to alleviate neurological deterioration in the G93A-SOD1 transgenic mouse model of ALS. Treatment of G93A-SOD1 transgenic mice with rosmarinic acid from 7 weeks of age at the dose of 400 mg/kg/day significantly extended survival, and relieved motor function deficits. Specifically, disease onset and symptom progression were delayed by more than one month. These symptomatic improvements were correlated with decreased oxidative stress and reduced neuronal loss in the ventral horns of G93A-SOD1 mice. These results support that rosmarinic acid is a potentially useful supplement for relieving ALS symptoms.

Key words: Rosmarinic acid, ALS, neuroprotection, antioxidant

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the development of voluntary muscle paralysis that usually begins focally. This disease eventually leads to death, usually within 2–3 years of symptom onset [1, 2]. Important pathological features of ALS include the selective neuronal loss of lower motor neurons in the spinal cord and upper motor neurons in the brain in the absence of sensory symptoms [3, 4]. A subset of ALS cases have genetic causes, whereas over 90% of all ALS cases are sporadic [5, 6].

Approximately 20% of all familial ALS cases are caused by mutations in the SOD1 (also called Cu/Zn SOD) gene [7-9]. The SOD1 enzyme is 153 amino acids long and converts the superoxide anion to hydrogen peroxide [7, 8]. Transgenic mice overexpressing a mutant form of human SOD1 (G93A-SOD1) display various neurological deficits that resemble those of human ALS, including neuronal loss in the spinal cord [10]. ALS spinal cords show enhanced levels of lipid peroxidation, protein carbonylation, DNA oxidation, and peroxynitrite-mediated damage [11]. These results suggest that oxidative stress contributes to the pathophysiology of ALS. The definitive pathophysiological...
mechanisms underlying genetic and sporadic ALS cases are not yet clearly understood; however mitochondrial dysfunction [11-14], glutamate excitotoxicity [15-17], disrupted axonal transport [18, 19], inflammation [5, 20-22], and miRNA modifications and epigenetic mechanisms [23, 24] have all been postulated to play a role. Currently, riluzole is the only clinically available drug for treating ALS; however, but riluzole only delays the progression of ALS symptoms [25-28]. Therefore, new curative and/or additive treatments for ALS are urgently needed.

Rosmarinic acid is a phenolic compound that is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. It is found in plants, such as species of the Boraginaceae family and the subfamily Nepetoideae of the Lamiaceae family. Rosmarinic acid has a number of biological activities, including antiviral, antibacterial, anti-inflammatory and antioxidant [29]. The intraperitoneal administration of rosemary extract and of rosmarinic acid to presymptomatic G93A-SOD1 transgenic mice was recently reported to slightly delay the onset of motor dysfunction and to attenuate motor neuron degeneration [30]. However, the dose of rosmarinic acid used in their study was relatively low (0.13 mg/kg), so that it remains uncertain whether its weak therapeutic effects were due to the use of a low dose. Moreover, the temporal profiles of behavioral changes were not compared with those achieved with riluzole, making it difficult to interpret the therapeutic value of rosmarinic acid. In the present study, we compared the effects of high doses of rosmarinic acid (30 or 400 mg/kg/day) with those of riluzole on G93A-SOD1 transgenic mice with respect to behavioral, biochemical and histological parameters.

MATERIALS AND METHODS

Animals
Transgenic G93A-SOD1 mice carrying the G93A mutation in human SOD1 [10] were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). G93A-SOD1 mice were maintained by crossing male G93A-SOD1 mice with female C57BL/J6×SJL F1 hybrid as previously described [31]. Mice were housed in pairs in a standard clear plastic cage with free access to food and water. The environment was temperature-controlled (23–24°C) and humidity-controlled (50–60%) and maintained under a 12 h light/dark cycle. All animals were handled in accordance with the animal care guidelines of the Ewha Womans University School of Medicine.

Rosmarinic acid and riluzole treatments
Treatments of G93A-SOD1 mice with rosmarinic acid and riluzole were carried out as previously described [31]. Drug treatments and behavioral assessments of the present study were conducted as a sister experiment of our previous study [31]. G93A-SOD1 mice were divided into four experimental groups according to their diet plans: lab chow alone (Tg-control; n=6 animals), lab chow containing rosmarinic acid (30 or 400 mg/kg/day) (Tg+RA30 and Tg+RA400; n=13 animals for each dose), and lab chow containing riluzole (35 mg/kg/day) (Tg+Rilu; n=8 animals). Lab chows containing supplements were prepared by grinding regular lab chow, mixing the lab chow powder (grindate) with rosmarinic acid or riluzole, remolding, and gamma-ray-irradiating the remodeled grindate. Control lab chow was prepared from regular lab chow by the same procedure. Lab chow mixtures and their control were prepared on a weekly basis. G93A-SOD1 mice were administered rosmarinic acid or riluzole from 7 weeks of age to 16 weeks of age. The amount of food consumed by each mouse was carefully monitored and the treatment doses of rosmarinic acid or riluzole were maintained as indicated.

Mortality test
At the terminal stage of symptoms, G93A-SOD1 mice become completely paralyzed and death follows within a few hours. The time of death was defined as the date on which this symptomatic sequence occurred.

Evaluation of behavioral performance
Hindlimb extension reflex test
The hindlimb extension reflex test was performed as previously described [31]. Briefly, mice were suspended by the tail, and hindlimb extension reflex deficits were scored from 0 to 2 as follows: 2, normal extension reflex in both hind limbs; 1.5, imbalanced extension in the hind limbs; 1.0, extension reflex in only one hindlimb; 0.5, the absence of any hindlimb extension; and 0, total paralysis.

Rota-rod test
The rota-rod test was performed as previously described [31]. Briefly, mice were placed individually on the rotating cylinder of a rota-rod apparatus (Dae-Jong Co. Inc., Seoul, Korea) revolving at a constant speed of 16 rpm. The motor coordination and balance of each mouse were assessed by measuring total riding time on the rotating cylinder. Each animal was given three trials, and the longest latency time before falling was recorded. An arbitrary cut-off time of 300 sec was applied.

Paw grip endurance test
The paw grip endurance (PaGE) test was performed as previously
described [31]. The wire lid of the mouse cage was gently presented to a subject mouse to elicit gripping. Next, the lid was swiftly turned downwards such that the mouse held its own body against the pull of gravity by only its forelimbs and hindlimbs. The time that each mouse could successfully grip the lid was recorded. Each mouse was given up to three trials to hold onto the lid, and the longest latency time before falling was recorded. An arbitrary cut-off time of 90 sec was applied.

**Grip strength test**

The grip strength test was performed as previously described [31]. This test was developed as a supplemental method for the PaGE test. While the PaGE test evaluates the ability of a mouse to support its own body weight using only forelimbs and hindlimbs against gravity, body weight varies between individual mice, in part because of the heterogeneous C57BL/J6×SJL F1 hybrid genetic background. Therefore, the PaGE test is limited in its ability to accurately measure forelimb and hindlimb muscle strength of an individual mouse. To overcome this limitation, mice were presented with an iron grid with a fixed weight of 24 g. The iron grid was 5.5 cm×8 cm, and was constructed with an iron wire 1.0 mm in diameter. To elicit gripping, the iron grid was presented to each mouse. By lifting the grid up, the mice was required to hold its own body with its forelimbs and hindlimbs against the pull of gravity. The time that each mouse held onto the grid was recorded. Each mouse was given up to three trials to hold onto the grid for 10 sec, and the longest latency time was recorded.

**Cell culture**

The SH-SY5Y neuroblastoma cell line was cultured as previously described [31]. Briefly, SH-SY5Y cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-BRL), penicillin (20 units/ml), and streptomycin (20 mg/ml) at 37°C in a humidified incubator. Cells were maintained in a 95% air and 5% CO₂ atmosphere.

The viability of SH-SY5Y against 800 µM hydrogen peroxide (H₂O₂) in the presence of rosmarinic acid (15 µg/ml), trolox (200 µM) or vitamin C (200 µM) in serum-free DMEM. Cell viability was determined using the WST-1 assay as previously described [31].

**Histological analyses**

Histological analyses of lumbar cords (L3~5) were performed as previously described [31, 32]. Briefly, lumbar cords were surgically removed and post-fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Fixed cords were cut coronally into 40 µm-thick sections with a vibratome (Leica VT 1000S; Leica Instruments, Nussloch, Germany).

Cord sections were stained with 1% cresyl violet and the numbers of stained cells were determined using the TOMORO ScopeEye 3.6 system (Olympus, Japan) as previously described [31, 32]. For immunohistochemistry, free-floating sections were blocked with 4% bovine serum albumin in PBST (PBS containing 0.1% Tween-20, pH 7.4) for 1 h. Sections were then incubated with anti-HNE (Alpha Diagnostic Intl. Inc., TX, USA) or anti-ChAT (Chemicon, Temecula, CA, USA) antibodies at 4°C overnight. The sections were then washed with PBST and incubated with biotinylated secondary antibodies diluted 1:200 in PBST. Immunoreactive regions were visualized using an ABC Elite kit (Vector Laboratories; Burlingame, CA, USA). Anti-HNE stained sections were incubated with TRITC-conjugated secondary antibody (Sigma-Aldrich Inc., St. Louis, MO, USA) diluted 1:200 in PBST. After washing the sections were mounted with VECTASHIELD® Mounting Medium (Vector Laboratories). Anti-ChAT stained sections were washed with PBST and incubated with biotinylated secondary antibody diluted 1:200 in PBST. Immunostained sections were visualized using an ABC Elite kit (Vector Laboratories, Burlingame, CA, USA). Images were captured with an Olympus BX 51 microscope equipped with a DP71 camera and DP-B software (Olympus Co., Tokyo, Japan). Fluorescence intensities, which reflect the extent of anti-HNE staining, were measured using TOMORO ScopeEye 3.6 software (Techsan Community, Seoul, Korea) as previously described [32].

**Statistical analysis**

Data were analyzed using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Two-sample comparisons were carried out using Student’s t-test, while multiple comparisons were made using one-way ANOVA followed by the Newman-Keuls multiple range test. All data are presented as means±SEM.

**RESULTS**

**Rosmarinic acid increases survival of G93A-SOD transgenic mice**

The G93A-SOD1 transgenic mice (G93A-SOD1 mice) exhibited various neurological symptoms from 7 weeks of age. Some of untreated G93A-SOD1 mice died as expected at around 12 weeks of age. In contrast, both groups (30 or 400 mg/kg/day from 7 weeks of age) of rosmarinic acid-treated G93A-SOD1 mice survived longer and began to die at 13 and 15 weeks, respectively (Fig. 1A). At 16 weeks of age, the survival rate of untreated G93A-SOD1 mice was 0%, while the survival rates of rosmarinic acid-
treated G93A-SOD1 mice (30 and 400 mg/kg/day) were 57% and 66%, respectively. None of riluzole-treated G93A-SOD1 mice had died by this stage. Thus, the survival of G93A-SOD1 mice was moderately extended by rosmarinic acid administration; however, it was lower than that of the riluzole-treated group. During the period of rosmarinic acid treatment (from 7 weeks to 16 weeks of age), the weekly body weight measurements of the G93A-SOD1 mice were slightly lower than those of the non-transgenic controls, whereas the body weights of rosmarinic acid-treated G93A-SOD1 mice were not significantly different from those of the untreated G93A-SOD1 mice or the riluzole-treated G93A-SOD1 mice (Fig. 1B).

Rosmarinic acid relieves motor function deficits in G93A-SOD1 mice

We next examined whether high doses of rosmarinic acid relieve motor function deficits in G93A-SOD1 mice. G93A-SOD1 mice were treated with rosmarinic acid (30 or 400 mg/kg/day) from 7 weeks of age to 16 weeks of age. Beginning at 8 weeks of age, a set of behavioral tests were carried out twice a week in the following order: rota-rod test, PaGE test, grip test, and hindlimb extension reflex test.

G93A-SOD1 control mice showed behavioral deficits in the hindlimb extension reflex test when beginning from 8 weeks of age; these symptoms progressively deteriorated over time. In contrast, G93A-SOD1 mice treated with rosmarinic acid (30 or 400 mg/kg/day) showed a delay of 4–5 weeks in the onset and progression of neurological symptoms compared with the G93A-SOD1 control mice (Fig. 2A). Rosmarinic acid was more effective at 400 mg/kg than at 30 mg/kg throughout the whole test period until 16 weeks of age, but it was slightly less effective than riluzole.

Beginning from 9 weeks of age, G93A-SOD1 control mice showed impaired performance while running at 16 rpm in the rota-rod test; moreover their performance progressively worsened over time. The administration of rosmarinic acid (30 or 400 mg/kg/day) to G93A-SOD1 mice delayed the onset and progression of neurological symptoms by 3–5 weeks compared with G93A-SOD1 control mice throughout the test period (Fig. 2B). Rosmarinic acid was more effective at 400 mg/kg than at 30 mg/kg until 14 weeks of age. However, no significant differences were observed between the two doses at 15–16 weeks of age; both doses were less effective than riluzole.

The paw grip endurance (PaGE) test requires mice to hold their own bodies against the pull of gravity with their hindlimbs by grasping a metal lid. In this test, G93A-SOD1 mice were impaired in their ability to hang onto the lid beginning from 9 weeks of age. Moreover, their performance progressively deteriorated over time. Administration of rosmarinic acid (300 mg/kg/day) delayed the onset and progression of neurological symptoms by 3–5 weeks during the test period, although its effects were weaker than those by riluzole (Fig. 2C). Moreover, 30 mg/kg rosmarinic acid was far less effective in improving behavioral deficits compared with 400 mg/kg/day.
In the grip strength test, mice were required to hold an iron grid (5.5 cm×8 cm) with a fixed weight of 24 g using forelimbs and hindlimbs. G93A-SOD1 mice showed an impaired ability to hold the grid, beginning at 10 weeks of age, with the holding time gradually decreasing over time. At 15 weeks, G93A-SOD1 mice were not able to properly hold the iron grid. Administration of rosmarinic acid (400 mg/kg/day) delayed the onset and progression of neurological symptoms by 5 weeks, although the effects of rosmarinic acid were slightly weaker than those of riluzole (Fig. 2D). The effects of 30 mg/kg rosmarinic acid were much less dramatic compared to the effects of 400 mg/kg.

**Rosmarinic acid suppresses oxidative stress in the spinal cords of G93A-SOD1 mice**

We next tested whether rosmarinic acid exerts neuroprotective effects against oxidative stress-induced cell death. SH-SYSY cells treated with 800 µM H₂O₂ for 24 h were only 30% viable, whereas the viabilities of H₂O₂-treated cells were improved to 38% and 57% in the presence of 15 µg/ml and 30 µg/ml of rosmarinic acid, respectively (Fig. 3A).

We next examined whether rosmarinic acid suppresses the accumulation of oxidative stress in the spinal cords of G93A-SOD1 mice. Spinal cord sections of untreated G93A-SOD1 mice that survived to 16 weeks of age were also prepared. Immunohistochemical staining with anti-HNE antibody showed that the levels of HNE (4-hydroxy-2-nonenal), a product of lipid
peroxidation, were significantly reduced in the ventral horns of the spinal cords (L3~L5) of rosmarinic acid-treated (400 mg/kg/day) G93A-SOD1 mice compared with control G93A-SOD1 mice (Fig. 3B-F).

**Rosmarinic acid reduces neuronal loss in the spinal cords of G93A-SOD1 mice**

We examined whether rosmarinic acid protects against neuronal loss in the spinal cords of G93A-SOD1 mice. Cresyl violet-staining revealed that G93A-SOD1 mice at 16 weeks of age had only 28% of the number of cresyl violet-stained cells in the ventral horns of the spinal cords (L3~L5) compared with non-transgenic control mice. In contrast, rosmarinic acid-treated (400 mg/kg/day) G93A-SOD1 mice had an increased number of cresyl violet-stained cells compared with non-transgenic control mice, 55.7% of that in non-transgenic control mice (Fig. 4A–D, and 4I).

Immunohistochemical analysis with anti-choline acetyltransferase (ChAT) revealed that untreated G93A-SOD1 control mice had 32.1% of the number of ChAT-positive cells in the ventral horns of the spinal cords (L3~L5) compared with non-transgenic control mice, while rosmarinic acid-treated (400 mg/kg/day) G93A-SOD1 mice exhibited an increased numbers of ChAT-positive neurons, 50.3% of that in non-transgenic control mice (Fig. 4E–H, and 4J).

**DISCUSSION**

We demonstrated that supplementation of rosmarinic acid inhibited neuronal loss in the ventral horns in G93A-SOD1 mice (Fig. 4). This inhibition was accompanied with improvement of neurological symptoms and extended survival of G93A-SOD1 mice (Fig. 1 and 2). Consistent with these findings, the administration of rosmarinic acid markedly reduced the accumulation of HNE in the spinal cords of G93A-SOD1 mice (Fig. 3). These results suggest that rosmarinic acid confers protective effects against neurological symptoms and neurodegeneration in G93A-SOD1 mice. This finding might be due to its ability of anti-oxidant activity. In fact, oxidative stress is an important cause of ALS [11, 33-36]. However, previous trials with vitamin A [37], vitamin E [38, 39], vitamin D [40-42], riboflavin [43], resveratrol [44], folic acid [45], N-acetylcysteine [46], and CoQ10 [47, 48] have shown complicated results and yielded little or even no therapeutic benefit, antioxidants alone might be insufficient to inhibit the pathological progression of ALS.

We recently reported that SK-PC-B70M, an oleanolic-glycoside saponin fraction extracted from the root of *Pulsatilla koreana* [32], delays the progression of neurological deficits in G93A-SOD1 mice.
Neuroprotective Effects of Rosmarinic Acid in ALS Mice

In contrast, the present study showed that rosmarinic acid delayed the onsets of symptomatic mortality as well as neurologic symptoms by more than 1 month and these effects were produced by treatment with rosmarinic acid orally. Drug treatments and behavioral assessments of the present study were conducted as a sister experiment of our previous study [31], in which the survival rate of SOD1-G93A mice at 16 weeks of age was 25%, although survived SOD1-G93A mice at this stage expressed severe neurological symptoms. So it is possible that a certain portion of SOD1-G93A mice in our experimental condition may survive longer than 16 weeks of age. Even though rosmarinic acid was found to delay symptomatic onset by more than one month, rosmarinic acid was not more effective than riluzole. Nonetheless, rosmarinic acid may still have a potential as a supplement for treating ALS. Further studies will investigate whether combination treatments of rosmarinic acid and riluzole yield greater therapeutic benefits compared with riluzole treatment alone.

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